Modulation of Synaptic transmission: Quantitative Analysis of Gβγ specificity to adrenergic α_{2a} receptor and SNARE

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To my beloved parents, Dong Shin Yim and Chung Hun Park,
and
my dearest sister, Yun Chung Yim

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ABBREVIATIONS

5HT	Serotonin
AMPA	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
α_{2a}AR	A2a adrenergic receptor
ADRA2A	A2a adrenergic receptor gene
AID	A interaction domain
ATP	Adenosine triphosphates
AUC	Area under the curve
BLAST	Basic alignment search tool
BME	b-mercaptoethanol
BONT/C	botullium neurotoxin Type C
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAPS	3-(cyoclohexylamino)-1-propannonesulfonic acid
cGMP	cyclic guanosine monophosphate
CHAPS	3-[{3-cholamidopropy;} dimethylammonio]-1-propanesulfonate
CID	collision induced dissociation
CNS	central nervous system
CRB	cerebellum
CSP	cysteine string proein
CTX	cortex
CV	coefficient of variation
DSP	3,3-`dithiobis[sulfosuccin imydlpropinate]
DTT	dithiothreitol
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycoltetracetic acid
EPSC	excitatory postsynaptic current
FACS	fluroscently-activated cell sorting
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate ehydrogenase

GGL	G protein g-like
GDP	Guanosine diphosphate
GIRK	G protein coupled inward rectifier potassium channels
GPCR	G protein coupled receptors
GRK2	G protein-coupled receptor kinase 2
GTP	Guanosine triphosphate
GTPγS	Guanosine 5`-O-[gamma-thio]riphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIPPO	hippocampus
HRP	horseradish peroxidase
IC_{50}	half maximum inhibitory concentration
Inp	input
IP	immunprecipitation
K_{ir}	inwardly rectifying potassium channel
LC-MS/MS	liquid chromatography tandem mass spectrometery
LOD	limit of detection
LOQ	limit of quantification
LTD	long-term depression
LTP	long-term potentiation
LRP	labeled reference peptide
mGluR	meroboropic glutamate receptor
MHD	Munc homology domain
MRM	Multiple reaction monitoring
NMDAR1	N-methyl-d-aspartate receptor-1
PMSF	phenylmethanesulfonyl fluoride
RGS	regulator of G protein singaling
PLC	phospholipase C
PRE	presynaptic
PSD-95	postsynaptic density 95
PSD	postsynaptic density

R7BP	R7family binding protein
RGS	regulators of G protein signaling
RIPA	radioimmunoprecipitation assay
RT	retention time
RWG	resonant standard
SD	standard deviation
SERT	serotonin transporter
SID	stable isotope dilution
SM	Sec-1/Munc-18
S/N	Signal to noise
SNAP	Synaptosomes-associated protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
Str	striatum
Sup	depleted supernatant
TBS	tris buffered saline
TCA	tricholoroacetic acid
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
TX-100	triton x-100
QC	quality control
VAChT	vesicular acetylcholine transporter
VAMP	vesicle-associated membrane protein
VDCC	voltage-dependent calcium channels

CHAPTER 1

INTRODUCTION

1.1 Synaptic Transmission

Synaptic transmission, the communication between neurons utilizing chemical neurotransmitters, hormones and enzymes, is essential for any physiological function. There are approximately 86 billion neurons in the human brain and more than 105 synaptic contacts may exist (Azevedo et al. 2009, Napper and Harvey 1988). These neurons communicate using vesicles. Presynaptic vesicles undergo a series of steps known as docking and priming (Fig. 1). Docked vesicles are vesicles that are an un-measureable distance away from the plasma membrane although they have direct contact with the active zone (Weimer et al. 2006, Verhage and Sorensen 2008, Fernandez-Busnadiego et al. 2010). Once docked vesicles are primed by SNARE and other synaptic proteins (see Organization of Synaptic Terminals section for more details), they are now either slowly or readily releasable pool vesicles (Becherer and Rettig 2006), which can fuse with the plasma membrane in calcium (Ca^{2+}) dependent and independent manners (Weimer et al. 2006, Becherer and Rettig 2006, Malsam, Kreye, and Sollner 2008). While the Ca^{2+} independent exocytosis remains unclear, Ca^{2+} dependent exocytosis is well understood.

At chemical synapses, depolarizing electrical signals are rapidly converted into chemical signals at the synapse by the opening of voltage-dependent Ca^{2+} channels (VDCC) which increases the Ca^{2+} concentration at the presynaptic terminal of neurons and activates Ca^{2+} dependent exocytosis (Fig. 1)(Zhai and Bellen 2004, Schoch and Gundelfinger 2006, Sudhof 2004, Purves D 2008). Both small neurotransmitter vesicles, filled with acetylcholine, glutamate, or the monoamines, and large dense-core vesicles, filled with neuropeptides or neurohormones, are released in the Ca^{2+} dependent manner of evoked release, evoked asynchronous release, or spontaneous mini release (Park and Kim 2009, Pang and Südhof 2010). In evoked release, vesicles fuse within a millisecond of action potential arrival whereas vesicles fuse in a delayed manner for evoked asynchronous release (Pang and Südhof 2010). Moreover, a single vesicle fuses with the plasma membrane in absence of a depolarizing stimulus for spontaneous mini release (Pang and Südhof 2010). Approximately 100 to 200 vesicles, filled with neurotransmitters, neuropeptides, or neurohormones fuse with the plasma membrane and release their contents into the synaptic cleft to activate receptors and channels, such as AMPA, NMDA, and GPCRs, on the postsynaptic membrane to mediate and modulate excitatory or inhibitory postsynaptic currents in postsynaptic cells (Fig.1)(Südhof 2013, Sudhof 2004, Dresbach et al. 2001).

1.2 Architecture of the synaptic terminal

Chemical synapses are structurally complex and made with numerous different synaptic proteins. To understand molecular mechanisms of chemical synaptic functions in the brain, synaptosomes are often used. Synaptosomes, isolated nerve terminals, have a presynaptic nerve terminal, postsynaptic density, synaptic vesicles, and mitochondria

(Evans 2015). From the proteomic analysis of brain synaptosomes, approximately 300,000 proteins and 60 vesicle trafficking proteins, such as SNAP25, VAMP2, syntaxin1, CSP, complexin1/2, and Munc18, are detected at the presynaptic terminal of an average synaptosomes (Wilhelm et al. 2014). These various presynaptic proteins, such as Sec-1/ Munc-18 (SM) proteins Munc-18 and Munc-13, and the calcium sensor, synaptotagmin, together regulate exocytosis (Südhof 2013) (Fig. 1).

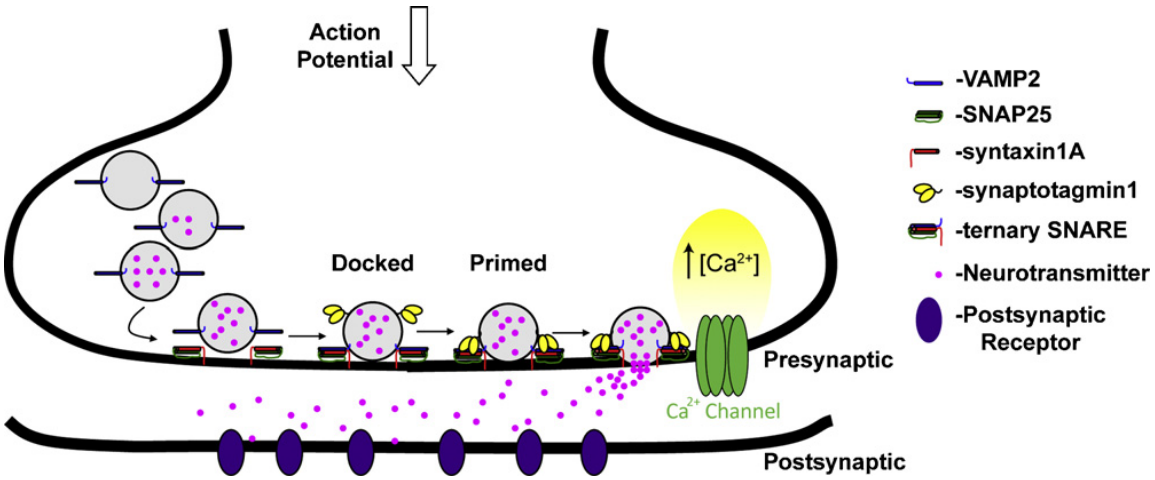


Figure 1. Calcium dependent exocytosis at the presynaptic terminal.

In neurons, synaptic vesicles are loaded with neurotransmitters and docked to the plasma membrane via VAMP2 and t-SNARE (SNAP25 and syntaxin 1A) interaction. Further zippering of SNARE by Synaptotagmin1 (Syt1), Munc-13, and Munc-18 primes the vesicles ready to be fused. Upon the arrival of an action potential, VDCC facilitates an increase of intracellular Ca^{2+} concentration. Syt1, as a Ca^{2+} sensor, senses this change in calcium and initiates fusion of the vesicle and presynaptic membranes, resulting in release of neurotransmitter into the synaptic cleft to activate its respective receptor on postsynaptic terminals. Figure adapted from Betke, KM. *et al*. 2012 *Prog. Neurobiol.*96:304-21.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins

SNARE proteins, the essential exocytotic machinery, are ubiquitously used for exocytosis and other vesicle trafficking tasks in organisms ranging from yeast to humans (Parpura and Mohideen 2008, Jahn and Scheller 2006b) and the SNARE hypothesis is a central hypothesis in our current understanding of exocytosis. In this hypothesis, we believe the formation of a four-helix SNARE generates force to drive the fusion of amphiphilic vesicles and the cell plasma membrane (Südhof 2013). The exocytotic SNARE is further divided into t-SNARE (target) and v-SNARE (vesicle) in the brain. The t-SNARE is made with syntaxin 1A and synaptosome associated protein of 25 kDa (SNAP-25), while the v-SNARE is synaptobrevin/VAMP (vesicle-associated membrane protein)-2.

Syntaxin 1A is made with three conserved domains: an N-terminal helical H_{abc} domain which regulates SNARE assembly and priming, the H_3 or SNARE forming domain which contributes a single helix to the four-helix bundle of SNARE, and a C-terminal hydrophobic transmembrane domain anchored to the presynaptic membrane (Fig. 2A). Syntaxin 1A can exist in a 'closed' or 'opened' conformation depending on its H_{abc} domain interaction with SM proteins such as Munc-18 (Sudhof 2004, Teng, Wang, and Tang 2001). Synaptobrevin, VAMP2, is also a transmembrane protein made with a single α-helical SNARE and cytoplasmic domains (Fig. 2A). It is localized at the vesicles (Hong 2005, Seagar et al. 1999). Lastly, SNAP25 consists of two α-helical SNARE domains without a transmembrane domain and contributes 2 helices to the four-helix SNARE protein (Matteoli et al. 2009). Localized at the presynaptic membrane, SNAP25 anchored to the membrane via the posttranslational palmitoylation of 4 cysteine residues

within the linker region (Jahn and Scheller 2006b) (Fig. 2A). In addition to SNAP25, SNAP23 and SNAP29 are also shown to be involved in the exocytosis of many non-neuronal tissues or specific cell types (Ravichandran, Chawla, and Roche 1996, Zhu, Yamakuchi, and Lowenstein 2015, Mendez and Gaisano 2013, Williams et al. 2015, Su et al. 2001). The formation of ternary SNARE is initiated by the formation of t-SNARE with syntaxin 1A and SNAP25. After the formation of a three-helix t-SNARE, ternary SNARE is assembled by the three-helix t-SNARE interaction with VAMP2 as the vesicle is primed at the plasma membrane (Fig. 2A). A stable, four-helical ternary SNARE is formed and brings the vesicle closer to the presynaptic membrane, zippering to allow the fusion to occur (Fig. 2B).

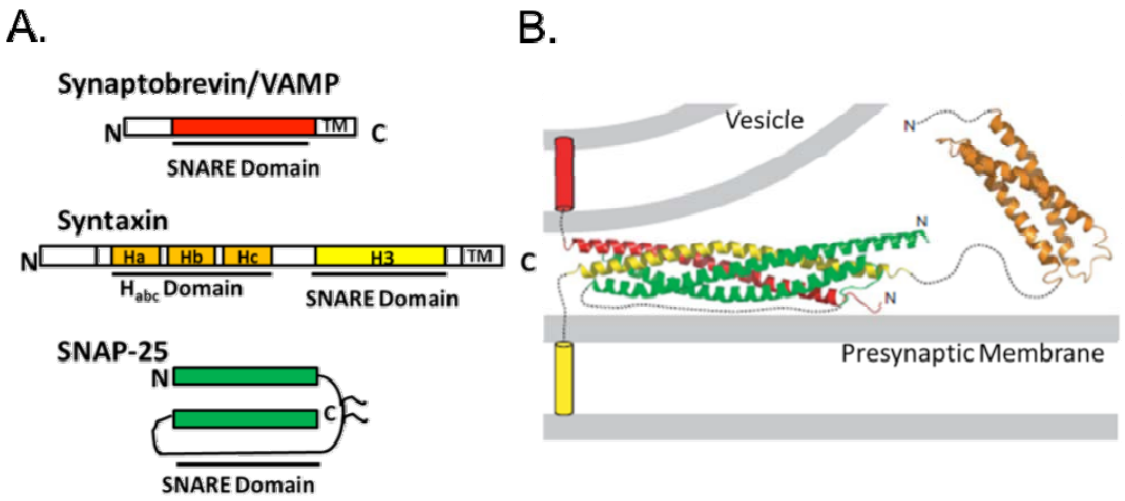


Figure 2.The ternary SNARE.

Domain of synaptobrevin (VAMP), syntaxin, and SNAP25 (A). Cartoon showing the crystal structure of the SNARE complex with the NMR structure of syntaxin Habc domain (B). Figure adapted from Rizo, J. *et al*. 2006. *Trends Cell Biol*. 16(7): 339-50.

Sec-1/Munc-18(SM) proteins

In addition to SNARE proteins, other synaptic proteins, such as Munc-18 and Munc-13, are identified to be involved in the exocytotic machinery (Südhof 2013, Rizo and Xu 2015). Munc-18 initiates the assembly of SNARE protein by interacting with a 'closed' conformation of syntaxin 1A (Rizo and Sudhof 2002, Dulubova, Sugita, Hill, Hosaka, Fernandez, Südhof, et al. 1999, Misura, Scheller, and Weis 2000) (Fig.3). This interaction causes a conformational change in syntaxin 1A to an 'opened' state allowing SNARE protein formation (Rizo and Sudhof 2002, Dulubova, Sugita, Hill, Hosaka, Fernandez, Südhof, et al. 1999, Misura, Scheller, and Weis 2000) (Fig.3). Because the number of docked vesicles was reduced in Munc 18-1 knockout mice (Voets et al. 2001), Munc-18 may be involved in vesicle docking. In addition, Munc-18 maintains SNARE proteins in a correct orientation and allows energy transduction to the membranes by remaining attached to SNARE protein and interacting with the N-terminus of syntaxin 1A (Rizo and Rosenmund 2008, Südhof and Rothman 2009).

Unlike Munc-18 which participates in the ternary SNARE formation, Munc-13, especially the central MUN domain, is primarily involved in the priming process. In the absence of Munc-13, both evoked and spontaneous neurotransmitter release were abolished although vesicle docking remained unaffected (Augustin et al. 1999, Richmond, Davis, and Jorgensen 1999, Aravamudan et al. 1999). Currently, the MUN domain of Munc-13 is hypothesized to facilitate the conformational change of syntaxin 1A for the Munc18-ternary SNARE interaction to stabilize the SNARE protein complex (Ma et al. 2011, Rizo and Südhof 2012).

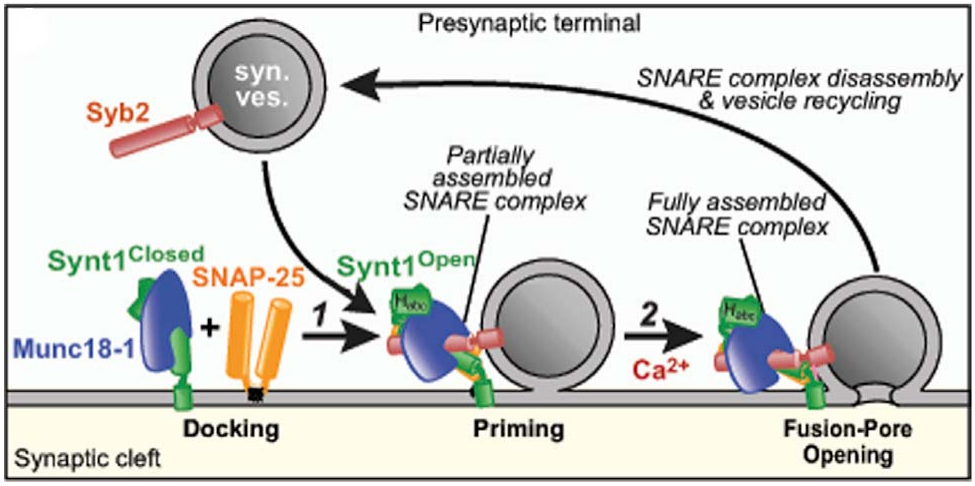


Figure 3. The role of Munc-18 in vesicles fusion.

Prior to the priming of docked vesicles, Munc-18 binds to the closed conformation of syntaxin 1. Partial SNARE is assembled with 'opened' syntaxin 1 and vesicles are primed and attach to the membrane. Munc-18 remains bound to the open confirmation via the N-peptide of syntaxin1. Once the full SNARE is assembled, the fusion pore opens and neurotransmitters are released. Figure adapted from Sudhof, T. *et al*. 2013 *Neuron*. 80:675-690.

Synaptotagmins

Synaptotagmins (Borisovska et al.), Ca^{2+} sensors, are also essential for vesicle fusion to occur (Brose 1992). In humans, over 17 Syt isoforms are found (Südhof and Rothman 2009). However, Syt1 is known as the one that is crucial for fast, synchronous, Ca^{2+} dependent neuronal exocytosis (Rizo, Chen, and Arac 2006, Geppert, Goda, Hammer, Li, Rosahl, Stevens, and Südhof 1994, Tang et al. 2006, Schonn et al. 2008, Gao et al. 2000). Localized at the synaptic and large dense core vesicles membrane, Syt1 has an N-terminal transmembrane domain and two cytoplasmic, PKC like C2 domains: C2A and C2B respectively. These domains bind to the lipid membrane and Ca^{2+} and play an important role in exocytosis (Perin et al. 1990, Chapman et al. 1995). Syt1 interacts with both syntaxin1A and the C-terminus of SNAP25 of SNARE, in a Ca^{2+} dependent manner. On Syt1, Arg198 and Lys200 of C2A domain and Lys297 and Lys301 of C2B domain are important for Ca^{2+} dependent SNARE binding (Lynch et al. 2007). In addition, anionic phospholipids are important for Syt1`s function (Chapman and Jahn 1994a, Chapman et al. 1998, Fernandez et al. 2001). When Syt1 is deleted, 10-fold enhancement of spontaneous release was found (Littleton et al. 1993, Broadie et al. 1994). Mutation of Ca^{2+} binding sites on the C2A domain inhibit synchronous release by 50%, but Ca^{2+} binding impaired C2B domain mutants fully block synchronous release (Mackler et al. 2002, Nishiki and Augustine 2004, Shin et al. 2009). While Syts are essential for exocytosis, further characterization of their molecular functions in synchronous release is necessary.

As Syts play major roles in regulating synchronous release with both SNARE interactions and phospholipid binding, other calcium sensors such as Doc2 and Syt7 may play important roles in regulating asynchronous release, release in the absence of

action potentials (Sun et al. 2007). Doc2 is shown to act as the Ca^{2+} sensor for asynchronous release in cerebellar slices (Groffen et al. 2010) and cultured hippocampal neurons (Groffen et al. 2010, Yao et al.). Although further studies are needed, calcium sensors are important in both asynchronous and synchronous release.

Other accessory proteins

In addition to SNARE, SM proteins, and Syts, other accessory proteins, such as complexin, tomosyn, Rab3-interacting molecule (RIM), and cysteine string protein (CSP), are required for fusion events to occur. These proteins interact with each SNARE protein, the t-SNARE dimer, or the ternary SNARE.

Complexin is a regulatory protein involved in the priming of vesicles and exocytosis (Tang et al. 2006, Chicka and Chapman 2009, Hobson et al. 2011, McMahon et al. 1995). Made with the N-terminus accessory helix and a central helix (Bracher et al. 2002), it interacts with the ternary SNARE by binding as a fifth α-helix to the SNARE bundle of ternary SNARE (Bracher et al. 2002, Chen et al. 2002, Zhou et al. 2017). In a Ca^{2+} independent manner, complexin, as a clamp, interacts with syntaxin and VAMP2 of incompletely "zippered" SNARE until Ca^{2+} binds to Syt1 (Bracher et al. 2002, Chen et al. 2002, Giraudo et al. 2006, Maximov et al. 2009). Moreover, complexin was found to be important for fast synchronous release but not asynchronous or spontaneous release (Reim et al. 2001, Xue et al. 2007). In Mun13-1 knockout mice, complexin levels were also decreased, suggesting its interaction with Munc 13-1 in addition to Syt1 to regulate exocytosis (Augustin et al. 1999). Complexin is suggested to inhibit fusion at low concentrations of Ca^{2+} and promotes exocytosis in a Syt1 dependent manner as the

concentration of Ca^{2+} increases (Diao et al. 2012, Lai et al. 2014).

Presynaptically co-localized with syntaxin1, synaptophysin, and bassoon, Tomosyn binds to t-SNARE and prevents the association of VAMP2 to t-SNARE to form ternary SNARE (Ashery et al. 2009, Fujita et al. 1998, Hatsuzawa et al. 2003b). It has three distinct domains: two sets of 7 WD40 repeats at the N-terminus domain, C-terminal VAMP like SNARE binding domain, and a hypervariable linker domain (Pobbati et al. 2004, Ashery et al. 2009). Both N- and C- termini are important in *in vivo* regulation of exocytosis (Burdina et al. 2011). The C-terminal VAMP like SNARE motif particularly binds to t-SNARE and inhibits the priming step in both neuroendocrine cells and neurons (Baba et al. 2005, McEwen et al. 2006, Yizhar et al. 2004, Gracheva et al. 2006). Interestingly, knock down of tomosyn causes a redistribution of synaptic vesicles (Gracheva et al. 2006) whereas its overexpression o increases vesicles at the plasma membrane (Yizhar and Ashery 2008). This suggests that tomosyn and Munc-13 may act through syntaxin in an antagonist manner (Ashery et al. 2009) regulating vesicle docking and priming by the formation of "zippered" SNARE. Better understanding of tomosyn and its exact molecular mechanisms and its interaction with various synaptic proteins are necessary to understand its effect in vesicle docking, priming, and exocytosis.

Rab3-interacting molecule (RIM), a major scaffold protein in fusion machinery, binds to Munc13, VDCC, and the vesicle bound GTPase Rab 3/27 in a GTP dependent manner (Südhof 2013). It is made up of 5 domains: an N-terminal domain with zinc finger, a central PDZ domain, two C-terminal C2 domains where interestingly Ca^{2+} does not bind, and a conserved proline-rich linker between two C2 domains (Sudhof 2012). The interactions between RIM and Munc13 and VDCC allow vesicles to be close to the plasma membrane for the tight spatial regulation of calcium concentration at the active

zone (Südhof 2013).

Cysteine string protein (CSP) is a member of Hsp40 family of co-chaperone localizes at the synaptic vesicles (Burgoyne and Morgan 2015). With an N-terminal phosphorylation site, a HPD motif in the J domains, and a cysteine-rich C-terminal region, CSP directly interacts with syntaxin (Nie et al. 1999, Wu et al. 1999, Chamberlain et al. 2001), VAMP (Seagar et al. 1999, Boal et al. 2004), Syt (Evans and Morgan 2002a, Boal et al. 2011), dynamin 1 (Rozas et al. 2012), and G protein subunits (Magga et al. 2000, Natochin et al. 2005). In CSPα knockout mice, SNAP25 is found to be reduced. Moreover, overexpression of CSP increases the abundance of SNAP25 (Graham and Burgoyne 2000). Although only 2.8 CSP molecules per synaptic vesicles and 941 CSP molecules per synapse on average were found (Burgoyne and Morgan 2015), CSP may be a neuroprotective chaperone of SNAP25 by its interaction with Hcs70 (Johnson, Ahrendt, and Braun 2010).

Lastly, Gβγ dimers, made with G protein β and γ subunits, interact with ternary SNARE and each individual SNARE protein to mediate inhibition of exocytosis by $G_{i/o}$ GPCRs (Blackmer et al. 2001, Blackmer et al. 2005, Gerachshenko et al. 2005, Hamid et al. 2014, Photowala et al. 2006b, Yoon et al. 2007, Yoon, Hamm, and Currie 2008, Wells et al. 2012). Although Gβγ specificity to each effector remain unknown, the interaction with each effector, such as VDCC, and SNARE, and its importance in synaptic transmission are under active study (see GPCR mediated regulation of synaptic transmission section for more details). These accessory proteins described above are of the main synaptic proteins that affect synaptic transmission, but many other proteins are also play a role. Numerous presynaptic proteins are involved in the architecture of synaptic

terminals, particularly the active zone, and their importance in the regulation of exocytosis still remains unclear. Further understanding will be necessary to fully understand the architecture and mechanism of exocytosis.

1.3 GPCRs: structure and function

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 (Oldham and Hamm 2008, Eglen and Reisine 2009, Millar and Newton 2010). Encoded by approximately 800 genes in the human genome, GPCRs are some of the most successful drug targets and various GPCR ligands such as neurotransmitters and peptide hormones have been identified (Fredriksson et al. 2003, Venkatakrishnan et al. 2013). However, many of them still remain as orphan receptors with no identifiable ligands (Oldham and Hamm 2008, Eglen and Reisine 2009, Millar and Newton 2010).

All GPCRs share a common structural motif of seven hydrophobic transmembrane helices (7TM). They consist of seven transmembrane-spanning α–helices, an extracellular amino terminus (N-terminus), an intracellular carboxyl terminus (C-terminus), and three inter-helical loops on each side of the membrane (Oldham and Hamm 2008). Depending on the GPCR subfamily, the amino terminus may vary the most (Kobilka 2007). Based on their amino acid sequence similarities, GPCRs can be grouped into five major families and numerous subfamilies (Fredriksson et al. 2003). These five main families are: Rhodopsin (ClassA), Secretin (Class B), Glutamate (Class C), Adhesion, and Frizzled taste 2 (Class F) (Schioth and Fredriksson 2005). The rhodopsin

family is the largest and most diverse family that is activated by a variety of ligands including monoamine neurotransmitters and acetylcholine, as well as photons of light. It can be further divided into 4 subgroups: α, β, γ, and δ, although common amongst them is a relatively short N-terminus. The structure of 16 different Class A GPCRs has been determined (Katritch, Cherezov, and Stevens 2013). The beta 2 adrenergic receptor is the most well studied GPCR structurally (Rasmussen et al. 2011, Cherezov et al. 2007, Hanson et al. 2008, Wacker et al. 2010, Warne et al. 2008, Moukhametzianov et al. 2011, Warne et al. 2012, Warne et al. 2011). The secretin family has long N-terminal domains with conserved cysteine bridges where typically peptide ligands bind (Archbold et al. 2011, Schioth and Fredriksson 2005): the glutamate family has a large Venus fly trap at the N-terminus, where their ligands bind. The glutamate receptors, GABA receptors, and taste 1 receptors belong to the glutamate family (Kniazeff et al. 2011). Comparatively, the adhesion family is comprised of orphan receptors without known ligands. Receptors of the adhesion family generally have many N-terminal serine and threonine residues for glycosylation, and proteolytic sites for the cleavage of the extracellular N-terminus (Fredriksson et al. 2003). Lastly, the frizzled taste 2 family (F2DR) is a combination of frizzled receptors and taste-2 receptors. F2DR is involved in controlling cell fate, proliferation, and polarity for the Wnt glycoprotein and the taste 2 receptors in tongue and palate suggests a probable role in bitter taste (Fredriksson et al. 2003).

Upon ligand binding, GPCRs go through a conformational change to increase the receptors` affinity for the G protein heterotrimer (Gαβγ), resulting in the formation of a transient ligand-GPCR-G protein ternary complex (Oldham and Hamm 2008). Receptor activation results in outward movement of helix VI by 14 Ă and extension of helix V by

two helical turns, breaking the salt bridge formed by the (E/D)RY ionic lock motif between R (Rosenbaum, Rasmussen, and Kobilka 2009, Purves D 2008) of helix III and E (Cherezov et al. 2007, Simon, Strathmann, and Gautam 1991) of helix VI ((Rasmussen et al. 2011, Palczewski et al. 2000, Farrens DL1 1996). In addition, changes occur to a rotamer toggle switch W (Cherezov et al. 2007, Lim et al. 2001b) and a conserved NPxxY motif (Oldham and Hamm 2008, Rosenbaum, Rasmussen, and Kobilka 2009, Oldham and Hamm 2007). Overall, these changes in configuration allow Gαβγ binding to the GPCRs by the residues on the αN helix of the Gα subunit, the β2-β3 loop, and the α5 helix, but not the Gβγ subunit (Rasmussen et al. 2011). As a catalytic activator of Gαβγ, GPCRs then transduce extracellular signals into various intracellular responses by the release of GTP-bound Gα (GTP-Gα) and Gβγ (Buranda et al. 2007) to modulate downstream effectors (Fig. 4) (Cabrera-Vera et al. 2003, Gautam et al. 1998a, Clapham and Neer 1997a, Vanderbeld and Kelly 2000a, Blackmer et al. 2005, Gerachshenko et al. 2005, Smrcka 2008a). A great diversity of GPCR function is observed throughout the different families of receptors.

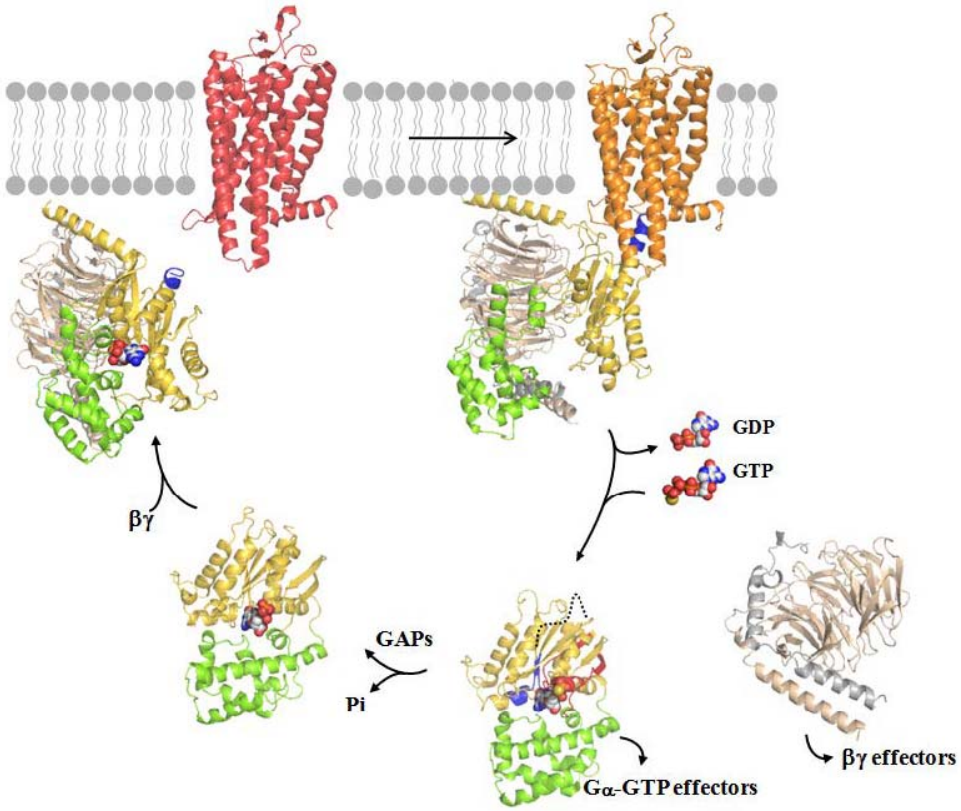


Figure 4. The heterotrimeric G protein activation by G protein coupled receptors.

Heterotrimeric GDP bound Gαβγ binds to a ligand-bound GPCR (red) to form a high affinity ternary complex (orange). Then, GDP is released from Gα subunit (yellow and green), and GTP binds to the nucleotide binding pocket to promote Gαβγ dissociation, Gα-GTP and Gβγ (brown and gray) subunits. These subunits interact with their effectors to initiate downstream signals. Then, GTPase-activating proteins (GAPs) hydrolyze GTP to GDP and promote the re-association of Gαβγ. Illistration drawn by Drs. Ali Kaya and Heidi Hamm.

1.4 Heterotrimeric G proteins

Heterotrimeric G proteins consist of α, β and γ subunits that transduce extracellular signals. In humans, there are 16 genes and 11 splice variants accounting for 27 unique Gα subunits (Yokoyama S 1992, Wilkie et al. 1992) while 5 and 12 genes encode 5β and 12γ subunits respectively (Downes and Gautam 1999, Hildebrandt 1997, Simon, Strathmann, and Gautam 1991). Depending on their function, Gα subunits are classified into four families, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ (Simon, Strathmann, and Gautam 1991). Gαs activates whereas $G\alpha_{i/o}$ inhibits adenylyl cyclase (AC), $G\alpha_q$ activates phospholipase Cβ, and $G\alpha_{12/13}$ activates RhoGEFs (Simon, Strathmann, and Gautam 1991, Siehler 2009). However, all Gα subunits share a similar tertiary structure composed of a GTPase domain and a helical domain (Oldham and Hamm 2008)(Fig. 5A). Unlike the GTPase domain which is conserved among all members of the Gα subunits for the GTP hydrolysis, the helical domain is unique to each Gα subunit (Oldham and Hamm 2008). Consisting of 6α helices, it blocks nucleotide release by covering the nucleotide-binding pocket (Oldham and Hamm 2008)(Fig. 5A). Moreover, all Gα subunits are post-translationally modified, either palmitoylated or myristolated, at the N-terminus to tether at the membrane (Oldham and Hamm 2008, Smotrys and Linder 2004, Chen CA 2001).

There are 5 different Gβ and 12 different Gγ subunits (Downes and Gautam 1999, Hildebrandt 1997, Simon, Strathmann, and Gautam 1991). Gβ subunits are made up of an α helix of approximately 20 amino acids and 7 blades of four anti-parallel β strands, a propeller like WD repeat (Clapham and Neer 1997b, Gautam et al. 1998b, Smrcka 2008a, Sondek et al. 1996) (Fig. 5B). $G\beta_{1\text{-}4}$ share up to 90% amino acid sequence

identity whereas $G\beta_5$ is only 50% identical (Betty et al. 1998b, Smrcka 2008a). In contrast, Gγ subunits are very divergent, sharing only 30-70% sequence identity (Smrcka 2008a, Betty et al. 1998b). Made up of two α-helices, the C-terminal α helix interacts with the surface of blade 5 and a small section of the N-terminal region on Gβ while the N-terminal α helix forms a coiled-coil interaction with the N-terminal helix of the β subunit (Clapham and Neer 1997b)(Fig. 5B). Each Gγ subunits can be post-translationally modified in many different ways at the processed C-terminal cysteine which is carboxymethylated and modified with a farnesyl or geranylgeranyl moiety via a thioether bond. These modifications mediate membrane localization of Gβγ (Cox 1995, Clarke 1992, Cook et al. 2006). Together, Gβ and Gγ subunits form Gβγ dimers with the help of chaperonins including containing TCP-1 (CCT), phosducin-like protein 1 (PhLP1), and dopamine receptor-interacting protein 78 (DRip78) (Dupre et al. 2009, Khan et al. 2013), and once assembled, act as signaling units for GPCRs. This functional dimer cannot be dissociated except with denaturants and neither subunit can signal on its own (Smrcka 2008a).

Although there are 60 (5 Gβ x 12 Gγ subunits) different theoretical combinations of Gβγ dimers (Dingus et al. 2005, Dingus and Hildebrandt 2012b), numerous *in vitro* assays and yeast-two hybrid analyses (Table. 1) have indicated that not all theoretical Gβγ dimers exist, are equally expressed, or interact with Gα subunits, receptors, effectors, and downstream signaling factors (Hildebrandt 1997, Smrcka 2008b, Pronin and Gautam 1992a, Yan, Kalyanaraman, and Gautam 1996b, Robishaw and Berlot 2004, Schwindinger et al. 2003a, Schwindinger et al. 2004, Schwindinger et al. 2010b, Schwindinger et al. 2011a, Khan et al. 2013). Some Gβ and Gγ subunits are ubiquitously expressed, while

others are localized in specific brain regions and cell types (Betty et al. 1998b, Cali et al. 1992, Jones, Lombardi, and Cockett 1998, Largent et al. 1988, Liang, Cockett, and Khawaja 1998, Zhang, Lai, and Simonds 2000). Because most cell types express multiple Gβ and Gγ subunits, specific expression levels and localization may influence intracellular signaling cascades through the formation of specific Gβγ dimers. Moreover, each Gβ and Gγ subunit shows widely varying affinities for each other (Smrcka 2008a, Stephens 2009, Hildebrandt 1997). While $G\beta_1$ and $G\beta_4$ dimerize with all Gγ subunits, $G\beta_2$ and $G\beta_3$ are unable to dimerize with $G\gamma_1$ and $G\gamma_{11}$(Dingus and Hildebrandt 2012a). $G\beta_2\gamma_1$ shows a stronger association than $G\beta_2\gamma_4$ (Pronin and Gautam 1992b, Smrcka 2008a, Zhang et al. 2009). Different affinities between Gβ and Gγ subunits, in combination with the expression and localization of individual Gβ and Gγ subunit, may determine which Gβγ dimers are active in a given cell (Betty et al. 1998b).

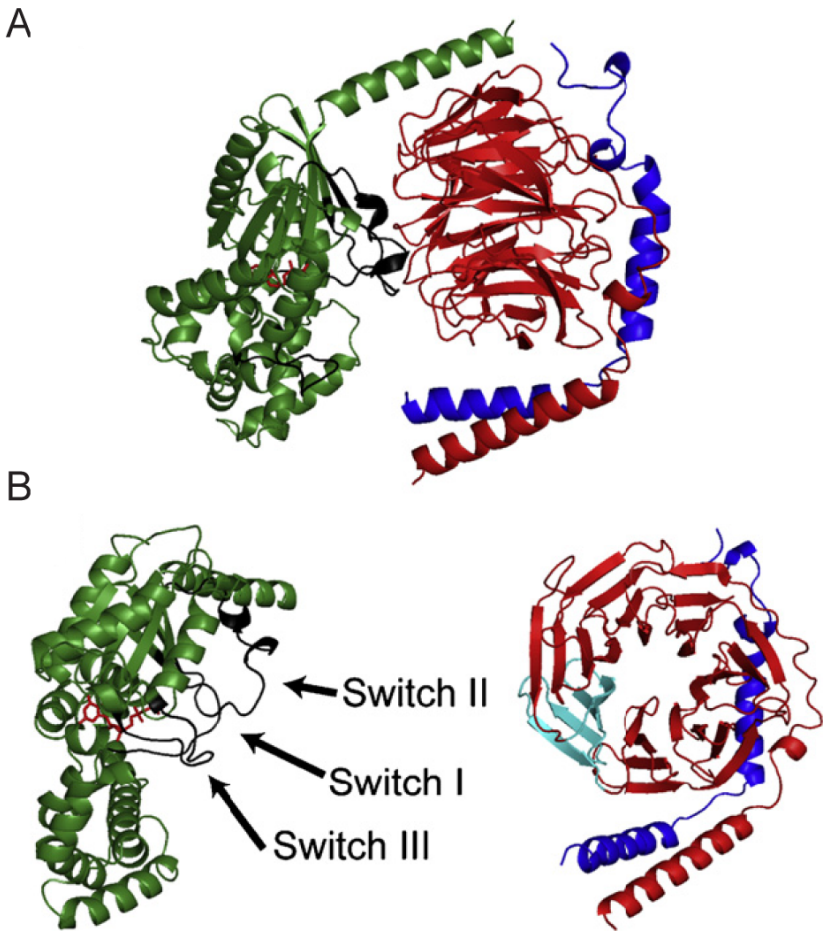
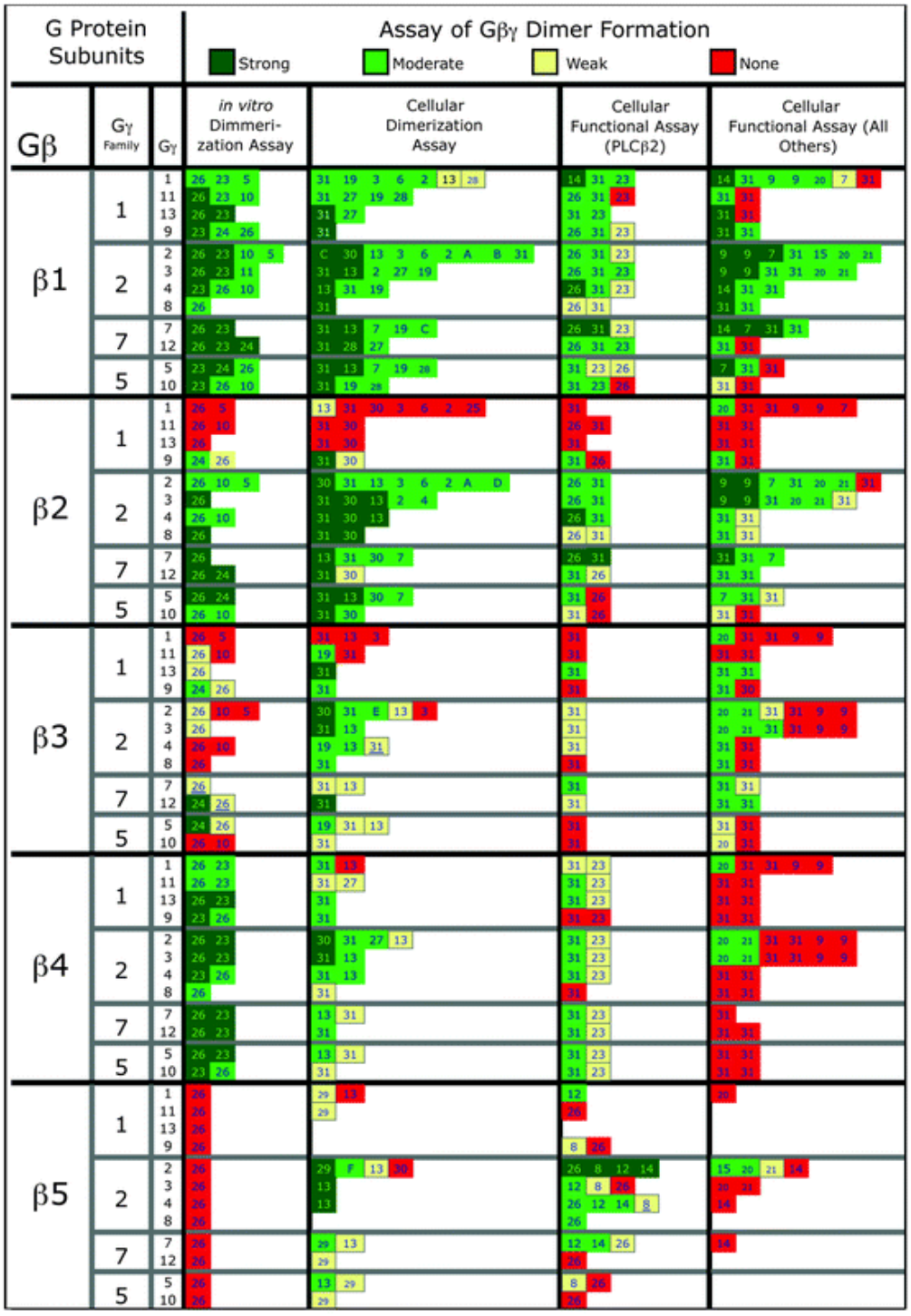


Figure 5. The structure of heterotrimeric G proteins.

The structure of the Gαβγ (PDB ID: 1GOT)(Lambright et al. 1996) is shown (**A**). The Gα subunit (green) has two domains, a GTPase domain and an α helical domain. In the GDP-bound form, Switches I–III form the major interface that interacts with the Gβγ dimer (**B**). The structure of the Gβ subunit (red) is an N-terminus α-helix followed by a 7 bladed propeller β sheets. A single blade (cyan) is a WD40 repeat. The structure of the Gγ subunit (blue) is two tandem α-helices that form interactions with the N-terminus α helix of Gβ and a surface of the Gβ propeller, opposite of Gα interaction site. Figure adapted from Betke, KM. *et al*. 2012 *Prog. Neurobiol.*96:304-21.

Table 1. Summary of studies of Gβγ dimer formation

Table from Dingus, J. *et al*. 2012 *Subcellular Biochemistry* 63, DOI 10.1007/978-94-007-4765-4_9. The numbers refer to the references in Dingus, J. *et al*. 2012 *Subcellular Biochemistry* 63, DOI 10.1007/978-94-007-4765-4_9.



To date, numerous genetic deletion studies and knockout animal studies have suggested specific roles for different Gβ and Gγ subunits in intact cells and mice (Gautam et al. 1998b, Khan et al. 2013). $G\beta_1$ is involved in neural development; its knockout leads to perinatal lethality with reduced cortical thickness, brain volume, and impaired neural progenitor cell proliferation (Okae and Iwakura 2010). $G\beta_2$ is involved in neuronal excitability by the modulation of Ca^{2+} channels (Wolfe et al. 2003, DePuy et al. 2006). In contrast, $G\beta_5$ specifically interacts with the Gγ-like domain of the regulator of G protein signaling (RGS) proteins (RGS 6, 7, 9 and 11) in addition to Gγ subunits (Liang, Cockett, and Khawaja 1998). $G\beta_5$ knockout mice show impaired development, motor learning, and hyperactivity (Zhang et al. 2011, Chen et al. 2003). $G\gamma_3$ knockout mice show an increased susceptibility to seizures and resistance to diet induced obesity while $G\gamma_7$ knockout mice have reduced adenylyl cyclase activity and increased startle response (Pronin and Gautam 1992b, Schwindinger et al. 2003a). Unique physiological phenotypes of each Gβ and Gγ subunit suggests a great deal of specificity in Gβγ dimerization and signaling (Albert and Robillard 2002, Lim et al. 2001a, Lindorfer et al. 1998).

Interestingly, various Gβγ dimers have been reported to have different affinities to their effectors. $G\beta_1\gamma_2$ has a 40-fold higher affinity for SNARE and a 20-fold higher inhibition of exocytosis than $G\beta_1\gamma_1$ (Blackmer et al. 2005). We speculate that the difference in the Gγ subunit, per se, or in the post-translation modification of $G\gamma_1$ and $G\gamma_2$ may mediate the change in affinity (Cook et al. 2006). Such expression and affinity diversity of Gβ and Gγ subunits and the affinity of Gβγ-effector interactions may also suggest that specific dimers could permit specialized roles in signal transduction pathways through

association with particular GPCRs. For example, $G\beta_2\gamma$ and $G\beta_4\gamma$ dimers may specifically 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 (Hosohata et al. 2000, Asano et al. 1999, Kleuss et al. 1992). The expression and subcellular localization of Gβ and Gγ subunits in the CNS may determine which Gβγ dimers exist, their roles in regulating signaling cascades, and their impact in neurological diseases and GPCR targeted drug mechanisms.

Gβγ dimers interact with a wide variety of effectors such as adenylyl cyclases, phospholipase Cβ, PI3 kinase, calmodulin, and components of the mitogen-activated protein kinase cascade (Clapham and Neer 1997b, Cabrera-Vera et al. 2004, Tang and Gilman 1991, Myung et al. 1999, Vanderbeld and Kelly 2000b, Goldsmith and Dhanasekaran 2007). In the central nervous system (CNS), Gβγ dimers also interact with voltage-dependent calcium (VDCC) and inward-rectifying potassium (GIRK) channels, and soluble NSF attachment protein receptors (SNARE) to regulate neurotransmitter release at the synapse (Fig.6) (Herlitze et al. 1996, Huang et al. 1995, Blackmer et al. 2001, Gerachshenko et al. 2005, Yoon et al. 2007, Currie 2010, Sadja and Reuveny 2009, Wells et al. 2012). The Gβγ-effector interaction sites were hypothesized to be localized at the Gα interface (Ford et al. 1998). Mutagenesis studies revealed the binding regions of effectors within the blades of the Gβ propeller and its N-terminus coiled-coil, the Gα subunit interface, and regions on Gγ subunit (Clapham and Neer 1997b, Smrcka 2008a, Myung et al. 2006, Panchenko et al. 1998, Peng et al. 2003). For example, GIRK channels were found to interact just outside of the Gα binding site. Thr86, Thr87, and Gly131 on $G\beta_1$ are important for the Gβγ-GIRK interaction (Zhao et al. 2003). While the

Gα subunits` interface likely represents a core site of Gβγ and its effector binding, other regions may also play important roles in facilitating Gβγ mediated downstream signaling. Although many of these Gβγ-effector interactions and downstream signaling cascades are well understood, it is still unclear which combination of Gβγ dimers are present *in vivo* and what factors control the specificity of Gβγ dimers to their effectors.

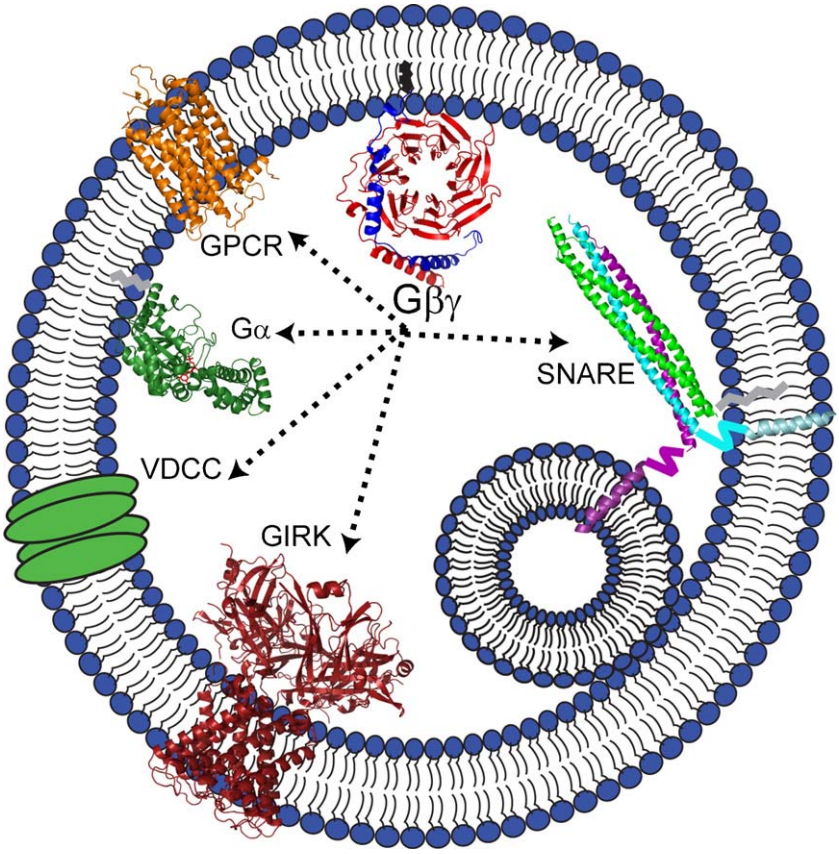


Figure 6. The effectors of Gβγ to regulate the exocytosis.

In addition to GPCRs and Gα subunits, Gβγ dimers are known to interact with numerous effectors to regulate exocytosis. The structures of Gβγ effectors except VDCC are shown. SNARE (SNAP25 (green)/Syntaxin 1A (cyan)/VAMP2 (purple)) (PDB ID: 1SFC)(Sutton et al. 1998b). GIRK channel (PDB ID: 2X6A)(Clarke et al. 2010). Figure adapted from Betke, KM. *et al*. 2012 *Prog. Neurobiol.*96:304-21.

1.5 GPCRs mediated modulation of synaptic transmission

GPCRs are key regulators of synaptic transmission by the actions of GTP-Gα and Gβγ (Betke, Wells, and Hamm 2012a). $G\alpha_s$ and $G\alpha_q$ coupled GPCRs mainly modulate the synaptic transmission by GTP-Gα. GTP-$G\alpha_s$ and $G\alpha_q$ stimulate AC and phospholipase Cβ and lead to protein kinase A (PKA) or protein kinase C (PKC) activation. These lead to the phosphorylation of proteins involved in vesicle recruitment, docking, and fusion and cause either inhibition or facilitation of synaptic transmission (Brown and Sihra 2008). For example, the phosphorylation of K^+ channels by PKA perturbs cell voltage and activates VDCC (Brunelli M 1976, Castellucci et al. 1980). The S10 of CSPα is also phosphorylated by PKA and reduction of its binding to syntaxin, and Syt occur to prolong the opening of the fusion pore (Evans et al. 2001, Evans and Morgan 2002b). Similar to PKA, PKC phosphorylates Munc-18 at S313 (Morgan et al. 2005), the I-II linker of VDCC (Zamponi et al. 1997), and SNAP25 SN2 helix at S187 (Shimazaki et al. 1996). In addition, activated GPCRs recruit arrestins and MAP kinase signaling to influence exocytosis (DeFea 2011).

Unlike $G\alpha_s$ and $G\alpha_q$ coupled GPCRs, $G\alpha_{i/o}$ coupled GPCRs ($G_{i/o}$ coupled GPCRS), such as D_2 dopamine, M_4 muscarinic, α_{2a} adrenergic, and opioid receptors, are known to inhibit the evoked exocytosis by the actions of both GTP-Gα and Gβγ (Stephens 2009, Betke, Wells, and Hamm 2012a). GTP-$G\alpha_i$ inhibits AC and reduces cAMP and activity of PKA on a slower timescale while Gβγ modulate exocytosis through interactions with VDCC and GIRK channels and SNARE in a fast timescale (Fig.6)(Betke, Wells, and Hamm 2012a, Miller 1998, Fernández-Alacid et al. 2009, Blackmer et al. 2005, Gerachshenko et al. 2005, Hamid et al. 2014). Gβγ from both auto-, receptors activated by

neurotransmitter released from the activated synapse, and hetero-, receptors activated by neurotransmitters from neighboring nerve terminals (Fig.7), $G_{i/o}$ coupled GPCRs modulate synaptic transmission (Table. 2)(Betke, Wells, and Hamm 2012a, Mongeau et al. 1998, Millan, Lejeune, and Gobert 2000, Forray, Bustos, and Gysling 1999).

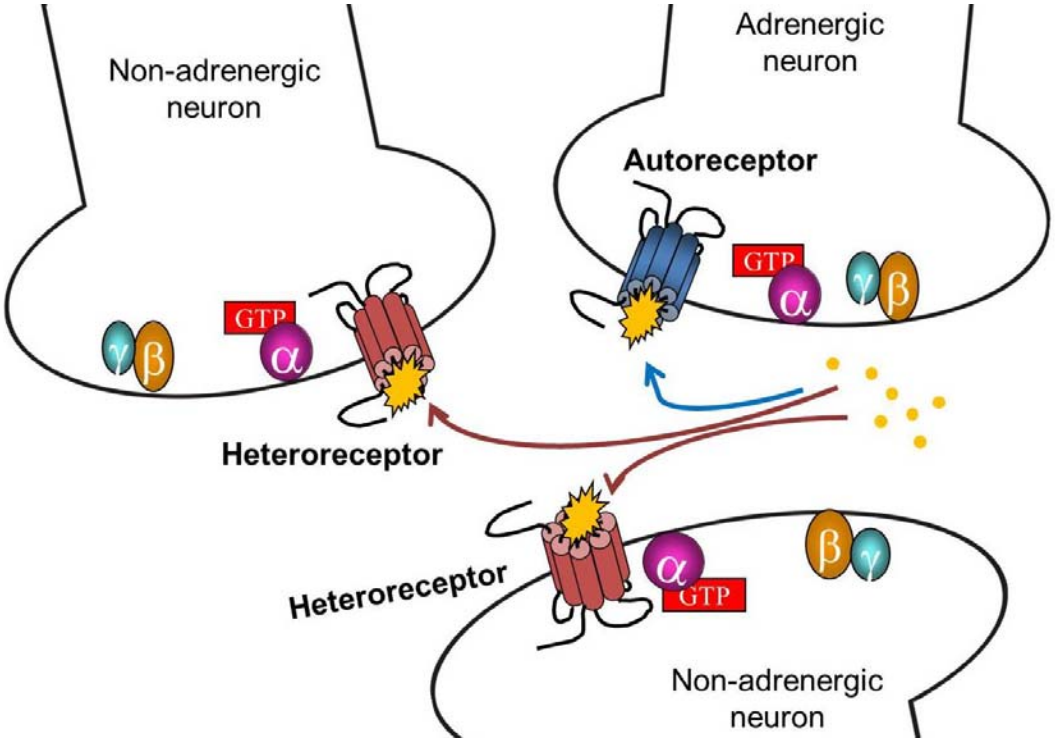


Figure 7. Auto- vs. hetero-GPCRs.

Autoreceptors, located at presynaptic terminal, are receptors that are sensitive to the neuron`s own transmitter while heteroreceptors are receptors that are activated by neurotransmitters from neighboring nerve terminal to mediate effects from other neurons. Heteroreceptors can be at both pre- and post-synaptic terminals. For example, α_{2a} adrenergic receptors (α_{2a} ARs) at adrenergic neurons are autoreceptors while α_{2a} ARs at non-adrenergic neurons are heteroreceptors.

Table 2. The mechanisms of inhibitory GPCRs mediated modulation of synaptic transmission in the central nervous system.

Listed are examples of $G_{i/o}$ coupled GPCRs known to inhibit synaptic transmission via VDCC or SNARE. Receptors are separated depending on localization to the synapse or soma of neurons in the central nervous system. Figure adapted from Betke, KM. *et al*. 2012 *Prog. Neurobiol.*96:304-21.

	Synapse		Soma
	VDCC	Fusion Apparatus	VDCC
M2/M4 Ach receptor	(Higley et al., 2009)	(Bellingham and Berger, 1996, Scanziani et al., 1995)	(Allen and Brown, 1993, Delmas et al., 1998)
Group II mGlu receptor		(Capogna, 2004, Glitsch, 2006, Scanziani et al., 1995)	
Group III mGlu receptor	(Giustizieri et al., 2005)	(Gereau and Conn, 1995)	
$GABA_B$ receptor	(Dittman and Regehr, 1996, Isaacson, 1998, Wu and Saggau, 1995)	(Dittman and Regehr, 1996, Harvey and Stephens, 2004, Wu and Saggau, 1995)	
μ opioid receptor	(Heinke et al., 2011)	(Heinke et al., 2011)	(Wilding et al., 1995)
CB1 cannabinoid receptor	(Brown et al., 2004)		
A1 adenosine receptor	(Dittman and Regehr, 1996, Scholz and Miller, 1992, Umemiya and Berger, 1994)	(Scholz and Miller, 1992)	(Umemiya and Berger, 1994)
D2 dopamine receptor	(Hamilton and Smith, 1991, Higley and Sabatini, 2010)	(Nicola and Malenka, 1997)	(Cabrera-Vera et al., 2004)
α_2 adrenergic receptor	(Boehm, 1999)	(Delaney et al., 2007)	(Boehm, 1999)

Given that most cell types express multiple Gβ and Gγ subunits, a greater understanding of Gβγ-effector regulation is necessary to fully elucidate the physiological importance of Gβγ in regulating synaptic transmission and how specificity is achieved *in vivo* at particular synapses in relevance of each mechanism to modulate synaptic transmission. Considerable research has shown that unique Gβγ combinations may play specific roles in mediating interactions with both receptors and effectors (Betty et al. 1998a, Lim et al. 2001a, Albert and Robillard 2002, Lindorfer et al. 1998) and even, that isoforms may show tissue-dependent specificity for an individual effector (Robishaw and Berlot 2004, Schwindinger et al. 2003a, Schwindinger et al. 2004, Schwindinger et al. 2010b, Schwindinger et al. 2011b). For example, activated alpha 2a adrenergic receptors (α_{2a}-ARs) are found to interact with $G\alpha_{i1}$, $G\beta_1$, $G\beta_2$, $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_7$ using fluorescence resonance energy transfer (FRET) assay (Gibson and Gilman 2006, Richardson and Robishaw 1999b). Moreover, M_4 Muscarinic receptor was found to inhibit L-type Ca^{2+} channel by $G\alpha_o$, $G\beta_3$, and $G\gamma_4$ (Krumins and Gilman 2006). Each $G_{i/o}$ GPCR and effector may utilize unique Gβγ as its signal unit and that the Gβγ specificity may affect the inhibition of exocytosis.

The regulation of G protein-Activated Inward Rectifying Potassium (GIRK) channels by Gβγ.

Somatodendritic GIRK channels in atrial cells and neurons act to hyperpolarize the cell membrane by facilitating an efflux of K^+ out of the cell, making it harder to reach the threshold for initiation of an action potential (Clapham and Neer 1997b, Fernández-Alacid et al. 2009, Lüscher and Slesinger 2010, Lüscher et al. 1997,

Yokogawa et al. 2011, Kulik et al. 2006). Today, 4 mammalian GIRK (GIRK 1–4) have been identified to form various functional heterotetramers in brain (Peng et al. 2003, Finley et al. 2004, Sadja and Reuveny 2009, Dascal 1997). The activity of GIRK channels is regulated by the activated Gi/o coupled GPCRs, such as opioid, $5HT_{1a}$ serotonin, and $GABA_B$ receptors (Ikeda et al. 2003, Ponce et al. 1996, Sadja, Alagem, and Reuveny 2003) to prevent exocytosis. Gβγ from these receptors directly bind to the cytoplasmic channel domain and rotate the domain and the inner helices to open the channel for the K^+ efflux (Sadja and Reuveny 2009, Huang et al. 1995, Logothetis et al. 1987, Whorton and MacKinnon 2013). As with many Gβγ effectors, GIRK channels interact with the Gα binding interface of Gβγ. Leu55, Lys78, Ile80, Lys89, Trp99, Asp228, Asp246, and Trp332 of Gβ (Ford et al. 1998, Albsoul-Younes et al. 2001) interact with Gln248, Thr249, Glu251, Gly252, Glu253, and Leu344 of GIRK`s cytoplasmic channel domain (Whorton and MacKinnon 2013, Yokogawa et al. 2011, He et al. 1999, Finley et al. 2004). In addition to residues on Gβ subunits, the residues on Gγ subunits are important for the Gβγ modulation of GIRK channels. $G\gamma_2$ 35–71 are required for the full stimulation of GIRK4 currents by $G\beta_1\gamma_2$ (Peng et al. 2003).

The Gβγ specificity affects the inhibitory GPCR mediated GIRK channel regulation. Gβγ dimers containing $G\beta_{1\text{-}4}$ enhance GIRK channel activity while those containing $G\beta_5$ suppress it (Lei et al. 2000, Lei, Talley, and Bayliss 2001, Reuveny et al. 1994, Wickman et al. 1994, Takao et al. 1994, Yamada et al. 1994). Although $G\beta_5$ containing Gβγ dimers are less likely to be available compared to other Gβγ dimers as it prefers RGS instead of Gγ subunits, $G\beta_5$ containing dimers are found to compete

with other $G\beta_{1-4}$ containing $G\beta\gamma$ on its binding to the cytoplasmic domains of the GIRK channels and prevent channel activation (Lei et al. 2003). Interestingly, $G\beta_5$ in complex with RGS is also found to interact with the cytoplasmic domains of GIRK channels and facilitate their coupling to $GABA_B$ receptors (Xie et al. 2010). This may be with presynaptic GIRK channels (Ladera et al. 2008, Michaeli and Yaka 2010). Similarly, D_2 receptor mediated activation of presynaptic GIRK channels are also found in ventral tegmental neurons (Michaeli and Yaka 2010). Presynaptic GIRK may inhibit exocytosis by decreasing the amplitude and duration of action potentials. However, it is unclear whether presynaptic GIRK uses $G\beta\gamma$ in a similar manner as it is pertussis-toxin resistant (Ladera et al. 2008). Given the diversity of binding sites on both the channel and the $G\beta\gamma$, it is possible that multiple $G\beta\gamma$ bind to GIRK at the same time to elicit the conformational change of GIRK for its activation (Sadja and Reuveny 2009).

The regulation of presynaptic voltage-dependent calcium channels (VDCC) by Gβγ.

The most well understood mechanism of inhibitory GPCRs mediated modulation of exocytosis is the $G\beta\gamma$ and VDCC interaction (Zamponi and Currie 2013, Ikeda 1996b, Hille 1994, Dolphin and Scott 1987). Composed of $\alpha 1$, β, $\alpha_2\delta$, and γ subunits, VDCC controls the calcium concentration of presynaptic terminal and is critical in the regulation of vesicle exocytosis (Catterall, Goldin, and Waxman 2005). As stated earlier, VDCC are activated upon presynaptic terminal depolarization and increases the Ca^{2+} concentration at the terminal, which causes Syt1 to bind to SNARE and trigger exocytosis. α_2 adrenergic, GABA, somatostatin, and opioid receptors are shown to function via this mechanism (Dolphin and Scott 1987, Womack and McCleskey 1995). At present, five classes of

VDCC have been identified in mammals based on their pharmacological and electrophysiological properties, with low voltage (T type) and high voltage (N , P/Q, and R types) subtypes (Catterall, Goldin, and Waxman 2005). Out of these VDCC, Gβγ directly interacts with N or P/Q type VDCC to inhibit Ca^{2+} influx into presynaptic cells and inhibit Ca^{2+} dependent exocytosis (Currie 2010, Herlitze et al. 1996, Ikeda 1996b, Tedford and Zamponi 2006, Catterall, Goldin, and Waxman 2005, Tedford et al. 2006). Additionally, R type VDCC is used for opioid receptor mediated Gβγ modulation of synaptic transmission (Berecki, Motin, and Adams 2016).

The Gβγ-VDCC interaction involves the N-terminal loop (Canti et al. 1999), the I–II loop (Zamponi et al. 1997, Herlitze et al. 1996), and the C-terminal loop (Qin et al. 1997, Furukawa et al. 1998, Li, Zhong, et al. 2004) of the pore-forming α1 subunit of VDCC. Within the I–II loop, two binding sites, a QXXER motif and G protein interaction domain, of Gβγ dimers were found (Herlitze et al. 1996, Zamponi et al. 1997, De Waard et al. 1997). Although two binding sites were found, the kinetic data suggest that Gβγ and channel interacts in a 1:1 ratio (Zamponi and Snutch 1998). On the Gβ subunit, Lys78, Met101, Asn119, Thr43, Asp186, and Trp322 Gα binding residues are important for the Gβγ-VDCC interaction (Ford et al. 1998). Interestingly, Gβγ binding on a QXXER motif, similar to that found on AC2 and GIRKs, involves PKC as a modulator. PKC reduces the somastatin receptors` mediated inhibition of Ca^{2+} currents (Swartz 1993) while phosphorylated Ser and Thr in α1 416–434 reduce Gβγ binding of VDCC (Zamponi et al. 1997).

Additionally, syntaxin 1A may regulate VDCC and the interaction between VDCC, syntaxin, and Gβγ (138,145). The N-terminus of syntaxin 1A interacts with the II-III linker

of N and P/Q types VDCCs to anchor them to SNARE (Bennett, Calakos, and Scheller 1992, Leveque et al. 1994, O'Connor et al. 1993, Jarvis and Zamponi 2001). Upon syntaxin binding, VDCCs undergo a hyperpolarizing change in the voltage dependence of inactivation (Bezprozvanny, Scheller, and Tsien 1995, Rettig et al. 1996). Syntaxin1A-VDCC-Gβγ complex may promote the inhibition of VDCC as Gβγ and VDCC bind different domains of syntaxin 1A (Jarvis et al. 2000a). Overall, the regulation of VDCC by Gβγ of inhibitory GPCRs is widespread and an important regulatory factor controlling neurotransmitter release.

The inhibition of synaptic transmission via the Gβγ-SNARE interaction.

Upon Ca2+ influx, Syt1 binds to the SNARE, vesicle fusion machinery, and promotes both vesicle priming and fusion of the vesicle and targets the plasma membrane by disordering the lipid bilayer in a Ca^{2+} dependent manner (Blackmer et al. 2001, Gerachshenko et al. 2005, Blackmer et al. 2005) (Fig.8). However, Gβγ subunits are known to compete with Syt1 for its binding to SNARE (Blackmer et al. 2005, Yoon et al. 2007). The direct interaction between Gβγ subunits and SNARE affects the exocytotic mechanism downstream of Ca^{2+} entry (Fig.8) (Gerachshenko et al. 2005). At low Ca^{2+} concentrations, the Gβγ bound to SNARE competes with Syt1 causing the inhibition of exocytosis. At high Ca^{2+} concentrations, the affinity of Ca^{2+}-Syt1 for SNARE increases and it competes with Gβγ to override the inhibition (Yoon et al. 2007). In addition, *in vitro* binding studies have demonstrated direct Gβγ-SNARE interaction sites at the H_3 domain of syntaxin and the carboxyl-terminus of SNAP25 (Yoon et al. 2007, Wells et al. 2012). Exactly how the Gβγ-SNARE interaction prevents exocytosis is not known, but Gβγ may

inhibit the tight zippering of the SNARE complex necessary to drive membrane fusion, and inhibit vesicle exocytosis.

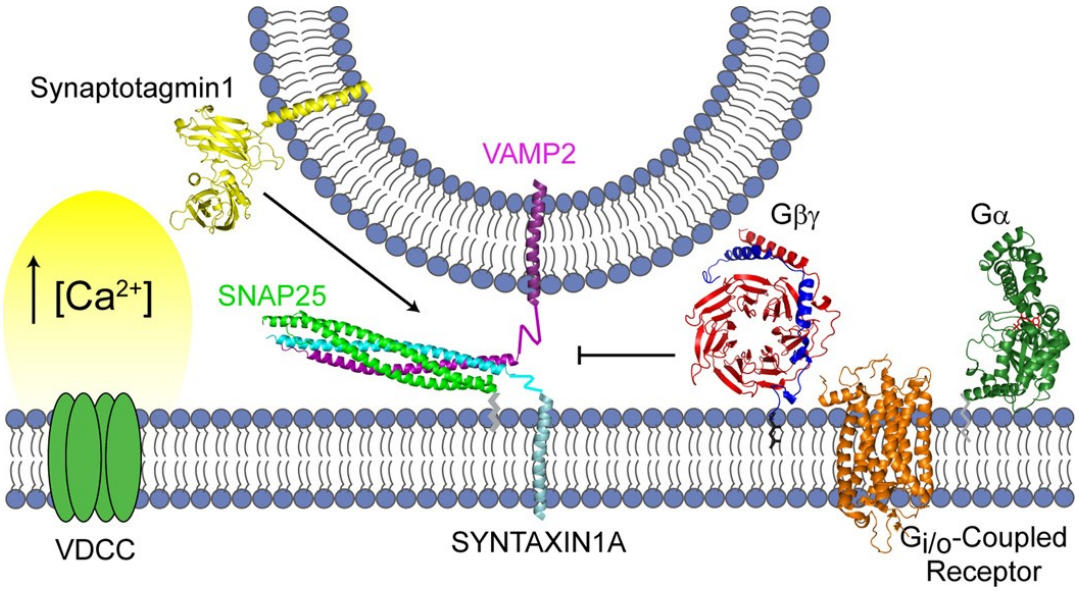


Figure 8. Gβγ regulation of the presynaptic vesicle release.

Synaptic vesicles are primed by a tethering interaction between VAMP2 on the vesicle and the SNAP25/syntaxin 1A dimer at the plasma membrane. At low intracellular concentrations of calcium, activation of $Gi_{/o}$-coupled receptors results in release of Gβγ that will bind to the SNARE proteins and prevent binding of synaptotagmin. However, high enough intracellular calcium concentrations, such as with repetitive neuronal stimulation, synaptotagmin is able to compete with Gβγ for binding to SNARE, and thereby promote fusion of the vesicles with the plasma membrane. Figure adopted from Betke, KM. *et al*. 2012 *Prog. Neurobiol.*96:304-21.

The first insight into direct interaction between Gβγ and SNARE proteins was derived from studies utilizing botulinum toxin A (BoNT/A). BoNT/A cleaves the C-terminal 9 amino acids from SNAP-25 (Schiavo et al. 1993, Binz et al. 1994). Treatment of presynaptic reticulospinal neurons with BoNT/A disrupted the ability of serotonin receptors to inhibit excitatory postsynaptic action potentials (Gerachshenko et al. 2005). Furthermore, a 14-amino acid peptide based on the C-terminus of SNAP-25 was able to block Gβγ-mediated inhibition (Gerachshenko et al. 2005, Blackmer et al. 2005). This interaction between Gβγ and SNAP-25 has been confirmed at the molecular level with reduction of binding between BoNT/A cleaved SNAP-25 compared to uncleaved SNAP-25 with Gβγ (Yoon et al. 2007, Zhao et al. 2010). In addition to binding to SNAP-25, Gβγ also binds individually to the other SNARE proteins, syntaxin 1A and synaptobrevin, as well as to the t-SNARE dimer (SNAP-25 with syntaxin 1A) and to the ternary SNARE complex (Yoon et al. 2007). These findings of presynaptic Gβγ inhibition have been identified in neurons (Delaney, Crane, and Sah 2007b, Blackmer et al. 2001, Zhang, Upreti, and Stanton 2011), chromaffin cells (Yoon, Hamm, and Currie 2008), and pancreatic β cells (Zhao et al. 2010).

Currently, little is known about the *in vivo* physiological importance and regulation of Gβγ-SNARE interaction. Only few $G_{i/o}$ coupled GPCRs are examined and found to modulate synaptic transmission using the Gβγ-SNARE interaction (Table. 2). For example, α_{2a}ARs and κ-opioid receptors modulate noradrenaline or glutamate release by the Gβγ–SNARE interaction at the central nucleus of the amygdala (Delaney, Crane, and Sah 2007a) and vasopressin-expressing magnocellular neurosecretory cells (Iremonger and Bains 2009). Recently, $5HT_{1B}$ and $GABA_B$ receptors at the same

presynaptic terminal of CA1 pyramidal neurons in the hippocampus inhibit the evoked release through Gβγ, but two different effectors of Gβγ. $GABA_B$ receptors use VDCC while $5HT_{1B}$ receptors use SNARE implying the selectivity of Gβγ to its effectors (Hamid et al. 2014). The physiological importance of the Gβγ-SNARE interaction may be found in the regulation of long term potentiation (LTP) or long term depression (LTD). In addition to $G\alpha_i$, Gβγ may affect the long term alterations of presynaptic signals associated with LTP and LTD and alter learning and memory, development of neural networks, and fine-tuning of synaptic connections (Zhang, Upreti, and Stanton 2011). The C-terminus of SNAP25 is critical for mGluR mediated presynaptic depression by the Gβγ-SNARE interaction and stimulus-evoked LTD, but not LTP, and cleavage of residues 198–206 on SNAP25 completely prevented the induction of stimulus reduced LTD and alters the extracellular calcium concentrations (Zhang, Upreti, and Stanton 2011). Taken together, the Gβγ–SNARE interaction mediated modulation of synaptic transmission can affect various normal neural processes, and neurological and neuropsychiatric diseases.

Implications of G protein Modulation of Synaptic Transmission in Diseases

Complex regulatory mechanisms converge on the exocytotic apparatus to ensure precise control of neurotransmitter release and modulation of synaptic transmission. Significant advances have been made toward identifying the protein-protein interactions involved in exocytosis (Betke, Wells, and Hamm 2012b, Bruns and Jahn 2002, Fasshauer 2003, Mahmoud, Yun, and Ruiz-Velasco 2012, Sudhof 2004, Tjolsen et al. 1992, Ungar and Hughson 2003, Verhage and Toonen 2007, Stephens 2009). However, gaps in our

understanding of the physiological implication of Gβγ mediated modulation of synaptic transmission in various neurological and neuropsychiatric diseases remain.

Various mutations and dysregulations of $G_{i/o}$ coupled GPCRs with Gβγ mediated synaptic transmission mechanisms are identified and used as drug targets in various neurological and neuropsychiatric diseases. In Alzheimer's disease, activation of M_2 autoreceptors by anticholinesterase treatment contributes to disease progression by reducing acetylcholine release (Albrecht et al. 1999). Similar to this, D_2 autoreceptors mediated inhibition of dopamine release by dopamine agonists is suggested to contribute to the deterioration of cognitive function in Parkinson's diseases (Arnsten et al. 1994, Breitenstein et al. 2006). Moreover, risperidone targeting the D_2 dopamine, $5HT_{1A}$ serotonin, and α_{2A} adrenergic (α_{2a}ARs) receptors is often used as a treatment of schizophrenia, anxiety disorders, and attention deficit hyperactivity disorder, respectively (Hunter 1995, Janssen et al. 1988, Taylor et al. 1985). $G_{i/o}$ coupled GPCRs, such as M_4 muscarinic and mGluR2, 3, and 4 metabotropic glutamate receptors, are often targeted to generate drugs for various neurotransmitter imbalance diseases like schizophrenia, Parkinson's disease, psychosis, dystonia, cognitive deficits, sleep wake disorders, and attention deficit disorder. However, we do not fully understand the relative importance of their role as a modulator of synaptic transmission and Gβγ specificity to each receptor and effector. A better understanding of the Gβγ specificity and the Gβγ-SNARE interaction may provide insight into disease progression and reveal more disease selective targets for therapeutic intervention.

1.6 Conclusion

Overall, GPCRs mediated regulation of synaptic transmission in a complex and highly regulated process. Although numerous studies were done to fully understand this modulation, numerous questions, especially regarding the Gβγ-SNARE interaction, still remain as questions. Given the role of α_{2a}-adrenergic receptors (α_{2a}-ARs) in modulating neurotransmitter release in physiological and pathological settings and the importance of drugs modulating α_{2a}ARs in working memory, anxiety, and analgesia, my dissertation study is on the development of quantitative analysis of Gβ and Gγ subunits, Gβγ selectivity to α_{2a}AR and SNARE, and the microarchitecture of the Gβγ-SNARE interaction. I anticipate revealing the Gβ and Gγ specificities to α_{2a}ARs and SNARE for its Gβγ-SNARE interaction and determining the microarchitecture of exocytosis. Further understanding of the Gβγ-SNARE interaction may lead to discovery of novel therapeutic targets to help patients with hypertension, attention-deficit/hyperactivity disorder, post-traumatic stress disorder, and the need of analgesia.

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CHAPTER 2

QUANTIFICATION OF NEURONAL Gβ AND Gγ SUBUNITS IN BRAIN SYNAPTOSOMES

2.1 Introduction

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane proteins, encoded by approximately 800 genes in the human genome, and are some of the most successful drug targets (Fredriksson et al. 2003, Venkatakrishnan et al. 2013). Upon activation, GPCRs transduce extracellular signals into various intracellular responses by the activation and dissociation of heterotrimeric G proteins. Heterotrimeric G proteins, made up of Gα, Gβ, and Gγ subunits, dissociate after activation of GPCRs into a GTP-bound Gα (GTP-Gα) and a Gβγ dimer; each signals to a number of effectors. Gβγ dimers interact with adenylyl cyclases and phospholipase Cβ, in addition to PI3 kinase and components of the mitogen-activated protein kinase cascade (Clapham and Neer 1997b, Cabrera-Vera et al. 2004, Tang and Gilman 1991, Myung et al. 1999, Vanderbeld and Kelly 2000b, Goldsmith and Dhanasekaran 2007). In the central nervous system (CNS), Gβγ dimers also interact with voltage-dependent calcium (VDCC) and inward-rectifying potassium (GIRK) channels, and soluble NSF attachment proteins (SNARE) to regulate neurotransmitter release at the synapse (Herlitze et al. 1996, Huang et al. 1995, Blackmer et al. 2001, Gerachshenko et al. 2005, Yoon et al. 2007, Currie

2010, Sadja and Reuveny 2009, Wells et al. 2012). Although many of these Gβγ-effector interactions and downstream signaling cascades are well understood, it is still unclear which combination of Gβγ dimers are present *in vivo* and what factors control the specificity of Gβγ dimers to their effectors. Given the diversity of GPCRs, G proteins, and Gβγ effectors, and the importance of GPCRs as drug targets, examining the expression and subcellular localization of Gβ and Gγ subunits will aid in our understanding of not only the regulatory effects of Gβγ dimer specificity in physiology but also in disease pathophysiology such as depression, ADHD, and Parkinson`s disease (Betke, Wells, and Hamm 2012a).

In mammals, there are five different Gβ genes and twelve different Gγ genes encoding each subunit (Downes and Gautam 1999, Hildebrandt 1997, Simon, Strathmann, and Gautam 1991). $G\beta_{1\text{-}4}$ share up to 90% amino acid sequence identity whereas $G\beta_5$ is only 50% identical (Betty et al. 1998b, Smrcka 2008a). In contrast, Gγ subunits are very divergent, sharing only 30-70% sequence identity (Smrcka 2008a, Betty et al. 1998b). Made up of two α-helices, Gγ subunits can be post-translationally modified at the processed C-terminal cysteine which is carboxymethylated and modified with a farnesyl or geranylgeranyl moiety via a thioether bond. These modifications aid Gβγ dimers in membrane localization (Cox 1995, Clarke 1992). Together, Gβ and Gγ subunits form Gβγ dimers and once assembled, act as signaling units for GPCRs. Although we do not fully understand the selectivity of Gβγ dimers for various GPCRs and effectors, some studies have hypothesized that Gβ subunits determine the Gβγ-effector specificity, while Gγ subunits confer Gβγ-receptor specificity (Yan and Gautam 1997, Yan, Kalyanaraman, and Gautam 1996a, McIntire, MacCleery, and Garrison 2001,

Kleuss et al. 1993, Kisselev and Gautam 1993, Clapham and Neer 1997b, Gautam et al. 1998b, Smrcka 2008a, Oldham and Hamm 2007). To date, numerous genetic deletion studies and knockout animal studies have suggested specific roles for different Gβ and Gγ subunits in intact cells and mice (Gautam et al. 1998b, Khan et al. 2013). Various Gβ and Gγ subunits are implicated in neurodevelopmental disability, hypotonia, and seizures (Petrovski et al. 2016, Pronin and Gautam 1992b, Schwindinger et al. 2003a). These unique physiological phenotypes of each Gβ and Gγ subunits suggest a great deal of specificity in Gβγ dimerization and signaling (Albert and Robillard 2002, Lim et al. 2001a, Lindorfer et al. 1998). *In situ* hybridization studies of Gβ and Gγ subunits in the CNS indicate how the distribution of Gβ and Gγ subunits may affect Gβγ dimerization in specific brain regions and cell types (Betty et al. 1998b, Smrcka 2008a, Vanderbeld and Kelly 2000b). Some Gβ and Gγ subunits are ubiquitously expressed, whereas others are localized in specific brain regions and cell types(Betty et al. 1998b, Cali et al. 1992, Jones, Lombardi, and Cockett 1998, Largent et al. 1988, Liang, Cockett, and Khawaja 1998, Zhang, Lai, and Simonds 2000). Because most cell types express multiple Gβ and Gγ subunits, specific expression levels and localization may influence intracellular signaling cascades through the formation of specific Gβγ dimers.

Although there are 60 different theoretical combinations of Gβγ dimers (Dingus et al. 2005, Dingus and Hildebrandt 2012b), numerous *in vitro* assays and yeast-two hybrid analyses have indicated that not all theoretical Gβγ dimers exist, are equally expressed, or interact with Gα subunits, receptors, effectors, and downstream signaling factors (Hildebrandt 1997, Smrcka 2008b, Pronin and Gautam 1992a, Yan, Kalyanaraman, and Gautam 1996b, Robishaw and Berlot 2004, Schwindinger et al. 2003a, Schwindinger et al.

2004, Schwindinger et al. 2010b, Schwindinger et al. 2011a, Khan et al. 2013). Each Gβ and Gγ subunit shows widely varying affinities for one another (Smrcka 2008a, Stephens 2009, Hildebrandt 1997). While $G\beta_1$ and $G\beta_4$ dimerize with all Gγ subunits,$G\beta_2$ and $G\beta_3$ are unable to dimerize with $G\gamma_1$ and $G\gamma_{11}$(Dingus and Hildebrandt 2012a). $G\beta_2\gamma_1$ shows a stronger association than $G\beta_2\gamma_4$(Pronin and Gautam 1992b, Smrcka 2008a, Zhang et al. 2009). Different affinities between Gβ and Gγ subunits, in combination with the expression and localization of individual Gβ and Gγ subunit, may determine which Gβγ dimers are active in a given cell (Betty et al. 1998b). Interestingly, various Gβγ dimers have been reported to have different affinities to their effectors. $G\beta_1\gamma_2$ has a 40-fold higher affinity for SNARE and a 20-fold higher inhibition of exocytosis than $G\beta_1\gamma_1$(Blackmer et al. 2005). We speculate that the difference in the Gγ subunit, per se, or in the post-translation modification of $G\gamma_1$ and $G\gamma_2$ may cause the change in affinity. Such expression and affinity diversity of Gβ and Gγ subunits and the affinity of Gβγ-effector interactions may also suggest that specific dimers could permit specialized roles in signal transduction pathways through association with particular GPCRs. For example, $G\beta_2\gamma$ and $G\beta_4\gamma$ dimers may specifically 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(Hosohata et al. 2000, Asano et al. 1999, Kleuss et al. 1992). Thus, a greater understanding of the expression and subcellular localization of Gβ and Gγ subunits in the CNS will be particularly important in determining the physiologically relevant Gβγ dimers, the roles of each unique Gβγ dimer in regulating signaling cascades, and their impact in neurological diseases and GPCR targeted drug mechanisms.

Despite many attempts to quantify Gβ and Gγ protein level (Cali et al. 1992, Liang, Cockett, and Khawaja 1998, Zhang, Lai, and Simonds 2000, Morishita et al. 1998, Robishaw et al. 1989, Betke et al. 2014), it has been difficult to develop reliable subunit-specific detection methods due to the high sequence homology between subunits and lack of subunit-specific antibodies (Bigler et al. 1993). To overcome this issue, we previously developed a targeted multiple reaction monitoring (MRM)(Boja and Rodriguez 2012, Shi et al. 2012) mass spectrometric approach to identify neuronal Gβ and Gγ subunits. We found a regional and subcellular expression pattern of four Gβ ($G\beta_1$, $G\beta_2$, $G\beta_4$, and $G\beta_5$) and six Gγ ($G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_7$, $G\gamma_{12}$, and $G\gamma_{13}$) subunits in mouse crude synaptosomes of cortex, cerebellum, hippocampus, and striatum(Betke et al. 2014). However, we were unable to quantify and compare the expression level of each Gβ and Gγ subunit. Here, we use the quantitative MRM method of Gβ and Gγ subunits, in combination with the isotopic labeling of standards and Skyline analysis(Betke et al. 2014, MacLean et al. 2010), to generate a comprehensive, quantitative brain map of Gβ and Gγ subunits. We measured and compared the protein level and subcellular localization of neuronal Gβ and Gγ subunits and predicted the *in vivo* expression level of Gβγ dimers, further supporting the Gβγ specificity to particular GPCRs and effector proteins to maintain normal brain function.

2.2 Experimental Procedures

Synaptosome preparation. 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.

Crude synaptosomes were made from adult, male C57Bl6/J mice as described previously(Gray and Whittaker 1962, Whittaker, Michaelson, and Kirkland 1964, Betke et al. 2014). Briefly, whole brains were homogenized in 20 mL of a 0.32 M sucrose solution (0.32M sucrose, 4.2 mM HEPES pH 7.4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.95 μM pepstatin, and 200 μM PMSF). Homogenates were centrifuged at 1000 x g and 4°C for 10 min and supernatants containing synaptosomes (S1) were transferred to clean conical tubes. Pellets were resuspended in 20 mL of 0.32 M sucrose solution and centrifuged again. Pellets were discarded. Supernatants (S1) were combined and centrifuged at 10,000g and 4°C for 20 min to produce the crude synaptosome pellet. Crude synaptosomes were stored at -80°C.

Synaptosome lysate. Crude synaptosomes were gently resuspended in 4 mL of RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.948 μM pepstatin, and 200 μM PMSF) using a 25 gauge needle to lyse membranes. Lysate concentrations were determined with a BCA assay kit (Pierce) and diluted to 1 mg/mL using RIPA buffer. Diluted homogenate was placed on a rotator for 1 hr and maintained at 4°C. Homogenates were transferred to 2 mL Eppendorf and centrifuged at 14,000 rpm and 4°C for 10 min to separate the triton-soluble and insoluble fractions. Supernatants, the triton-soluble fractions, were collected. Protein concentrations were determined with a BCA assay kit (Pierce).

Subcellular fractionation. Subcellular fractions were prepared as previously described(Betke et al. 2014, Phillips et al. 2001) (Fig. 12). Briefly, crude synaptosomes were gently resuspended in 4 mL of hypotonic lysis buffer (20 mM Tris pH 6.0, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.95 μM

pepstatin, and 200 μM PMSF) and incubated on ice for 20 min to lyse membranes. Lysates were subjected to ultracentrifugation at 100,000 x g and 4°C for 2 hrs using a SW-55 Ti rotor (Beckman Coulter) to separate supernatants consisting of the synaptosomal cytosolic fractions from membrane fractions. Synaptosomal cytosolic fraction may contain crude vesicles(Lobur, Kish, and Ueda 1990). Supernatants were transferred to clean conical tubes. Pellets containing membrane fractions were resuspended in 2 mL of Tris pH8.0 buffer (20 mM Tris pH 8.0, 1% Triton X-100, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.95 μM pepstatin, and 200 μM PMSF) and incubated on ice for 20 min. Lysates were centrifuged at 10,000 x g and 4°C for 30 min and supernatants containing enriched presynaptic fractions were collected. Finally, pellets were resuspended in 400μL of a 1×PBS/ 0.5% SDS buffer and centrifuged at 10,000 x g and 4°C for 30 min. Supernatants containing enriched postsynaptic fractions were collected. Pellets were discarded. Protein concentrations of each fraction were determined with a BCA assay kit (Pierce).

Antibodies. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, MAB374, 1:20,000), mouse anti-SNAP25 (Santa Cruz, sc-376713, 1:500), mouse anti-syntaxin-1 (Santa Cruz, sc-12736, 1:2,000), mouse anti-N-methyl-D-aspartate receptor-1 (NMDAR1) (BD Pharmingen, 556308, 1:2,000), mouse anti-postsynaptic density-95 (PSD-95) (Neuromab, 75-028, 1:20,000), and rabbit anti-Gβ (Santa Cruz, sc-378, 1:15,000) were used. HRP-conjugated secondary antibodies were obtained from Perkin-Elmer and Jackson Immunoresearch and used at the following dilutions: goat anti-rabbit (1:20000), goat anti-mouse (1:10,000 for NMDAR1 and syntaxin, and 1:20,000 for PDS-95, GAPDH, and SNAP25) and mouse anti-rabbit light chain specific 1:7,500 (Gβ).

Immunoblot Analysis. To examine the fractionation of crude synaptosomes, western blot analysis was performed on 7 μg of synaptosomal cytosolic, presynaptic, and postsynaptic

fractions as described(Betke et al. 2014).

Protein purification of Gβγ dimers. $G\beta_1\gamma_1$ was purified from the bovine retina as described(Mazzoni, Malinski, and Hamm 1991b). Recombinant His_6-tagged $G\beta_5\gamma_2$ were expressed in Sf9 cells and purified using nickel-nitrilotriacetic acid affinity chromatography (Sigma-100 Aldrich, St. Louis, MO). Human $G\beta_1\gamma_2$, containing an N-terminally hexahistidine tagged β subunit, was expressed in High Five cells using a dual promoter insect cell expression vector described previously(Waldschmidt et al. 2016). $G\beta_1\gamma_2$ was purified from membrane extracts of High Five cells harvested 48 hrs post infection using nickel-nitrilotriacetic acid affinity, and anion exchange chromatography as described previously(Kozasa 2004). Fractions containing $G\beta_1\gamma_2$ were subsequently pooled and buffer exchanged into 20 mM HEPES pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 2 mM $MgCl_2$ and 1 mM DTT using a S200 column. $G\beta_1\gamma_2$ fractions were then concentrated to 5 mg/mL, as determined by Bradford analysis, in a 30kD cut-off Amicon Ultra-15 Centrifugal Filter Unit, flash frozen in liquid nitrogen, and stored at -80 ˚C until future use.

Heavy labeled proteotypic peptides detection analysis. To examine the detection of each heavy labeled proteotypic peptide, we selected one heavy labeled peptide with the largest area under the curve from Betke et al.(Betke et al. 2014) study (Bold in Table 3), for each Gβ and Gγ subunit (Table 3). 0.04, 0.2, 0.4, 4, 20, 40, 100 fmol of each heavy labeled peptide were pooled and mixed with bovine serum albumin (BSA), analyzed by TSQ Vantage triple quadrupole mass spectrometry (Thermo Scientific), and quantified using Skyline(MacLean et al. 2010) (data not shown).

Heavy labeled peptide cocktail. Based on the amino acid sequence of tryptic digested proteins and fragment ion signal intensities in the previous study(Betke et al. 2014), we have selected two proteotypic peptides for four Gβ ($G\beta_1$, $G\beta_2$, $G\beta_4$, and $G\beta_5$) and six Gγ

($G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_7$, $G\gamma_{12}$, and $G\gamma_{13}$) subunits detected in crude synaptosomes. Proteotypic peptides listed in Table 3 were synthesized via SPOT synthesis (JPT Peptide Technologies). The arginine or lysine at the C-terminal of these peptides was isotopically labeled with heavy ^{13}C or ^{15}N with a trypsin cleavable Qtag to quantify (JPT). Heavy labeled peptides are 8-10 Da heavier without changing the physiological properties and chemical reactivity compared to the non-labeled proteolytic peptides. One nmol of each peptide was resolubilized in 200 µL HPLC grade water, 200 µL of 10% acetonitrile to make a stock concentration of 5 pM/µL. According to the mass spectrometry signal strength of each proteotypic peptide, different amount of peptides were pooled to create a "heavy labeled peptide cocktail." The cocktails were mixed with BSA, 1.5 M Tris, and HPLC grade water before being reduced with 2.5 mM TCEP for 30 min and alkylated with 5 mM iodoacetamide for 30 min in the dark. The cocktail was then digested overnight with 0.5 mg of trypsin then stored at -80°C. Before each MRM analysis, the cocktail was acidified with 1.5 µL formic acid and added to samples as internal standards for quantification.

Quantitative MRM of Gβ and Gγ subunits. As shown in Fig. 9, samples containing Gβ and Gγ subunits were separated by 12.5% acrylamide, SDS-PAGE and stained with colloidal Coomassie Blue (Invitrogen). Using $G\beta_1\gamma_1$ and $G\beta_5\gamma_2$ as markers, Gβ and Gγ bands were excised and in-gel digested as described(Betke et al. 2014), and then resolubilized in the presence of the "heavy labeled peptide cocktail" and run on a TSQ vantage triple quadrupole mass spectrometer (Thermo Scientific) following the scheduled multiple reaction monitoring method as described(Betke et al. 2014). Gβ and Gγ subunits were run separately and data were analyzed in Skyline. Correct peaks were manually

chosen on the basis of retention time, dot plot values, relative distributions of transition ion, and heavy labeled peptide peaks. Samples were dropped from analysis if no correct peaks could be chosen. Furthermore, all chosen peaks with a signal-to-noise (S/N) ratio less than 5 for non-labeled peptides and 3.5 for labeled peptides were removed from further analysis. Using the light to heavy peak intensity ratio given in Skyline, the total volume of re-solubilized samples, injection volume, and the amount of heavy label peptide, Gβ and Gγ subunits present in samples were calculated. Quantified Gβ and Gγ subunits were then normalized by the total protein amount to account for variabilities between animals.

Validation of quantitative Gβ and Gγ subunits MRM. $G\beta_1\gamma_2$ was serially diluted, separated by 12.5 % acrylamide SDS-PAGE, trypsin digested, mixed with heavy labeled peptide cocktail used in crude synaptosome studies, and run on TSQ with the quantitative MRM method as described in Fig. 10. The heavy labeled peptide was made with $G\beta_1$ amino acids 198-209, and 284-301, and $G\gamma_2$ amino acids 21-27 and 47-62. As described above, we calculate the $G\beta_1$ and $G\gamma_2$ detected per peptide using the light/heavy ratio in Skyline. The light to heavy ratio represents a ratio between the peak intensity of light and heavy proteotypic peptides. The ratio given in Skyline was timed with the amount of heavy label peptides and divided by the percent injection volume to calculate the amount of $G\beta_1$ or $G\gamma_2$ present in each sample tube. Then, the quantified $G\beta_1$ and $G\gamma_2$ by each proteotypic peptide (square or circle) were averaged and depicted as triangle in Fig. 10. The result was plotted in logarithmic scales and analyzed by linear regression to determine the limit of quantification and to assess the analytical reliability.

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).

Table 3. List of heavy labeled proteotypic peptides

Name	Sequence position	Peptide Sequence	Precursor m/z	charge	Product ion m/z
$G\beta_1$	**198-209**	(R)LFVSGACDASAK(L)	617.3048	2	487.23, 787.35, 874.38, 973.45
	284-301	(R)LLLAGYDDFNCNVWDALK(A)	1068.0215	2	640.35, 898.40, 954.94, 1127.54
$G\beta_2$	**198-209**	(R)TFVSGACDASIK(L)	632.3101	2	508.25, 829.40, 916.43, 1015.50
	257-280	(R)ADQELLMYSHDNIICGITSVAFSR(S)	917.4432 / 922.7749	3	419.23, 490.26, 676.37, 777.41, 949.52
$G\beta_4$	**198-209**	(R)TFVSGACDASSK(L)	619.2841	2	495.23, 675.29, 803.34, 890.38, 989.44
	305-314	(R)SGVLAGHDNR(V)	518.2639 / 345.8450	2 / 3	340.16, 396.70, 446.24, 474.75, 608.28, 679.31, 792.40
$G\beta_5$	**129-139**	(K)VIVWDSFTTNK(E)	659.3501	2	705.37, 820.39, 1006.47, 1105.54
	318-327	(R)VSILFGHENR(V)	591.3187	2	498.27, 622.29, 769.36, 882.45
$G\gamma_2$	21-27	(K)MEANIDR(I)	429.7043 / 437.7018	2	527.28, 598.32, 727.36
	47-62	(K)EDPLLTPVPASENPFR(E)	896.4612	2	774.43, 927.46, 1123.58, 1224.62
$G\gamma_3$	3-17	(K)GETPVNSTMSIGQAR(K)	779.3819 / 787.3793	2	441.24, 554.33, 635.83, 643.82, 686.35,694.35, 960.48, 1074.52, 1090.52
	25-31	(K)IEASLCR(I)	429.7225	2	458.24, 545.27, 616.31, 745.35
$G\gamma_4$	3-17	(K)EGMSNNSTTSISQAR(K)	796.8641 / 804.8615	2	471.25, 584.34, 671.37, 873.47, 960.50
	51-66	(R)EDPLIIPVPASENPFR(E)	902.4794	2	927.46, 1123.58, 1236.66
$G\gamma_7$	**19-25**	(R)IEAGIER(I)	399.2232	2	484.28, 555.31, 684.36
	45-60	(R)NDPLLVGVPASENPFK(D)	852.9560	2	738.42, 897.46, 1053.55, 1152.61
$G\gamma_{12}$	5-15	(K)TASTNSIAQAR(R)	565.2954	2	655.38, 769.42, 957.50
	23-29	(R)LEASIER(I)	414.2285	2	514.29, 585.32, 714.37
$G\gamma_{13}$	**18-23**	(K)YQLAFK(R)	389.2229	2	373.23, 486.32, 614.38
	37-44	(K)WIEDGIPK(D)	483.2627	2	252.18, 422.29, 537.31, 666.35

Red highlighted arginine and lysine residues were heavy labeled by ^{13}C or ^{15}N. Bolded sequence position indicates proteotypic peptides with the strongest fragmentation ion intensity. These peptides were used for the heavy labeled proteotypic peptides detection analysis (data not shown).

2.3 Results

Development of the quantitative multiple reaction monitoring (MRM) method for Gβ and Gγ subunits. To develop quantitative MRM method for neuronal Gβ and Gγ subunits, we first selected two proteotypic peptides for four Gβ ($G\beta_1$, $G\beta_2$, $G\beta_4$, and $G\beta_5$) and six Gγ ($G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_7$, $G\gamma_{12}$, and $G\gamma_{13}$) subunits from previous study (Betke et al. 2014) and had heavy labeled versions of them synthesized (Table 3) (see Material and Method for more details). Non-neuronal Gβ and Gγ subunits, such as $G\beta_3$, were not examined in this study. Two proteotypic peptides were selected per protein (see Material and Method for more detail). No proteotypic peptides were selected for $G\gamma_5$ and $G\gamma_{11}$ as we were unable to identify a correct peak of sufficient signal intensity to pass acceptable signal to noise criteria in a previous study(Betke et al. 2014).

To understand the detection intensities and fragmentation efficiency of each heavy labeled proteotypic peptide, for each protein, we performed scouting runs to determine appropriate amounts of heavy labeled proteotypic peptide (data not shown). As expected, each heavy labeled peptide had different fragmentation ion signal intensity. Based on these results and previous targeted mass spectrometry experiments(Betke et al. 2014), we pooled appropriate concentration of heavy labeled peptides to make a heavy peptide cocktail that was digested with trypsin in the presence of BSA. This cocktail was added to all Gβ and Gγ subunits samples after trypsin digestion and extraction from gel matrix. Using the light to heavy ratio calculated by the area under the curve (Betke et al. 2014) of the sample and of heavy labeled peptides, we can quantify the protein level of neuronal Gβ and Gγ subunits present in the sample (Fig. 9).

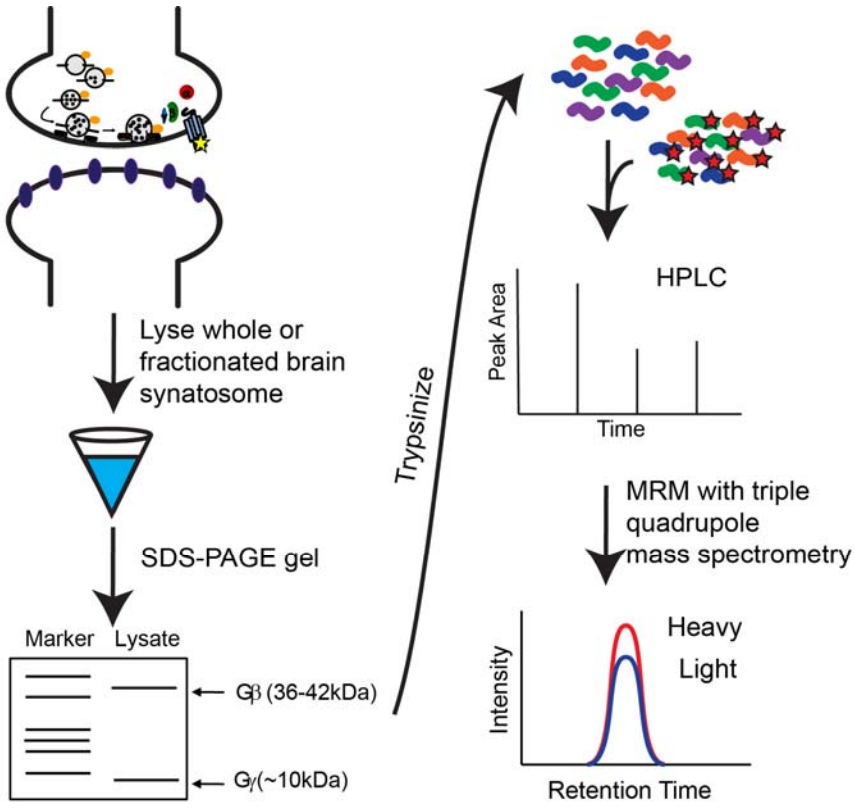

Figure 9. The workflow of quantitative multiple reaction monitoring (MRM) experiment.

Lysed whole or fractionated **s**ynaptosomes from wild-type adult mice were run on an SDS-PAGE gel to separate Gβ and Gγ subunits. Then, Gβ and Gγ subunits bands were excised, digested with trypsin, and resuspended with the heavy peptide cocktail (red star). Samples were injected to HPLC connected to Triple quadrupole mass spectrometer with the quantitative MRM method. The ratio of non-labeled proteotypic peptide (light) and heavy labeled proteotypic peptide was used to calculate the amount of Gβ and Gγ subunits present in samples. See results and experimental procedures sections for the detail descriptions.

Validation of quantitative MRM of Gβ and Gγ subunits. We next applied our method to examine serially diluted $G\beta_1\gamma_2$ (0.002 - 21.9 pmol) to determine the limit of detection, limit of quantification, and analytical accuracy of the method. Because $G\beta_1\gamma_2$ is the most abundant, easily expressed, and most commonly studied neuron Gβγ dimer, we chose it as our standard for validation. We plotted the quantification value per peptide (Gray and Whittaker) and averaged (black) against the fmol of $G\beta_1\gamma_2$ used. Here, we identified the limit of $G\beta_1$ and $G\gamma_2$ detection at 5.34 and 21.4 fmol of $G\beta_1\gamma_2$, respectively. Below the limit of detection, no peak passed the signal to noise test (see Material and Method for more details). The limit of $G\beta_1$ and $G\gamma_2$ quantifications were found at 85.5 and 42.7 fmol of $G\beta_1\gamma_2$ (Fig. 10). Because 42.7 fmol sample was excluded from the $G\beta_1$ quantification analysis, and 21.4 and 85.5 fmol samples had similar $G\beta_1$ level detected, we measured the limit of $G\beta_1$ quantification at 85.5 fmol (Fig. 10A). For all input quantities above the limit of quantification, we observed a linear increase in the amount of detected protein. Below the limit of quantification, the relationship between input and quantified protein was not linear. Using multiple proteotypic peptides for quantification has been shown to increase the confidence in quantification(Bantscheff et al. 2007). Although we detect and quantify $G\gamma_2$ between 42.7 to 342 fmol using one heavy labeled proteotypic peptide, we are confident with the $G\gamma_2$ quantification in this range (Fig. 10B). Moreover, a linear regression test confirmed that the detected and quantified $G\beta_1$ and $G\gamma_2$ with two heavy labeled proteotypic peptides were proportional to the $G\beta_1\gamma_2$ input (Fig. 10). Based on the slopes of these curves, we determined that our method systematically over-estimates the amount of $G\beta_1$ by ~3 fold (Fig. 10A) whereas we under-estimate the amount of $G\gamma_2$ by ~90 fold (Fig. 10B). Although we attempted to provide a comprehensive absolute quantitative map, our

data may not be absolute due to technical challenges associated with sample preparations. The presence of heavy labeled proteotypic peptides allows for quantification of non-labeled proteotypic peptides derived from proteolytic digestion of the target proteins. However, several factors impact whether this quantification reflects an accurate measure of the absolute quantity of the target protein. Differences in peptide yield due to sample preparation artifacts, trypsin efficiency, and post–translational modifications along with differences in peptide resolubilization efficiencies and stability can lead to systematic errors in quantification(Hoofnagle et al. 2016, Cook et al. 2006). For example, the use of SDS in the postsynaptic fraction`s resolubilization may affect the recovery and quantification of postsynaptic Gγ subunits (see Discussion for more detail). Because of these, we are unable to infer absolute protein level of neuronal Gβ and Gγ subunits, but we can accurately determine the expression pattern of neuronal Gβ and Gγ subunits in any given sample.

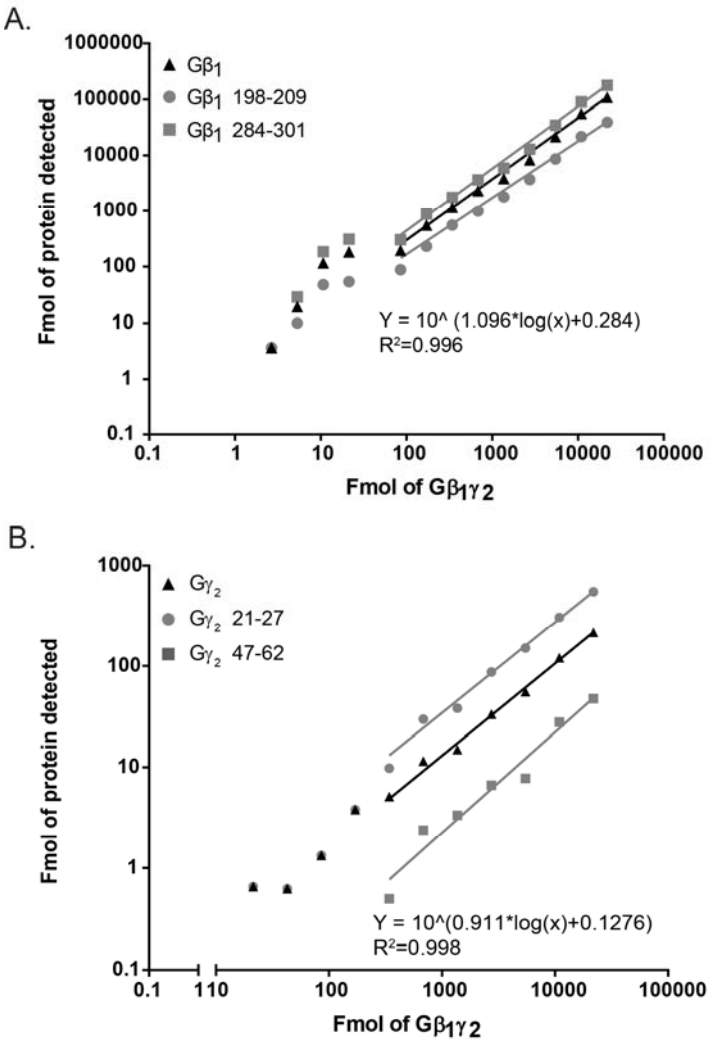

Figure 10. Quantification of $G\beta_1$ and $G\gamma_2$.

To validate the quantitative MRM of Gβ and Gγ subunits, 14 samples containing serial dilution of purified $G\beta_1\gamma_2$ dimer were examined for the quantification of $G\beta_1$ (**A**) and $G\gamma_2$ (**B**). After the signal to noise test, 13 and 11 samples were quantified for $G\beta_1$ (**A**) and $G\gamma_2$ (**B**), respectively. The subunit $G\beta_1$ (**A**) and $G\gamma_2$ (**B**) were quantified based on $G\beta_1$198-209 (circle in A) and 284-301(square in A) and $G\gamma_2$ 21-27 (circle in B) and 47-62 (square in B). These two proteotypic peptides per subunits were averaged and indicated as triangles (See Material and Method for more details). For $G\beta_1$, R^2 were 0.995 ($G\beta_1$198-209) and 0.997 ($G\beta_1$ 284-301). For $G\gamma_2$, R^2 were 0.982 ($G\gamma_2$ 21-27) and 0.999 ($G\gamma_2$ 47-62). The linear range represents the analytical reliability of the quantitative MRM method (R^2 of averaged =

0.996 (**A**) and 0.998 (**B**)).

The most abundant neuronal Gβ and Gγ subunits in brain synaptosomes. Using our quantitative MRM method, we first determined the protein level of each Gβ and Gγ subunit in crude synaptosomes (N=3) derived from whole mouse brain (Fig. 11). In ~13 mg of crude synaptosome lysate, we found $G\beta_1$ to be the most abundant neuronal Gβ subunit (Fig. 11A) supporting the previous finding(Okae and Iwakura 2010). $G\beta_4$ and $G\beta_5$, were the least abundant Gβ subunits in mouse crude synaptosomes (Fig. 11B). Moreover, $G\gamma_2$ is the most abundant Gγ subunit. $G\gamma_{13}$ was the least abundant Gγ subunit. $G\gamma_2$ was detected at 5.4 fold higher levels than $G\gamma_{13}$ (Fig. 11C). In crude synaptosomes, the level of expression for Gβ subunits was $G\beta_5$=$G\beta_4$<$G\beta_2$<$G\beta_1$, and for Gγ subunits was $G\gamma_{13}$=$G\gamma_4$<$G\gamma_7$<$G\gamma_{12}$<$G\gamma_3$<$G\gamma_2$. For the first time, we compared the protein level between each Gβ and Gγ subunits. This is important for estimating the quantity of each Gβγ dimer *in vivo*. For example, $G\beta_1\gamma_2$ may be one of the most abundant Gβγ dimers *in vivo.* Its interactions with receptors and effectors may be more physiologically relevant than those between $G\beta_4\gamma_4$ or $G\beta_4\gamma_{13}$ and their effectors.

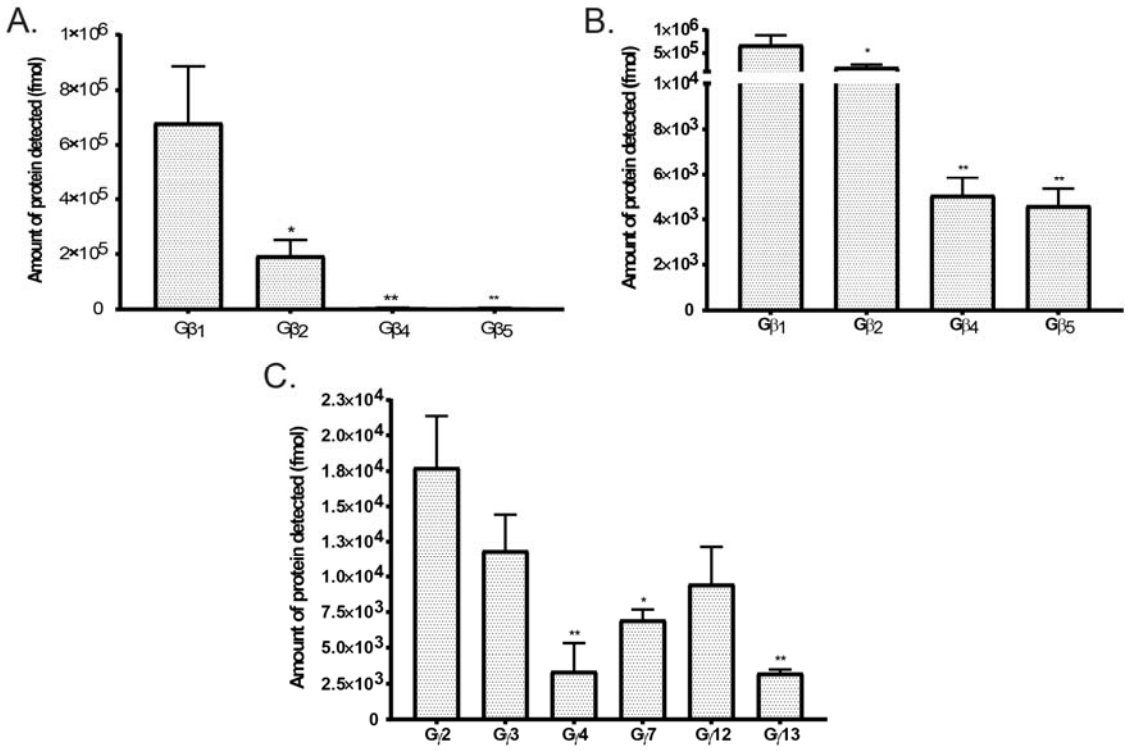

Figure 11. The protein level of Gβ and Gγ subunits in whole crude synaptosomes.

Quantification of Gβ subunits (**A and B**) and Gγ subunits (**C**) in ~13 milligrams (mg) of lysate (N=3). Sub-view at 1-10,000 fmol (**B**). Gβ1 was the most abundant Gβ subunit (P=0.040 compared to $G\beta_2$, and 0.007 compared to $G\beta_4$ and $G\beta_5$) when $G\gamma_2$ might be the most dominant Gγ subunit (P=0.050 compared to $G\gamma_7$, and 0.008 compared to $G\gamma_4$ and $G\gamma_{13}$). Data were presented as mean ± SEM and compared by a one-way ANOVA, *P<0.05, **P<0.01. *Post hoc* analysis was performed with Tukey's multiple comparison test.

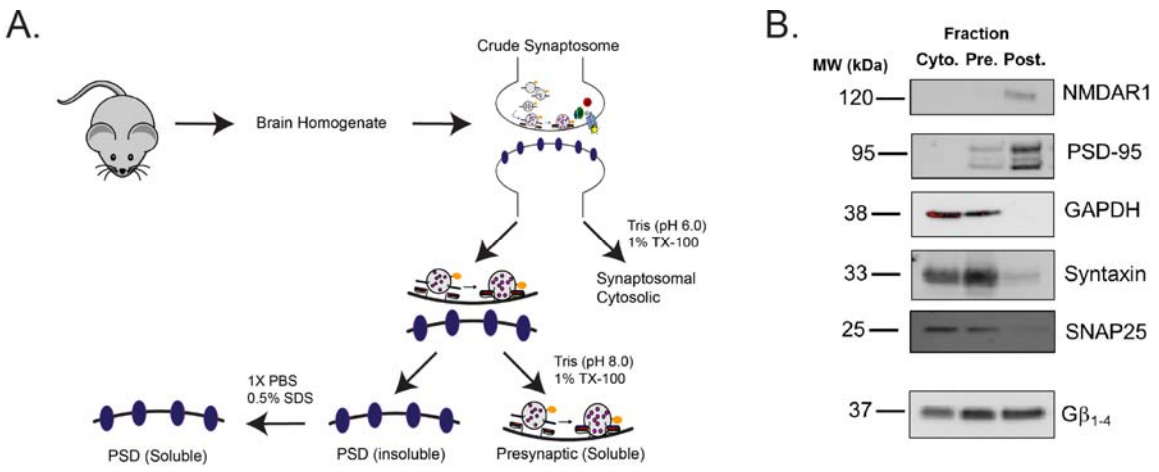

Figure 12. Subcellular fractionation of whole crude synaptosomes.

The workflow of fractionation experimental procedures (**A**), and representative western blot of fractionated samples (**B**). GAPDH was dominant in a synaptosomal cytosolic fraction. Syntaxin-1 and SNAP25 were dominant in a presynaptic fraction when postsynaptic density 95 (PSD-95) and NMDAR1 were dominant in a postsynaptic fraction. $G\beta_{1-4}$ were detected in synaptosomal cytosolic, pre-, and post-synaptic (PSD) fractions but concentrated at the membrane fractions.

Subcellular localization of Gβ and Gγ subunits. Because Gβγ dimers interact with a variety of effectors distributed throughout the cell, such as voltage gated calcium channels, potassium channels, and the SNARE complex(Herlitze et al. 1996, Huang et al. 1995, Blackmer et al. 2001, Gerachshenko et al. 2005, Yoon et al. 2007, Currie 2010, Sadja and Reuveny 2009, Wells et al. 2012), subcellular location of Gβγ dimers may also influence their effector selectivity. Here, we attempted to determine the subcellular localization of Gβ and Gγ subunits (N=8, otherwise noted in Fig. 13). Crude synaptosomes of wildtype mice were separated into synaptosomal cytosolic, presynaptic, and postsynaptic fractions as described previously (Betke et al. 2014, Phillips et al. 2001) (Fig. 12A). GAPDH was dominant in the synaptosomal cytosolic fraction. Syntaxin-1 and SNAP25 were dominant in a presynaptic fraction whereas postsynaptic density 95 (PSD-95) and NMDAR1 were dominant in a postsynaptic fraction (Fig.12B), confirming the efficacy of our fractionation method. In these fractions, we examined the protein levels of each Gβ and Gγ subunit. $G\beta_1$ was highly localized at the membrane, found in both pre- and post-synaptic fractions, whereas $G\beta_2$ was evenly distributed between all three fractions (Fig. 13A). Supporting the previous findings, both $G\beta_4$ and $G\beta_5$ were highly localized in the postsynaptic fraction (Fig. 13B)(Betke et al. 2014). Overall, more Gβ subunits were detected in membrane fractions than the synaptosomal cytosolic fraction (Fig. 12B and 13).

Gγ subunits also showed divergent localization patterns. The comparison was made between Gγ subunits in each fraction (Fig. 13C-E) and between the synaptosomal cytosolic and presynaptic fractions (Fig. 13C-D) to account for the potential differences in resolubilization, impacting the recovery and quantification of postsynaptic Gγ subunits (see Discussion for more detail). In the synaptosomal cytosolic fraction, $G\gamma_2$ and $G\gamma_3$ seem to be

abundant although no statistical significance was found (Fig. 13C). $G\gamma_2$ and $G\gamma_3$ may preferentially localize at the synaptosomal cytosolic fraction than the presynaptic fraction, P=0.074 and 0.076 (unpaired T-test) respectively. In the presynaptic fraction, $G\gamma_{12}$ might be the most abundant Gγ subunit while $G\gamma_4$ and $G\gamma_{13}$ were the least abundant Gγ subunits (Fig. 13D). Between the synaptosomal cytosolic and presynaptic fractions, $G\gamma_7$ showed no preference in a location, p= 0.866. In the postsynaptic fraction, $G\gamma_{12,}$ once again, is the most abundant Gγ subunits (Fig. 13E). Interestingly, $G\gamma_4$ and $G\gamma_{13}$ were also the least abundant Gγ subunits in the postsynaptic fraction (Fig. 13E). Although $G\gamma_2$ was one of the most abundant Gγ subunits in the crude synaptosomes, it was not the most abundant Gγ subunit in the presynaptic fraction where widely known Gβγ effectors are located.

Unlike Gβ subunits, more Gγ subunits were found in the postsynaptic fraction than in the synaptosomal cytosolic fraction. Although we do not fully understand this phenomenon, we hypothesize that this may be due to the difference in re-solubilization buffers used for pre- and post-synaptic fractions as stated earlier (see Discussion for more detail).

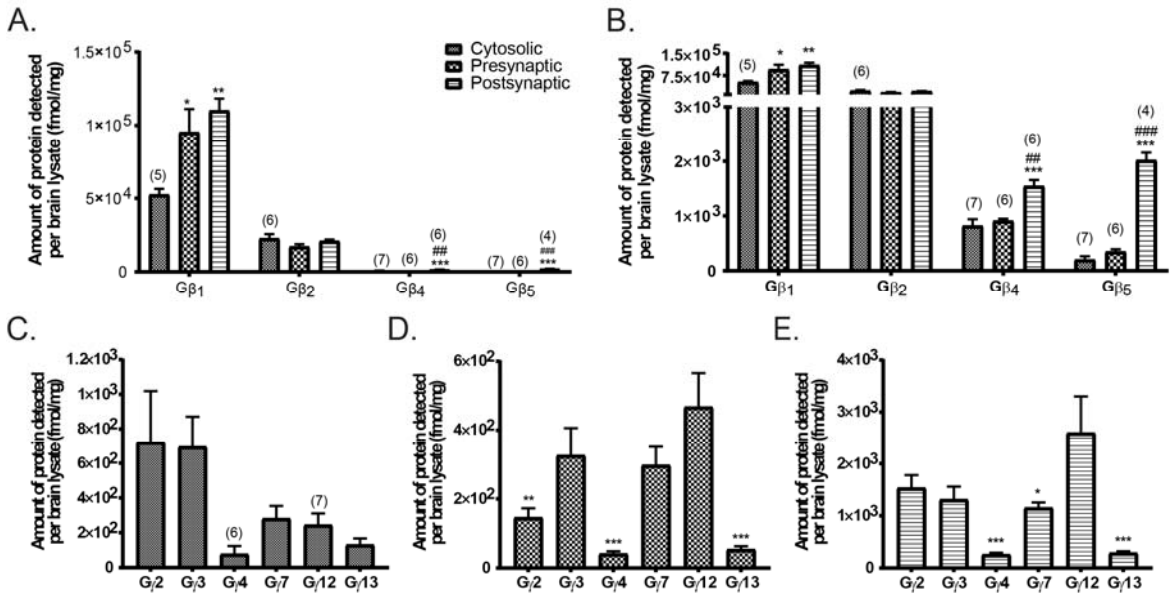

Figure 13. Subcellular localization of Gβ and Gγ subunits.

Quantification of Gβ subunit per mg of protein lysate (**A and B**) (N=8, otherwise noted). Sub-view at 1-3,000 fmol (**B**). (**C-E**) Quantification of Gγ per mg of protein lysate in synaptosomal cytosolic (**C**), presynaptic (**D**), and postsynaptic fractions (**E**) (N=8, otherwise noted). Data were presented as mean ± SEM and compared by a one-way ANOVA, $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$, $^{***,\#\#\#}P<0.001$. *Post hoc* analysis of Tukey`s multiple comparison test was performed. Statistical significances were found compared to synaptosomal cytosolic * and presynaptic$^{\#}$ fractions (**A and B**), and $G\gamma_{12}$ (**C -E**).

2.4 Discussion

Quantitative MRM of Gβ and Gγ subunits. Because no dependable subunit-specific antibodies exist due to high sequence homology between subunits, studies of Gβ and Gγ subunits at protein level have been limited. In this study, we present a method to perform a comprehensive, quantitative survey of Gβ and Gγ subunits in crude synaptosomes. With heavy labeled proteotypic peptides cocktails, we quantified and compared the protein expression level of each Gβ and Gγ subunit in whole and fractionated crude synaptosomes. Although our quantification method may only measure a subset of actual neuronal Gβ and Gγ subunits present in crude synaptosomes due to technical challenges stated below, our method can now be applied to further understand and model how the expression and subcellular neuronal localization of Gβ and Gγ subunits regulate dimerization and affect signaling pathways (Betke et al. 2014, Wojcik and Brose 2007, Blackmer et al. 2001). As the sequences of Gβ and Gγ subunits are highly conserved in mammals, the proteotypic peptides used in this method are conserved in human Gβ and Gγ subunits, thus our method is suitable to evaluate Gβγ expression in human tissues.

Sample preparation, detection intensity, and fragmentation efficiency of each proteolytic peptide may impact the detected quantity of Gβ and Gγ subunits. We enhanced the confidence in detection and quantification of all neuronal Gβ and Gγ subunits by using two heavy labeled proteotypic peptides per protein. Non-neuronal Gβ and Gγ subunits, such as $G\beta_3$, and those with insufficient proteotypic peptides, such as $G\gamma_5$, were not studied. Although $G\gamma_5$ was observed to be the most abundant Gγ subunit in a neuronal cell line(Kilpatrick and Hildebrandt 2007), we were not able to quantify $G\gamma_5$. Further study will be needed to identify proteotypic peptides in regions that are not post-translationally

modified and characterize the quantity and subcellular localization of $G\gamma_5$ in the brain. Each heavy labeled proteotypic peptide showed a different AUC demonstrating the importance of adjusting individual peptide amount for quantification (data not shown). Because we didn`t know the amount of non-labeled proteotypic peptides present in samples, we relied on previous data and detection of heavy labeled peptide alone to create an appropriate heavy labeled cocktail (data not shown)(Betke et al. 2014, Bantscheff et al. 2007). We were able to further adjust the heavy peptide cocktail for the subcellular localization study using the results of whole crude synaptosomes.

Purifying and creating validation curves for all neuronal Gβ and Gγ subunits was impractical due to reagent limitations, so instead we used $G\beta_1\gamma_2$, which is the most widely used and physiologically relevant Gβγ dimer, to validate our quantitative MRM method (Fig.10). As stated above, each proteotypic peptide has different physicochemical properties and is thus difficult to validate the quantification of neuronal Gβ and Gγ subunits with a $G\beta_1\gamma_2$ standard alone. However, since all neuronal Gβ subunits and all neuronal Gγ subunits were sampled together, we can extrapolate the experimental error of the quantification method. For example, any experimental manipulation affecting the proteotypic peptides of $G\beta_1$ likely affects the proteotypic peptides of the other neuronal Gβ subunits. Any systematic manipulation likely impacted all samples to a similar degree and thus accurately reflects the underlying expression pattern (Fig.10). Instead of serial diluting the amount of heavy labeled $G\beta_1$ and $G\gamma_2$ proteotypic peptides, we also used the heavy peptide cocktail of the subcellular localization study to have a better estimate of quantification error relevant to our experimental design. Because our quantitative method depends on the peak intensity and peak intensity ratio of light and heavy proteotypic

peptides, the experimental error may vary by the amount of heavy labeled proteotypic peptides present in the samples(Hoofnagle et al. 2016). It is important to predict the error with heavy labeled cocktail used in each experiment. Until we fully identify every Gβγ dimer present in the brain, we have determined that this is the best way to validate our quantitative method.

Overall, our data suggest that we underestimate the detection and quantification of neuronal Gγ subunits. The limit of $G\gamma_2$ detection was higher than $G\beta_1$ (Fig.10B). We also calculated a higher error in absolute quantification of $G\gamma_2$ compared to $G\beta_1$ (Fig.10B). Because Gγ subunits are ~8 kDa, we may experience a greater loss of Gγ than Gβ subunits in sample preparation. It is known that Gγ subunits are post-translationally modified, which may further complicate their detection. In validation experiments, the quantification of $G\gamma_2$ between 42.7 to 342 fmol was done using one heavy labeled proteotypic peptide because the 2nd peptide didn`t pass the signal to noise criteria (Fig. 10B). A previous study(Betke et al. 2014) showed that this peptide has higher signal intensity than the other peptide in lending us confidence in the $G\gamma_2$ quantification in this range (Fig. 10B). Despite these technical challenges, our quantitative MRM method is a valid and reliable method to quantify and compare the different protein levels of neuronal Gβ and Gγ subunits.

Expression and subcellular localization of Gβ subunits in brain synaptosomes. Similarly to previous studies, we determined that $G\beta_1$ was the most abundant Gβ subunit in whole crude synaptosomes(Betke et al. 2014, Betty et al. 1998b, Liang, Cockett, and Khawaja 1998). The expression of $G\beta_1$ was 3.5 fold more than $G\beta_2$ and ~130 fold more than $G\beta_4$ and $G\beta_5$ (Fig. 11A and B) supporting a critical role for $G\beta_1$ in Gβ subunit

signaling in processes in the brain. $G\beta_1$ is involved in neural development; its knockout leads to perinatal lethality with reduced cortical thickness, brain volume, and impaired neural progenitor cell proliferation(Okae and Iwakura 2010). Mutations in $G\beta_1$ are also found in patients with severe neurodevelopmental disability, hypotonia, and seizures(Petrovski et al. 2016). Compared to $G\beta_1$, significantly fewer $G\beta_4$ and $G\beta_5$ are available for dimerization in the brain (Fig.11B). Taken the result of previous study(Betke et al. 2014) in tandem with our quantitative data, $G\beta_5$ is the least abundant Gβ subunit present only in the striatum.

Our results may provide a partial rationale for Gβγ selectivity for effectors because Gβγ dimers containing $G\beta_1$, concentrated in presynaptic and postsynaptic fractions (Fig. 13A), may preferentially activate membrane bound effectors such as VDCC compared to Gβγ dimers containing $G\beta_2$. Dimers with $G\beta_1$ strongly inhibit VDCC activity(Arnot et al. 2000) compared to $G\beta_2$ and may affect VDCC activity in pain, Parkinson`s disease, and epilepsy(Simms and Zamponi 2014). Moreover, $G\beta_4$ and $G\beta_5$ were largely restricted to postsynaptic fractions suggesting that dimers with these subunits may primarily interact with postsynaptic effectors. While postsynaptic effectors of Gβγ dimers containing $G\beta_4$ remain unknown, $G\beta_5$ was shown to specifically interact with the Gγ-like domain of regulator of G protein signaling (RGS)7 family proteins instead of Gγ subunits(Liang, Cockett, and Khawaja 1998, Zachariou et al. 2003, Lopez-Fando et al. 2005, Anderson et al. 2010, Anderson, Lujan, and Martemyanov 2009, Anderson et al. 2007, Psifogeorgou et al. 2011, Masuho, Xie, and Martemyanov 2013, Gold et al. 1997). These RGS 7 family proteins were found to affect dopamine signaling in striatum for motor learning and locomotor responses(Anderson et al. 2010),

supporting the phenotype of $G\beta_5$ knockout mice(Zhang et al. 2011, Chen et al. 2003). Significant loss of RGS 7 family members were also found in $G\beta_5$ knockout mice(Chen et al. 2003). Because RGS9-2 - $G\beta_5$ complex was found at the membrane, predominantly at the postsynaptic fraction(Anderson et al. 2007, Song, Waataja, and Martemyanov 2006), whereas RGS7-$G\beta_5$ complex was found in the cytosolic fraction(Anderson et al. 2010, Anderson, Lujan, and Martemyanov 2009, Anderson et al. 2007, Gold et al. 1997), further studies will be needed to elucidate the *in vivo* presence of RGS9-2-$G\beta_5$ complex over RGS7-$G\beta_5$. In addition, further studies with various Gγ subunits will be needed to understand and verify the *in vivo* ramifications of these Gβ subunit differences in Gβγ localization.

Expression and subcellular localization of Gγ subunits in brain synaptosomes. We determined that $G\gamma_2$ was one of the most abundant Gγ subunits in whole crude synaptosomes, supporting previous studies(Betty et al. 1998b, Betke et al. 2014). In addition to $G\gamma_2$, $G\gamma_3$, and $G\gamma_{12}$ were the most abundant in whole crude syanptosomes. Together with our Gβ result, this may support the hypothesis that $G\beta_1\gamma_2$ is one of the most abundant dimers present in whole crude synaptosomes. Although the *in vivo* role of $G\beta_1\gamma_2$ has not been fully characterized, we speculate that its abundant expression may make $G\beta_1\gamma_2$ vital to a wide range of signaling pathways. Based on our quantitative results, we have gained further insight into the quantity and location of the following Gβγ dimers *in vivo*. Because significantly less $G\gamma_4$ and $G\gamma_{13}$ were detected in whole crude synaptosomes, $G\beta_4\gamma_4$ and $G\beta_4\gamma_{13}$ may be the least abundant Gβγ dimers. Although $G\beta_1$ and $G\beta_2$ are known to interact with $G\gamma_4$(Dingus and Hildebrandt 2012b, McIntire 2009), the low abundance of

$G\gamma_4$ disfavors the formation of $G\beta_1\gamma_4$ and $G\beta_2\gamma_4$ dimers.

In subcellular localization study, we overall detected a higher level of Gγ subunits in the postsynaptic fraction than presynaptic fraction. We speculate that this may be due to the presence of SDS in the re-solubilization buffer of the postsynaptic fraction pellet. In presence of 0.5% SDS, which has higher critical micelle concentration than Triton X-100(Johnson 2013, Linke 2009) in the re-solubilization buffer, we may be enhancing the recovery of Gγ subunits in the postsynaptic fraction. To account for this, the comparison was made between Gγ subunits in each fraction (Fig. 13C-E) and between the synaptosomal cytosolic and presynaptic fractions only (Fig. 13C-D). In both synaptosomal cytosolic and presynaptic fractions, 1% Triton X-100 was used.

Although no statistical significant differences were found within the synaptosomal cytosolic fraction and between the synaptosomal cytosolic and presynaptic fractions, $G\gamma_2$ and $G\gamma_3$ seem to be localized at the synaptosomal cytosolic fraction (Fig. 13C). Of these Gγ subunits, $G\gamma_{12}$ was the only Gγ subunit that was more likely to be abundant in the presynaptic fraction than the synaptosomal cytosolic fraction ($p=0.092$). Interestingly, a recent epigenetic study found increased $G\gamma_{12}$ expression in cigarette smokers compared to non-smokers(Joehanes et al. 2016), which may link $G\gamma_{12}$ to nicotinic cholinergic signaling. $G\gamma_{12}$ abundance at the membrane may also be influenced by the phosphorylation of $G\gamma_{12}$ by protein kinase C(Lindorfer et al. 1998, Morishita et al. 1998), which anchors it to the membrane by enhancing its interaction with Gα subunits and the adenosine A_1 receptor(Asano et al. 1999, Morishita et al. 1998, Yasuda et al. 1998). Phosphorylation of $G\gamma_{12}$ was not monitored in this study; therefore, further studies will be needed to determine if $G\gamma_{12}$ phosphorylation is required for its abundance at the membrane. In addition to $G\gamma_{12}$,

$G\gamma_3$ and $G\gamma_7$ might be the most abundant Gγ subunits in the presynaptic fraction (Fig. 13D) while $G\gamma_2$ and $G\gamma_3$ might be in the postsynaptic fraction (Fig. 13E). Unlike $G\gamma_3$ and $G\gamma_{12}$ that were abundant in both fractions, $G\gamma_7$ may preferentially interact with presynaptic effectors. Similar to the data of whole crude synaptosomes, $G\gamma_4$ and $G\gamma_{13}$ were the least abundant Gγ subunits in both membrane fractions (Fig. 13 E).

Together with Gβ subunit data, we can further speculate which Gβγ dimers may be present in each fraction. For example, $G\beta_1\gamma_2$ and $G\beta_1\gamma_3$ are more likely to be present in the synaptosomal cytosolic fraction than in presynaptic fraction. Because $G\gamma_3$ preferentially interacts with $G\beta_1$ and $G\beta_2$, $G\beta_1\gamma_3$ and $G\beta_2\gamma_3$ may be the dominant dimers in the synaptosomal cytosolic fraction(Schwindinger et al. 2004). Within the pre- and postsynaptic fractions, $G\beta_1\gamma_{12}$ may be one of the most abundant dimers. Further *in vivo* study will be needed to verify the quantity of each dimer stated above.

Gβγ selectivity for GPCRs and effectors. To date, very little is known about the *in vivo* selectivity of Gβγ dimers for particular GPCRs and effector proteins. Various *in vitro* studies hint at possible Gβγ preferences(Yan and Gautam 1997, Yan, Kalyanaraman, and Gautam 1996a, McIntire, MacCleery, and Garrison 2001, Kleuss et al. 1993, Kisselev and Gautam 1993, Schwindinger et al. 2010b). For example, $G\beta_1\gamma_3$ may associate with somatostatin GPCRs while $G\beta_3\gamma_4$ dimers interact with M4 muscarinic GPCRs to inhibit L-type Ca^{2+} channel(Kleuss et al. 1993, Krumins and Gilman 2006). An increased susceptibility to seizures in $G\gamma_3$ knockout mice may be due to the loss of activity mediated by somatostatin GPCRs(Tallent and Qiu 2008, Schwindinger et al. 2004). In addition, $G\beta_2\gamma_2$ interacts with the galanin receptor to inhibit a Ca^{2+} channel(Kalkbrenner et al. 1995). The wide difference in expression patterns within subcellular fractions could reflect the

unique contribution of each Gβ and Gγ subunit to different signaling pathways(Betke et al. 2014). However, further functional *in vitro* and *in vivo* studies will be required to validate the presence of particular Gβγ dimers and their selectivity for certain GPCRs and effectors. As our current method can only detect specific Gβ and Gγ subunits separately, further method development is needed to identify proteotypic peptides of each Gβγ dimer as a whole. Taking advantage of transgenic mice with cell-type specific promoters and HA or FLAG tagged GPCRs, and overexpression of viral vectors expressing particular Gβγ dimers, we hope to further elucidate Gβγ selectivity for GPCRs and effectors in future studies.

2.5 Conclusion

Here, we addressed the protein level and subcellular localization of neuronal Gβ and Gγ subunits using the quantitative MRM mass spectrometry in whole and fractionated crude synaptosomes. Our study supports the previous findings that $G\beta_1$ is the most abundant Gβ subunit while $G\gamma_2$ is one of the most abundant Gγ subunits in whole crude synaptosomes. $G\beta_1$ was mostly localized at the membrane while $G\beta_2$ was evenly distributed throughout the membrane and synaptosomal cytosolic fractions. In addition to $G\gamma_2$, $G\gamma_3$ and $G\gamma_{12}$ were also abundant in whole crude synaptosomes. $G\gamma_3$ and $G\gamma_{12}$ were abundant in both pre- and post-synaptic fractions. We highlight the *in vivo* distribution of neuronal Gβ and Gγ subunits using a new quantitative tool and thus provide insights into the Gβγ dimers assembly in normal brain function. Although further efforts will be necessary to further improve the quantification strategies and evaluate the selectivity of Gβ and Gγ subunits to form Gβγ dimers, this work represents an important advance in the

field of GPCR signaling, especially the selectivity of Gβγ dimers formation. Because the expression, localization, and affinity of the Gβ and Gγ subunits have been hypothesized to affect Gβγ dimerization and Gβγ-effector interaction, our study will have a far reaching impact for those interested in the regulatory effects of Gβγ dimer specificity not only in physiology but also in disease pathology.

CHAPTER 3

THE SPECIFICITY OF Gβ AND Gγ SUBUNITS TO α_{2a} ADRENERGIC RECEPTOR

3.1 Introduction

The adrenergic receptor family is comprised of α_1, α_2, and β receptors (Strosberg 1993). Within the adrenergic α_2 receptor (α_2AR) family, the $\alpha_{2a,}$ α_{2b}, and α_{2c} subtypes are $G_{i/o}$-coupled GPCRs (Bylund et al. 1994, David B. Bylund 1988) and are widely distributed in the peripheral and central nervous systems (CNS)(Gilsbach and Hein 2012, Daunt et al. 1997). In the CNS, α_{2a}AR activity is involved in working memory, hypotension, bradycardia, sedation, analgesia, and hypnosis(Gilsbach and Hein 2012). Moreover, 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 (Gribble 2010, Comings et al. 2000, Wakeno et al. 2008). In addition, the dysregulation of α_{2a}ARs, increasing the norepinephrine, enhances fear memory and impairs spatial working memory (Davies et al. 2003, Marrs et al. 2005).

α_{2a}ARs are expressed in both adrenergic and non-adrenergic neurons, and located in both pre- and post-synaptic(Gannon and Wang 2016) terminals. Presynaptic α_{2a}ARs in adrenergic neurons are called autoreceptors (auto-α_{2a}ARs) while α_{2a}ARs in non-adrenergic neurons are called heteroreceptors (hetero-α_{2a}ARs) (Gilsbach and Hein 2012). 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 (Gyires et al. 2009, Gilsbach and Hein 2012, Szabadi 2013, Gobert et al. 2004, Berridge and Waterhouse 2003). The disperse expression of α_{2a}ARs suggests multiple roles for these receptors in biological and physiological functions.

The physiological implications of α_{2a}ARs are further studied using α_{2a}AR agonists and various mouse models such as Hemagglutinin (HA)-α_{2a}AR knock-in (HA-α_{2a}AR) and FLAG-α_{2a}AR transgenic mice. HA-α_{2a}AR mice are generated utilizing a homologous recombination gene targeting strategy to express HA-α_{2a}ARs into the mouse endogenous *ADRA2A* gene locus (Fig. 14A) (Lu et al. 2009). Expression and distribution of HA-α_{2a}ARs in these mice are identical to those of wildtype mice (Lu et al. 2009), as they are expressed in both adrenergic and non-adrenergic neurons which represent both auto- and hetero-α_{2a}ARs. Moreover, FLAG-α_{2a}AR 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 (Fig. 14B) (Gilsbach and Hein 2012). These mice are then crossed with α_{2a}ARs knockout mice, such that only FLAG-α_{2a}AR autoreceptors are present. By comparing wildtype, FLAG-α_{2a}AR, and α_{2a}AR knock-out mice, others have characterized the different physiological functions of auto- and hetero-α_{2a}ARs. 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 (Fig. 15) (Gilsbach and Hein 2012). With the physiological importance of α_{2a}ARs, and different roles of auto-and hetero-α_{2a}ARs, the signaling mechanisms of α_{2a}ARs in both adrenergic and non-adrenergic neurons need further understanding.

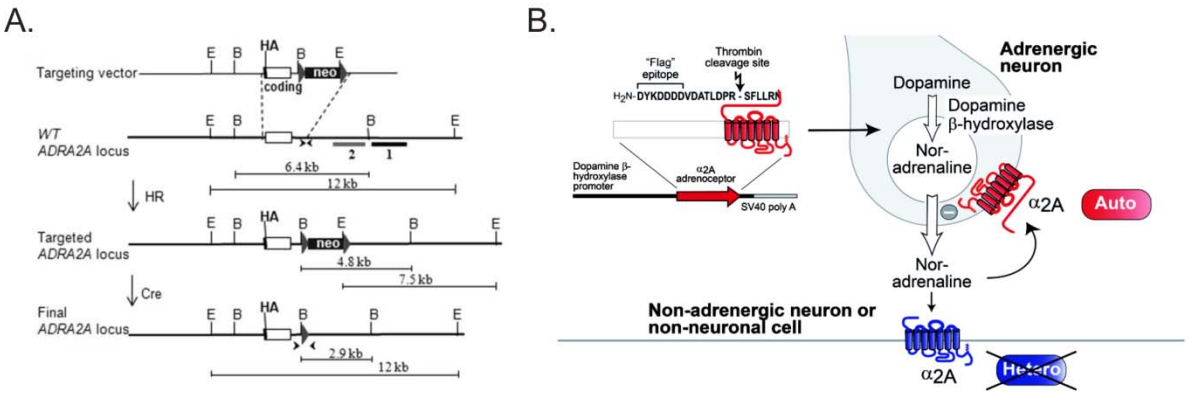

Figure 14. HA or FLAG tagged α_{2a}ARs mice.

Schematic showing the generation of HA-alpha2aAR knock-in (HA-α_{2a}ARs) and FLAG-alpha2aAR transgenic (FLAG-α_{2a}ARs) mice that are useful for studying the signaling mechanisms of auto- and hetero-α_{2a}ARs. Figure adapted from Lu, R. *et al*. 2009 *J. Bio.Chem*. 284.*19*:13233-43 and Gilsbach, R. et al. 2012 *Brit. J. of Pharm*. 165:90-102.

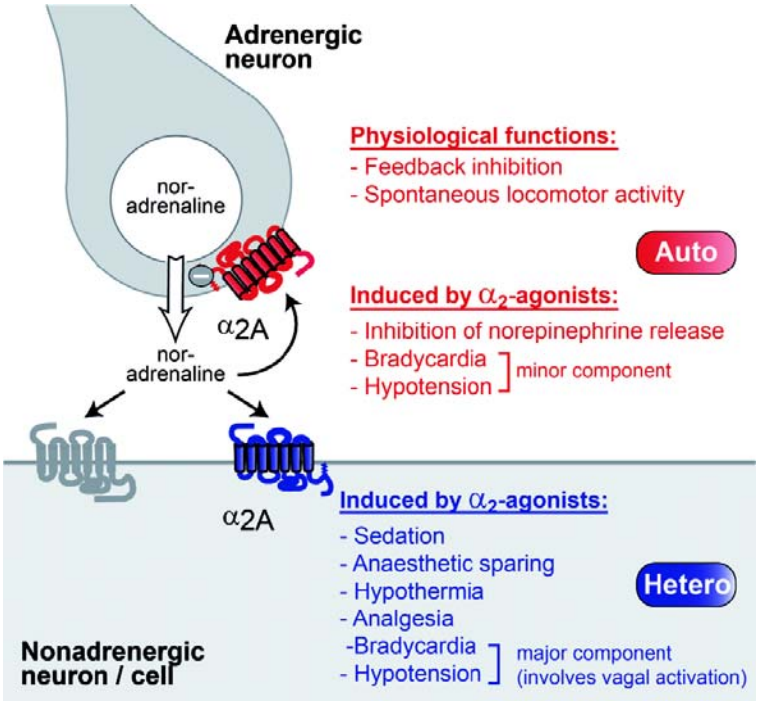

Figure 15. The differences in physiological functions of auto- and hetero-α_{2a}ARs.

Depending on the neuronal type, α_{2a}ARs are further divided into auto- and heteroreceptors and have different physiological functions. Figure adapted from Gilsbach, R. et al. 2012 *Brit. J. of Pharm*. 165:90-102.

Upon activation, α_{2a}ARs as inhibitory GPCRs inhibit voltage-dependent calcium channels (VDCCs) and adenylyl cyclase (AC), activate G-protein inward rectifying potassium (GIRK) channels, and induce phosphorylation of mitogen-activated protein kinases (MAPK) and Akt to activate respective downstream signaling cascades (Gannon and Wang 2016, Dolphin 2003, Herlitze 1996, Ikeda 1996a, Dascal and Kahanovitch 2015). While the activation of postsynaptic α_{2a}ARs causes membrane hyperpolarization and decreases the excitability of neurons, the activation of presynaptic α_{2a}ARs inhibits neurotransmitter release, either by inhibiting VDCCs or preventing synaptotagmin 1 (Syt1) from binding to exocytotic proteins (Delaney, Crane, and Sah 2007a, Boehm 1999). These downstream signaling cascades are regulated by the G proteins $G\alpha_{i/o}$ or $G\beta\gamma$,released from activated α_{2a}ARs, and their effectors (see Chapter 1 for more details). In previous study, we have found the change in abundance and localization of each neuronal Gβ and Gγ subunits (see chapter 2 for more details). Moreover, auto- and hetero-α_{2a}ARs have different physiological function (Gilsbach and Hein 2012). These suggest that these receptors may exhibit G protein, especially Gβγ, subunit specificity to mediate various downstream signaling pathways. Although $G\beta_1\gamma_2$ may be the most abundant neuronal Gβγ, other Gβγs may be utilized to specifically mediate 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 (Asano et al. 1999). Here, we test this hypothesis by using FLAG-α_{2a}ARs, HA-α_{2a}ARs, α_{2a}ARs knockout (α_{2a}ARs KO), and wildtype mice, together with various biochemical approaches such as a co-immunoprecipitation and the multiple reaction monitoring (MRM) method to quantify Gβ and Gγ subunits. We measured and compared the interaction of HA- or FLAG-α_{2a}ARs and neuronal Gβ and Gγ subunits, and predicted

the *in vivo* Gβγ specificity to auto- and hetero-α_{2a}ARs.

3.2 Experimental Procedures

Animals. Adult, male HA- and FLAG-alpah2a adrenergic receptors (α_{2a}ARs), α_{2a}ARs knockout (KO), and wildtype mice (Lu et al. 2009, Gilsbach and Hein 2012) were decapitated, and brain tissues were immediately homogenized to produce crude synaptosomes as described below. To block α_{2a}ARs, some mice were treated with intraperitoneal (i.p.) injection of saline or 10mg/kg BRL44408, a selective α_{2a}ARs antagonist, 30 minutes prior to the decapitation. 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. BRL44408 maleate (catalog 1133) was purchased from Tocris.

Antibodies. For the immunoprecipitation, Mouse anti-HA-agarose (Sigma, A2095) and mouse anti-FLAG (Sigma, F3165) were used. For the westernblot analysis, 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. HRP-conjugated secondary antibodies were obtained from Perkin-Elmer, Abcam, and Jackson Immunoresearch and used at the following dilutions: goat anti-rabbit (1:10,000), goat anti-mouse (1:10,000), Abcam anti-mouse light chain specific (1:5000), and mouse anti-rabbit light chain specific (1:7,500).

Synaptosome preparation. Crude synaptosomes were isolated from mouse brain tissue, as described previously (Gray and Whittaker 1962, Whittaker, Michaelson, and Kirkland 1964, Betke et al. 2014). Briefly, whole brains were sectioned at midsagittal plane to equally divide the right and left hemispheres. Both sections were separately homogenized in 20 mL of a 0.32 M sucrose solution (0.32M sucrose, 4.2 mM HEPES pH 7.4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.95 μM pepstatin, and 200 μM PMSF). Homogenates were centrifuged at 1000 x g and 4°C for 10 min, and supernatants containing synaptosomes (S1) were transferred to clean conical tubes. Pellets were resuspended in 20 mL of 0.32 M sucrose solution and centrifuged again. Pellets were discarded. Supernatants (S1) were combined and centrifuged at 10,000 x g and 4°C for 20 min to produce the crude synaptosome pellet (P2). One whole brain yielded two crude synaptosome pellets (P2).

Stimulation of Synaptosome. Crude synaptosomes (P2) were gently re-suspended in 2mL of resuspension buffer (10X DPBS (26.7mM KCl, 14.7mM KH_2PO_4, 1379mM NaCl, 80mM Na_2HPO_4•$7H_20$, pH 6.8-7), 1μM prazosin, and 1μM propranolol, transferred to 6ml culture vials, and placed on ice. As epinephrine (epi) is not an α_{2a}ARs selective agonist, we used prazosin and propranolol to block off-target effects from non-α_{2a}AR adrenergic receptors. For each set of crude synaptosomes per whole brain, one was used as an unstimulated control while the other was stimulated to examine α_{2a}ARs and G proteins (Gβ and Gγ) selectivity. For the control condition, 4mL of resuspension buffer were added to each vial and gently mixed. To stimulate α_{2a}ARs, 2ml of resuspension buffer were added to each vial first. Then, 2mL of stimulation buffer (resuspension buffer containing 200μM epi) were added to each vial to make the final concentration of epi to be 100μM in the

stimulation condition and gently mixed. All samples were placed in a 37°C water bath for 2 minutes (mins) and incubated with 2mM of the lipid soluble, thiol cleavable crosslinker, 3,3`-dithiobis [sulfosuccinimydlypropinate] (Michael J Hudspith) (Pierce 22585) for 2 hours (hrs) on ice. After 2 hrs, crosslinking reactions were quenched with 20mM Tris, pH 7.4 for 15 mins on ice. All samples were centrifuged at 10,000 x g at 4°C for 20 mins to regenerate the P2 pellet, then washed with 4mL of resupension buffer, and centrifuged again. Stimulated and non-stimulated crude synaptosomes were frozen in lipid nitrogen and stored at -80°C.

Synaptosome lysate. Crude synaptosomes were gently resuspended in 4 mL of RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 1.54 μM aprotinin, 10.7 μM leupeptin, 0.948 μM pepstatin, and 200 μM PMSF) using a 25 gauge needle to lyse membranes. Lysate protein concentrations were determined with a BCA assay (Pierce) and diluted to 1 mg/mL using RIPA buffer. Diluted homogenate was placed on a rotator for 1 hr and maintained at 4°C. Homogenates were transferred to 2 mL Eppendorf tubes and centrifuged at 14,000 rpm at 4°C for 10 min to separate the triton-soluble and insoluble fractions. Supernatants, the triton-soluble fractions, were collected. Supernatant protein concentrations were determined with a BCA assay.

Co-immunoprecipitation. Triton-soluble fractions were transferred to clean 2mL Eppendorf tubes and precleared for 1 hr at 4°C with 50μL of Protein G agarose beads (Pierce 20398). After the preclear, samples were centrifuged at 5,000 x g for 2 mins to pellet the beads. Protein concentrations of precleared lysates were determined with a BCA assay. Then, 300μL of precleared lysate per condition per genotype were saved as "inputs," and mixed with 100μL of 4X sample buffer containing dithiothreitol (DTT) and 5% βME.

Inputs were heated at 70°C for 5 mins and frozen at -80°C for Western blot analysis. The remainder of the precleared lysates was aliquoted to 1.5ml Eppendorf tubes. Precleared lysates from HA-α_{2a}AR and wildtype mice were aliquoted using 1ml per tube, while lysates from FLAG-α_{2a}-AR and α_{2a}-AR KO mice were aliquoted using 400ul per tube. Lysates were incubated with either an anti-HA or FLAG co-immunoprecipitation (coIP) antibody for 1 hr at 4°C. After 1 hr, 30µL of Protein G agarose beads was added to anti-FLAG coIP tubes only. All samples were rotated at 4°C overnight. The following day, all tubes were centrifuged at 5,000 x g for 2 mins to pellet the beads, and 300µL of supernatant per condition per genotype were transferred to clean, "supernatant" labeled 1.5mL Eppendorf tubes. Supernatants were mixed with 100µL of 4x sample buffer containing DTT and 5% 2-mercaptoethanol (βME), heated at 70°C for 5 mins, and frozen at -80°C for the Western blot analysis. Remaining supernatant was combined per condition and genotype and stored at -80°C. Beads were washed twice for 5 mins at 4°C with 500µL of coIP buffer (50mM Tris, pH 7.4, 150mM NaCl, 0.5% Triton X-100, 1.54µM aprotinin, 10.7µM leupeptin, 0.948µM pepstatin, and 200µM PMSF) and pelleted at 1,000 x g for 2 mins. Following the second wash, beads were resuspended in 500µL of coIP buffer, transferred to clean 1.5mL Eppendorf tubes, rotated for 5 mins in 4°C, and pelleted at 5,000 x g for 2 mins. Supernatants were aspirated, and beads were ready for the elution. (Fig. 16A).

Elution and trichloroacetic acid (TCA) precipitation of coIPs. Two different elution methods were used, depending on genotype. For HA-α_{2a}AR and wildtype samples, 100µL of 1X sample buffer with DTT and 5% βME were added to the samples, vortexed, and heated at 70°C for 5 mins. For FLAG-α_{2a}AR and α_{2a}AR KO samples, stock aliquots of

5mg/ml FLAG peptide (Sigma, F3290) were diluted with TBS to 0.377mg/ml, and 40µL the peptide was added to the coIP beads for a final amount of 15.09µg FLAG peptide. Samples were placed on a vortex shaker at medium speed for 30 mins and centrifuged at 5,000 x g for 2mins to pellet beads. Eluents were transferred to clean 1.5mL eppendorf tubes. The elution was repeated a second time, and the eluents were pooled together with the first elution. For Western blot analysis, one tube per condition per genotype was saved separately. Remaining eluent from same conditions were pooled per genotype and TCA precipitated to concentrate the G proteins for MRM analysis. One tube was saved per condition per genotype as Samples were incubated with 25% trichloracetic acid (TCA) (Sigma, T6399) on ice for 30 mins before being centrifuged at 14,000 rpm at 4°C for 30 mins. Following the centrifugation, supernatants were removed and pellets were washed with 500µL of cold acetone and centrifuged again at 14,000 rpm, 4°C for 15 mins before carefully aspirating off supernatants. The wash step was repeated twice and at the last centrifugation, supernatants were carefully removed using a pipette and dried down by the speed-vac for 5 mins. Dried pellets were resuspended in 100µL of 1x sample buffer with DTT and 5% βME, and heated at 70 °C for 5 mins. 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, coIP, and supernatant samples using 10% SDS-PAGE gels. Input and supernatant samples were diluted 1:1 with the 1X sample buffer to help the detection of G proteins in coIPs. Proteins were separated and transferred electrophoretically to nitrocellulose membrane in cold 1X CAPS transfer buffer (10mM CAPS, pH 11, 10% methanol). Following the transfer, membranes were Ponceau stained and cut between appropriate molecular weight markers. Membranes were blocked for 1 hr

in TBS with 0.1% Tween-20 (TBST) blocking solution with 5% milk on a shaker, and washed 5 times for 5 mins with TBST on a shaker. Membranes were incubated overnight at 4°C with appropriate primary antibodies in TBS with 5% Milk and 0.2% Tween-20 on a shaker. Membranes were subsequently washed 5 times with TBST, and incubated for 1 hr at room temperature with appropriate secondary antibodies in TBS with 5% Milk and 0.2% Tween-20 on a shaker. Membranes were washed with 0.1% TBST for three times, 10 mins per wash, followed by two 15 mins washes with TBS. Using Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer) and Bio-rad westernblot imager, Western blots were developed.

Limit of detection. To estimate the number of IPs that would be necessary for MRM experiments, a limit of detection (LOD) experiment was performed using a dilution series of purified $G\beta_1\gamma_1$ from 1pg to 10ng. As described in Chapter 2, targeted MRM methods were applied to the dilution series to estimate the LOD for our studies. Quantitative western blots were performed using a coIP sample and analyzed for densitometry using Image J(Schneider, Rasband, and Eliceiri 2012)

Heavy labeled peptide cocktail. As described in Chapter 2, a heavy labeled peptide cocktail was made (Table 3). In brief, using the mass spectrometry signal strength of coIP samples, the peptide quantity was further adjusted to create a "heavy labeled peptide cocktail."

Quantitative MRM of Gβ and Gγ subunits. Similarly to Chapter 2, coIP samples containing Gβ and Gγ subunits were separated, digested, and analyzed by a TSQ vantage triple quadrupole mass spectrometer (Thermo Scientific). Instead of purified $G\beta_5\gamma_2$ as marker, PageRuler™ unstained low range protein ladder (ThermoFisher) was used to

excise Gβ and Gγ bands. To allow comparisons between G proteins coIPed from multiple mice, quantitative Gβ and Gγ subunits detected (fmol) were normalized by divided by the amount of protein (mg) used in coIPs. The amount of protein used in coIPs 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).

3.3 Results

The interaction of adrenergic α_{2a} receptors and Gβγ. To study the Gβγ specificity to α_{2a}ARs, we used synaptosomes from wildtype, α_{2a}AR KO, HA- and FLAG-α_{2a}AR mice. Wildtype and α_{2a}-AR KO mice were used as controls of HA- and FLAG-α_{2a}-AR mice. Because no commercially available GPCR antibodies are specific enough to coIP α_{2a}ARs and Gβγ, we used HA- and FLAG-α_{2a}AR expressing mice to overcome this limitation. Synaptosomes from these mice were resuspended with 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 coIP experiments. The synaptosomes were then lysed and coIPed for HA- or FLAG-α_{2a}ARs and Gβγ (Fig. 16A), which was validated by Western blot. Input represents total proteins present in lysate after the preclear while supernatant (Sup) represents what proteins left in lysate after the co-immunoprecipitation with HA or FLAG specific antibodies (see Experimental Procedures

for more details). coIP lane represents proteins immuniprecipitated with HA or FLAG specific antibodies. Here, we detected both HA- and FLAG-α_{2a}ARs interacting with Gβγ only following α_{2a}AR stimulation (Fig.16B and C). In wildtype and α_{2a}ARs KO mice, no α_{2a}ARs and Gβγ interactions were detected following receptor stimulation (Fig.16B and C).

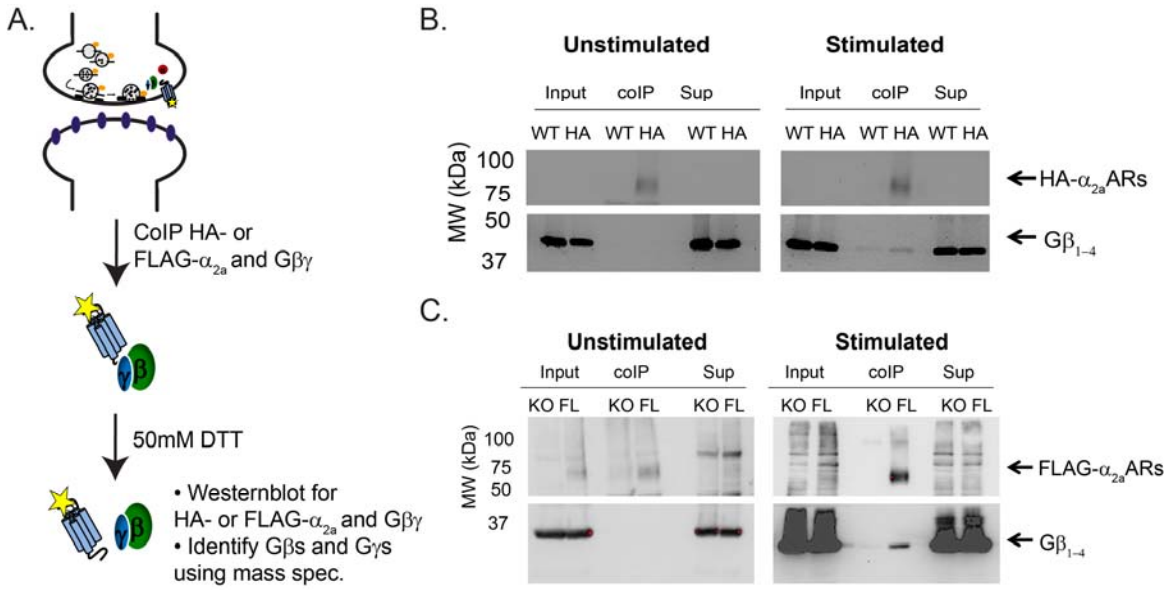

Figure 16. Co-immunoprecipitation of adrenergic α_{2a} receptors and Gβγ.

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, 100μM epinephrine). HA-α_{2a}ARs and FLAG-α_{2a}ARs are ~75kDa while Gβs are ~33kDa. HA-α_{2a}ARs and FLAG-α_{2a}ARs interact with Gβγ upon the activation of the receptors (stimulated). Sup: depleted supernatant

Limit of $G\beta_1$ detection and quantification. To determine the amount of coIPs needed to detect Gβ and Gγ subunits, we used a serial diluted, purified $G\beta_1\gamma_1$ and monitored 4 non-heavy labeled proteolytic peptides of $G\beta_1$ only to determine the limit of detection (LOD) (Table 4)(Betke et al. 2014). Because $G\beta_1\gamma_1$ is the easily purified from bovine retina, we chose it as our standard to detect LOD. Because $G\gamma_1$ is not neuronal, we only monitor $G\beta_1$. LOD was calculated by the area under the curve of detected $G\beta_1$ proteolytic peptides. Below 10pg of $G\beta_1\gamma_1$, we couldn`t confidently identify the presence of $G\beta_1$ in samples. Between 10pg to 250pg, we were able to detect $G\beta_1$ but total area under the curve (AUC) didn`t increase as the amount of purified $G\beta_1\gamma_1$ increase (Fig. 17). These suggest that we need more than 250 pg of $G\beta_1$. Moreover, we found that ~400-700ng of Gβγ was pulled down with FLAG-α_{2a}ARs per half brain of mice (10 coIPs) from the quantitative Western blot (data not shown). Without the heavy labeled proteolytic peptides, the limit of quantification for the quantitative MRM was not determined. However, previous limit of quantification experiment in chapter 2 suggest that we need more than 4ng of Gβγ for the quantification. With a half brain per condition, we can detect and quantify neuronal Gβ and Gγ despite the technical difficulty stated in chapter 2.

Table 4. **Proteotypic $G\beta_1$ peptides.**

Name	Sequence position	Peptide Sequence	Precursor m/z	charge	Product ion m/z
$G\beta_1$	24-42	(K)ACADATLSQITNNIDPVGR(I)	1008.4944	2	428.26,543.29, 884.46, 985.51
	138-150	(R)ELAGHTGYLSCCR(F)	762.3401	2	641.28, 858.36, 915.38, 1016.43
	198-209	(R)LFVSGACDASAK(L)	613.2977	2	483.22,779.34, 866.37,965.44
	284-301	(R)LLLAGYDDFNCNVWDALK(A)	1064.0144	2	632.34, 894.39, 950.93, 1119.52

The LOD of our studies was estimated by using targeted MRM methods on serially diluted $G\beta_1$ peptides. Bolded peptides were the ones that were heavy labelled for the quantitative MRM analysis.

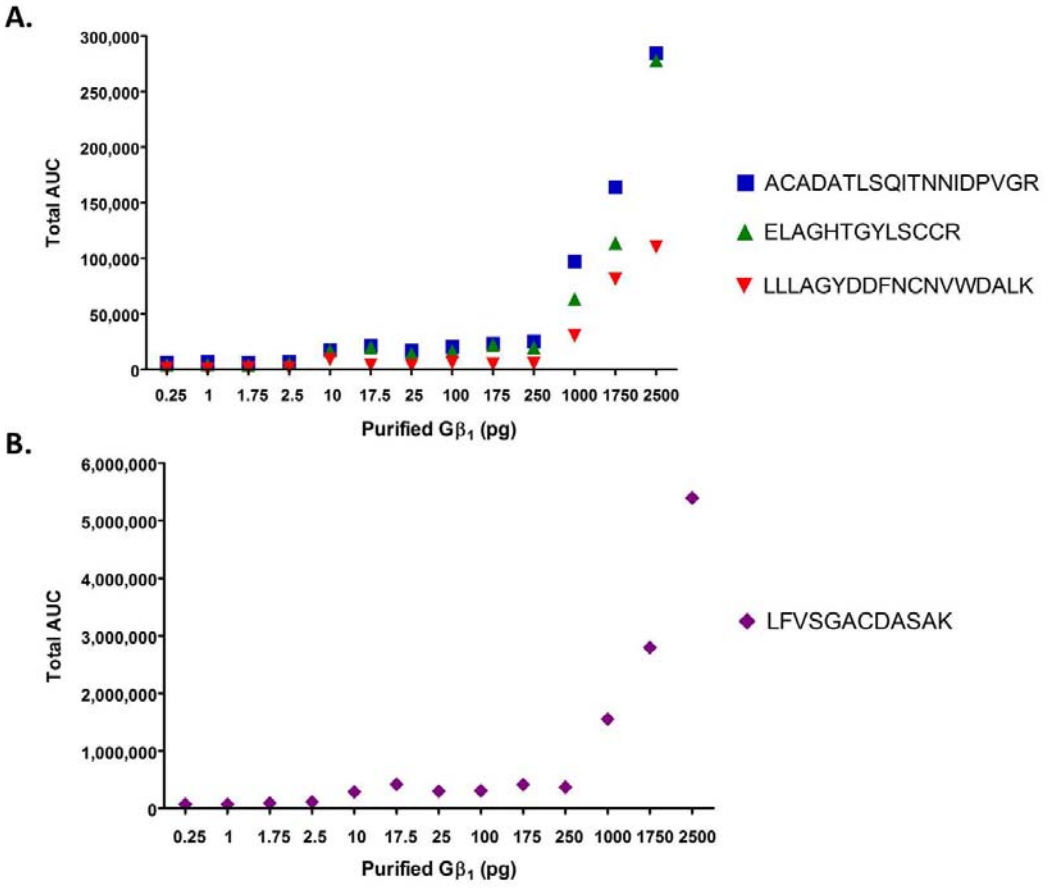

Figure 17. Estimation of the limit of detection for MRM experiments.

Total area under the curve (AUC) for $G\beta_1$ proteolytic peptides, ACADATLSQITNNIDPVGR, ELAGHTGYLSCCR, and LLLAGYDDFNCNVWDALK (A) and LFVSGACDASAK (B), were monitored by MRM across a dilution series of purified $G\beta_1$.

$G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_{12}$ specifically interact with adrenergic α_{2a} receptors. To determine which neuronal Gβ and Gγ subunits interact with adrenergic α_{2a}-ARs present in both adrenergic and non-adrenergic neurons, we applied the quantitative MRM method (see Chapter 2 for more details) to TCA-precipitated and trypsin-digested coIP samples (~5 coIPs per condition per genotype) of wildtype (WT) and HA-α_{2a}AR mouse synaptosomes (half brain per condition per genotype) (see Experimental Procedures). In this analysis, heavy labeled proteolytic peptides are used to quantify each neuronal Gβ and Gγ subunit (Table 3).

Using these mice, we examined Gβ subunit specificity to α_{2a}ARs present in both adrenergic and non-adrenergic neurons. We used both unstimulated WT (WT no epi) and HA-α_{2a}AR (HA-α_{2a}AR no epi) samples as controls to identify nonspecific interactions of Gβ subunits. In addition, we used stimulated WT (WT + epi) samples to detect non-α_{2a}AR mediated interactions. Comparing the amount of Gβ subunits detected per mg of lysate between WT +epi and HA-α_{2a}ARs+epi samples, $G\beta_2$ and $G\beta_4$ were significantly enriched with stimulated HA-α_{2a}ARs (Fig.18B and 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 further identified the specificity of Gγ subunits to α_{2a}ARs to determine possible Gβγ dimer interactions with α_{2a}ARs. From 6 detectable and quantifiable neuronal Gγ subunits (see Chapter 2 for more details), $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_{12}$ were enriched with HA-α_{2a}ARs upon epinephrine stimulation (Fig. 19 A, B, C, and E). We detected $G\gamma_2 > G\gamma_3 \approx G\gamma_4 > G\gamma_{12}$. $G\gamma_7$ and $G\gamma_{13}$ quantified in stimulated HA-α_{2a}ARs+epi samples were equal to or less than those quantified in control samples, suggesting these Gγs interact

nonspecifically (Fig 19. D and F). From the subunits we have detected, we postulate that there are 8 different combinations of Gβγ dimers ($G\beta_2\gamma_2$, $G\beta_2\gamma_3$, $G\beta_2\gamma_4$, $G\beta_2\gamma_{12}$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, $G\beta_4\gamma_4$, and $G\beta_4G\gamma_{12}$) which may interact with α_{2a}ARs in adrenergic and non-adrenergic neurons. Based on their detection level, $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$ may be more likely to be interact with α_{2a}ARs than other Gβγ dimers. 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.

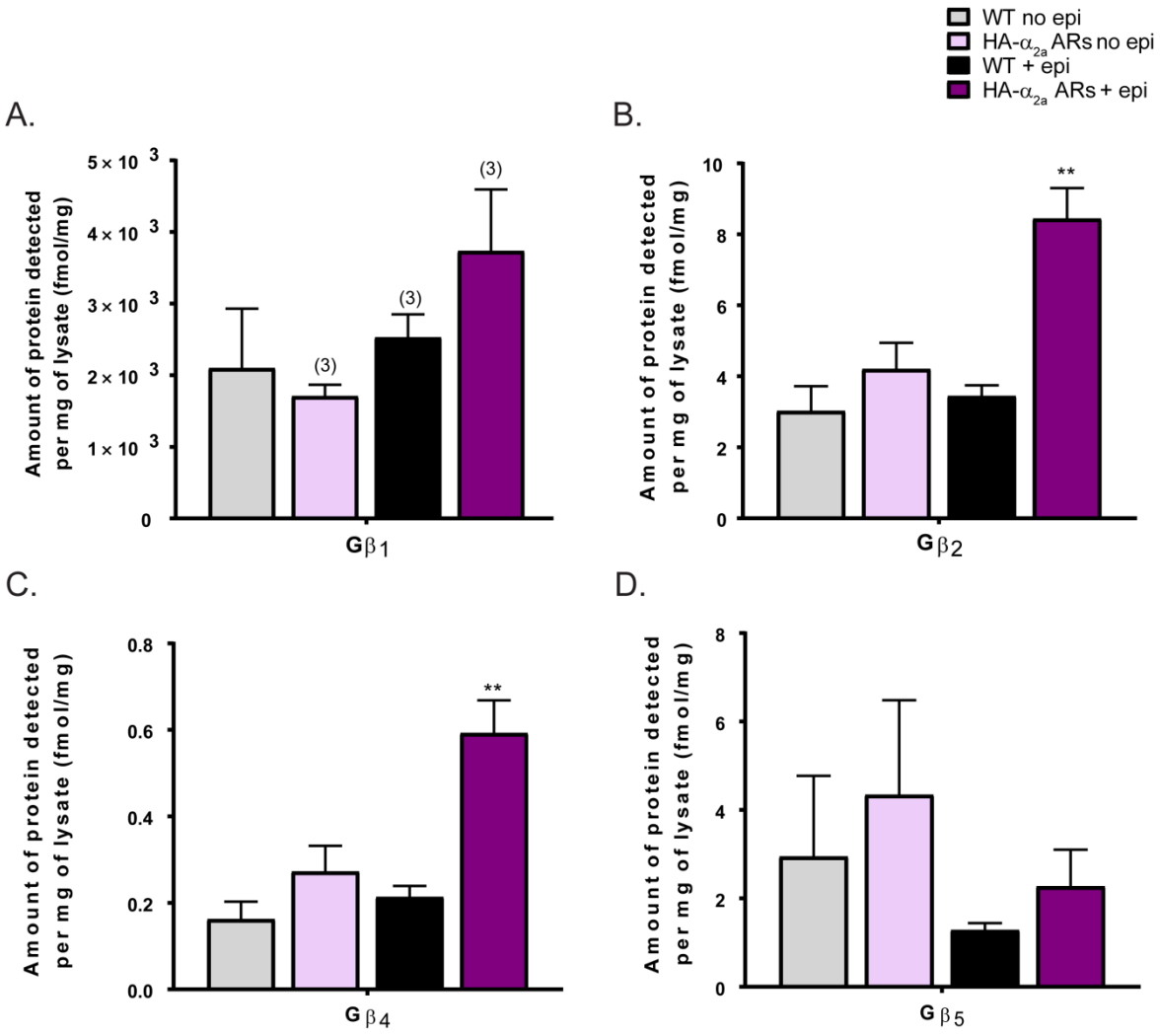

Figure 18. The specificity of Gβ subunit to α_{2a} adrenergic receptors.

Quantification of Gβ subunits interacting with α_{2a}ARs in both adrenergic and non-adrenergic neurons (N=4 unless otherwise noted). $G\beta_2$ and $G\beta_4$ specifically interact with α_{2a}ARs present in all neuronal 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.

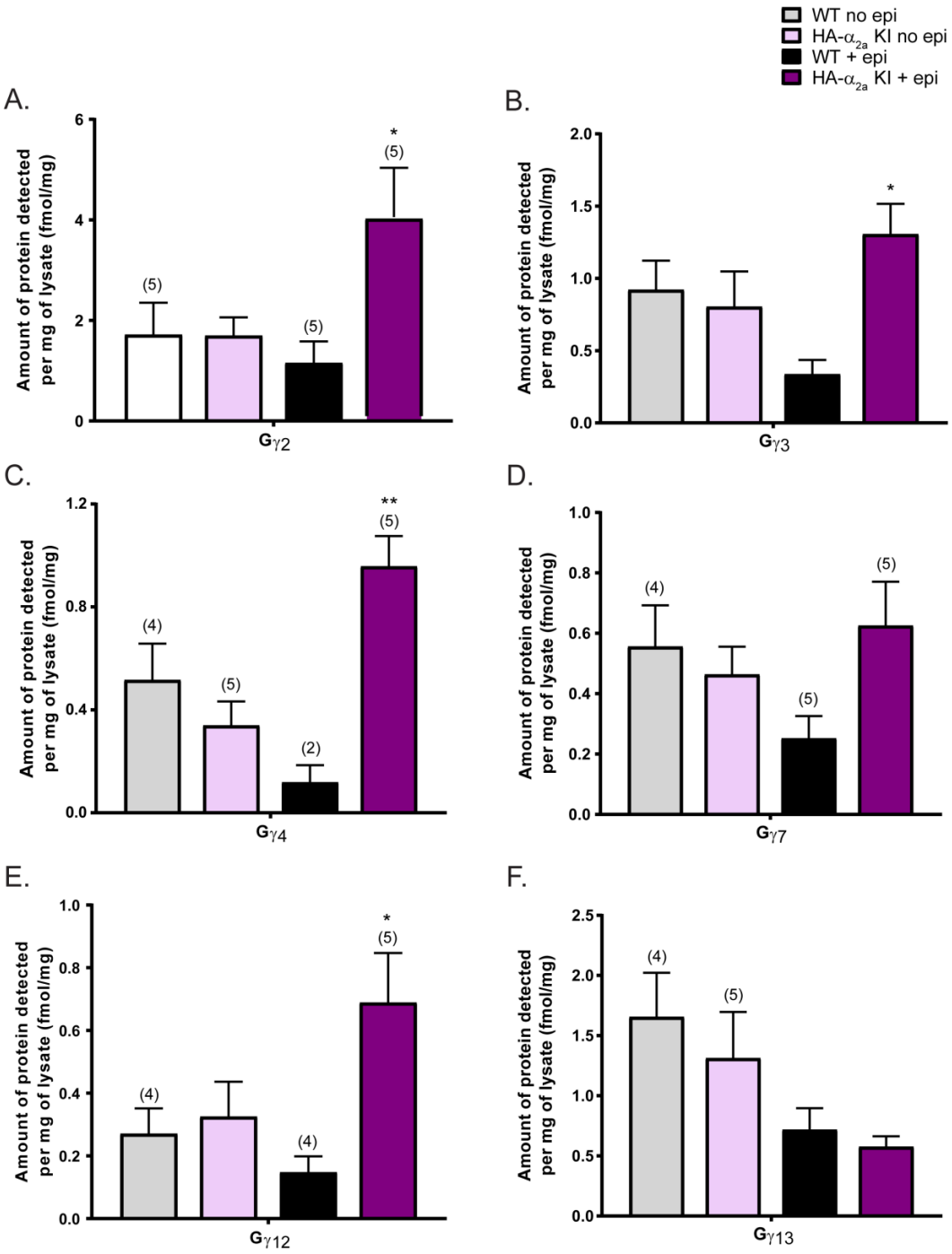

Figure 19. The specificity of Gγ subunit to α_{2a} adrenergic receptors.

Quantification of Gγ subunit interactions with α_{2a}ARs in both adrenergic and non-adrenergic neurons (N=4 otherwise noted on graph). $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, and $G\gamma_{12}$ specifically interact with α_{2a}ARs present in all neuronal 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.

$G\beta_2$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ specifically interact with auto-adrenergic α_{2a} receptors. To test whether both auto- and hetero-α_{2a}ARs utilize different $G\beta\gamma$ dimers upon activation, we again applied the quantitative MRM method to TCA-precipitated and trypsin-digested coIP samples (~10 coIPs per condition per genotype) of α_{2a}AR KO and FLAG-α_{2a}AR mouse synaptosomes (see Experimental Procedures for details).

As stated previously, FLAG-α_{2a}ARs are all auto-α_{2a}ARs, allowing us to study $G\beta$ and $G\gamma$ subunit specificities to autoreceptors. In addition, we can speculate $G\beta$ and $G\gamma$ subunit specificities to heteroreceptors by comparing the $G\beta$ and $G\gamma$ subunits detected in HA- and FLAG-α_{2a}ARs. Similarly to the previous experiment, α_{2a}AR KO no epi and FLAG-α_{2a}AR no epi samples were used as controls to identify nonspecific interactions, and α_{2a}AR KO +epi to detect non-α_{2a}AR associations. Here, $G\beta_2$, but not $G\beta_4$, showed a significant enrichment with auto-α_{2a}ARs (FLAG-α_{2a}ARs) (Fig.20B). Thus we can propose that $G\beta_4$ may be heteroreceptor specific. Again, $G\beta_5$ did not interact with auto-α_{2a}ARs (Fig.20D). Again, $G\beta_1$ did not interact with auto-α_{2a}ARs upon stimulation (Fig.20A).

In contrast to the 4 $G\gamma$ 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. 21A-C). Interestingly, we no longer saw enrichment of $G\gamma_{12}$ with FLAG-α_{2a}ARs (Fig. 21E). $G\gamma_{12}$ may be a hetero-α_{2a}AR-specific $G\gamma$ subunit. As expected from the previous HA-α_{2a}AR study, $G\gamma_7$ and $G\gamma_{13}$ did not interact with FLAG-α_{2a}ARs (Fig.21D and F). Although further validation is necessary, we can speculate that $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$ may be the possible $G\beta\gamma$ dimers interacting with auto-α_{2a}ARs in adrenergic neurons, while $G\beta_2\gamma_{12}$, $G\beta_4\gamma_2$, $G\beta_4\gamma_3$, $G\beta_4\gamma_4$, and $G\beta_4\gamma_{12}$ may interact with hetero-α_{2a}ARs. As we hypothesized, auto- and hetero-α_{2a}-ARs may

release different Gβγ dimers to mediate their physiological functions.

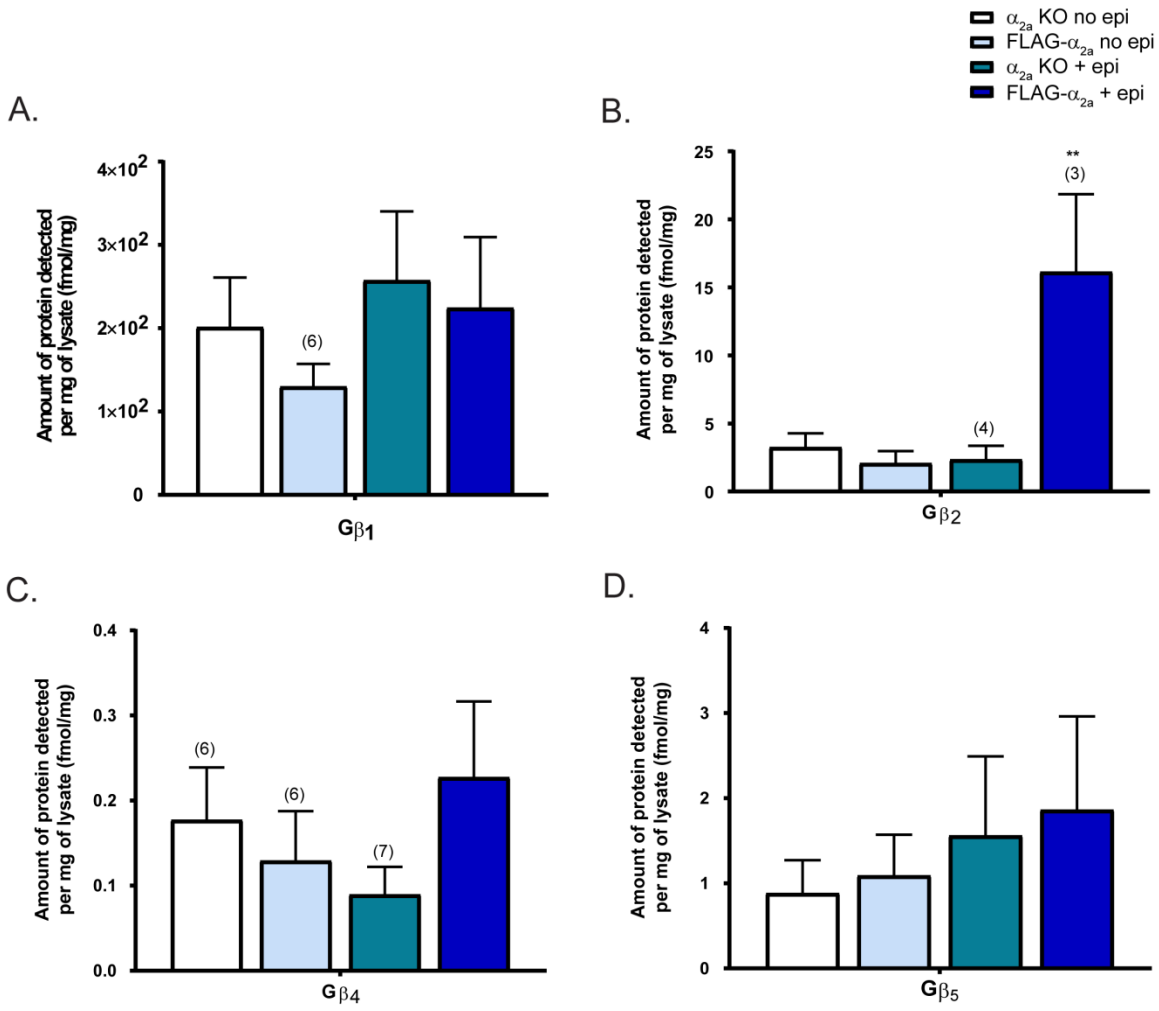

Figure 20. The specificity of Gβ subunits to auto−α_{2a} adrenergic receptors.

Quantification of Gβ subunits interacting with auto-α_{2a}ARs (FLAG-α_{2a}-ARs**)** in adrenergic neurons (N=5 unless otherwise noted). $G\beta_2$ 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.

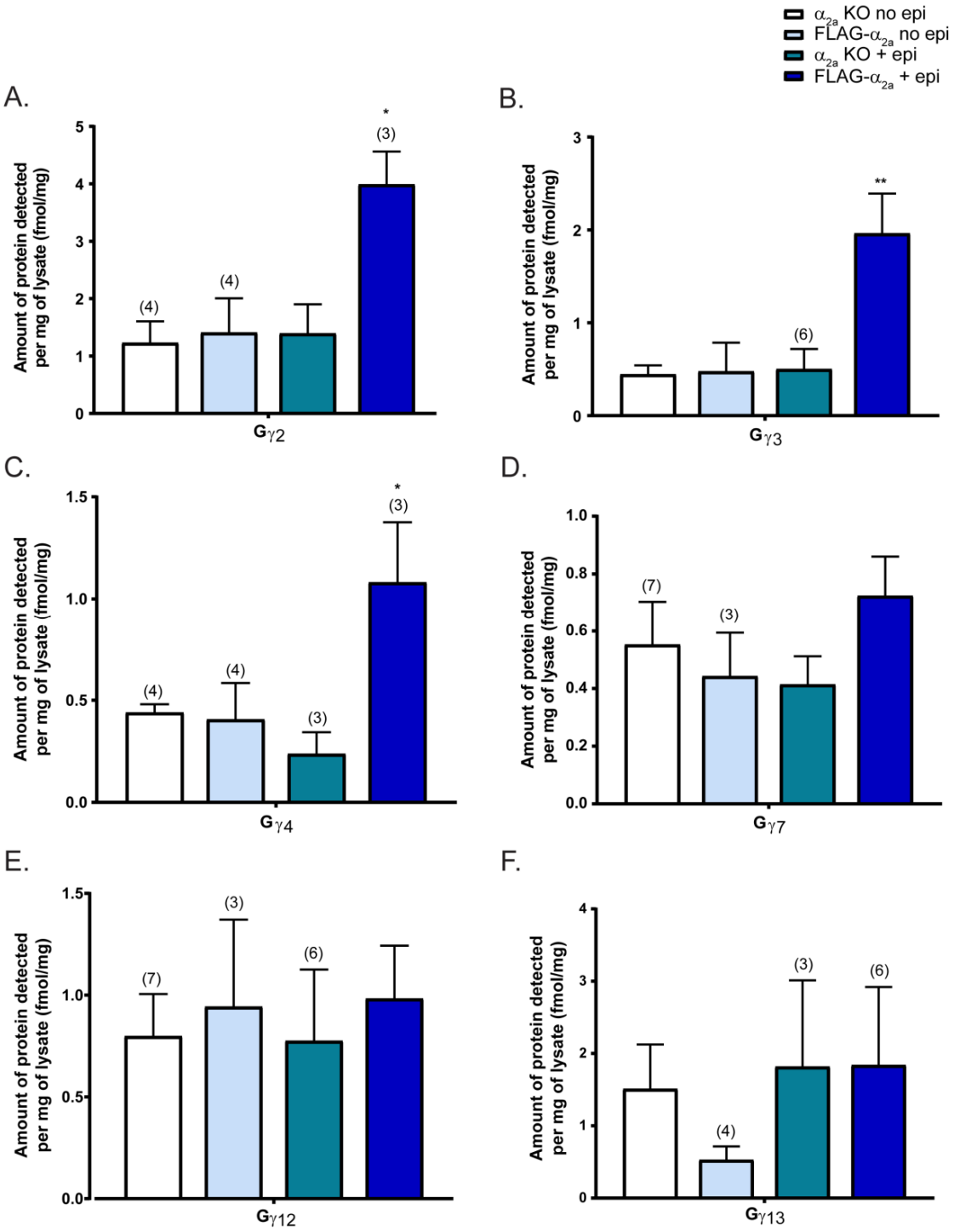

Figure 21. The specificity of Gγ subunits to auto-α_{2a} adrenergic receptors.

Quantification of Gγ subunits interacting with auto-α_{2a}ARs on adrenergic neurons (N=5 unless otherwise noted). $G\gamma_2$, $G\gamma_3$, 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.

3.4 Discussion

Upon the activation of $G\alpha_{i/o}$-coupled GPCRs such as α_{2a}ARs, Gβγ dimers are released and act as important signaling units to various downstream signaling cascades. One of its well understood roles is to regulate the inhibition of neurotransmitter release through actions on VDCCs, potassium channels, and members of the exocytotic machinery (Blackmer et al. 2005, Blackmer et al. 2001, Yoon 2007, Wells et al. 2012, Brown and Sihra 2008, Herlitze 1996, Michaeli and Yaka 2010, Fasshauer 2003) at the presynaptic terminal. However, we do not know whether all 32 possible Gβγs (combined from the known expression of 4 neuronal Gβs and 8 neuronal Gγs) are functional or play a role in defining the specificity of signaling pathways. In this study, we demonstrated that α_{2a}ARs preferentially interact with a subset of Gβ and Gγ subunits at neuronal terminals. Neuronal 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 effector interactions.

As previously addressed in Chapter 2, we experienced some technical challenges quantifying Gγ subunits. The amount of detected Gγ subunits was not equal 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 resolubilization efficiencies, all of which can lead to systematic errors in quantification (Hoofnagle et al. 2016). 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.

Comparing results obtained from HA-α_{2a}AR and FLAG-α_{2a}AR studies, we estimated theoretical Gβ and Gγ subunit interactions with hetero-α_{2a}ARs (Table 5). Although we were unable to determine the differences in coIP efficiency of HA- and FLAG- antibodies to calculate the relative Gβ and Gγ enrichment with hetero-α_{2a}ARs, 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. We found that $G\beta_2$ may be autoreceptor-specific, while $G\beta_4$ may be heteroreceptor specific. In previous study, we found $G\beta_1$ as the most abundant Gβ subunit in whole synaptosomes and at both pre- and post-synaptic fractions (Fig.11A and 13A). However, we did not find a statistical significance interaction between $G\beta_1$ and HA-α_{2a}ARs upon receptor activation (Fig. 18A). $G\beta_1$ may be specific to other receptors that are more abundance at the synaptic terminals. For Gγ subunits, $G\gamma_2$, $G\gamma_3$ and $G\gamma_4$ were determined to be auto-α_{2a}AR specific, while $G\gamma_{12}$ was hetero-α_{2a}AR specific. Overall, heteroreceptors may associate with heterotrimers paired with $G\beta_4\gamma_{12}$ to mediate heteroreceptor-specific phenotypes such as sedation and anesthetic sparing (Gilsbach and Hein 2012).

Table 5. Gβ and Gγ specificities to hetero-α_{2a}ARs.

G proteins	α_{2a}ARs	Auto-α_{2a}ARs	Hetero-α_{2a}ARs (estimated)
$G\beta_2$	√√	√√	x
$G\beta_4$	√	x	√
$G\gamma_2$	√√√	√√√	x
$G\gamma_3$	√√	√√	x
$G\gamma_4$	√	√	x
$G\gamma_{12}$	√	x	√

The number of √ increases as the abundance increases, 3 being most abundant and 1 being least abundant. √: interaction with receptor detected; x: no interaction was detected

In numerous *in vitro* studies, investigators have attempted to determine the specificity of Gβγ dimerization and their selectivity in interacting with various GPCRs and effectors (Kleuss et al. 1991, Albert and Robillard 2002, Robishaw and Berlot 2004). Similar to our observations (Fig.18 and 19), $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ were previously shown to be strongly associated with α_{2a}ARs (Gibson and Gilman 2006, Richardson and Robishaw 1999a). 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 $G\beta_2$. 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* neuronal 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 (Hosohata et al. 2000, Asano et al. 1999, Kleuss et al. 1992). 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 autoreceptor specific, while $G\beta_4\gamma_{12}$ may be heteroreceptor specific.

Interestingly, we detected a minor interaction between $G\gamma_{12}$ and α_{2a}ARs but not with auto-α_{2a}ARs (Fig. 19 and 21E). Although $G\gamma_{12}$ was one of most abundant Gγ subunits at the membrane fraction (Fig. 13D and E) in previous study, $G\gamma_{12}$ was not the most abundant Gγ subunits interacting with α_{2a}ARs. $G\gamma_{12}$ may interact with other GPCRs in addition to hetero-α_{2a}ARs. In addition, $G\beta_5$ showed no specific interaction with α_{2a}ARs (Fig.18 and 20 D), which confirmed 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 (Zachariou et al. 2003, Lopez-Fando et al. 2005, Anderson et al. 2010, Psifogeorgou et al. 2011, Masuho, Xie, and Martemyanov 2013) .

In addition to Gβγ, Gα may also define the selectivity of α_{2a}ARs. As a $G\alpha_{i/o}$ GPCR, α_{2a}ARs couple to $G\alpha_{i1\text{-}3}$ and $G\alpha_{o1\text{-}2}$. In a previous study by Richardson and Robishaw, $G\alpha_i$-containing heterotrimers were highly coupled to α_{2a}ARs. Further, $G\alpha_i$ subunits were demonstrated to mediate sedative anesthetic sparing effects, but not inhibition of evoked release (Albarran-Juarez et al. 2009), and $G\alpha i_1$ were found to preferentially associate with $G\beta_1\gamma_3$ over $G\beta_1\gamma_1$ or $G\beta_1\gamma_{10}$ (Richardson and Robishaw 1999a). 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.

3.5 Conclusion

Here, we demonstrated that presynaptic α_{2a}-ARs exhibit specificity in their interactions with a subset of Gβ and Gγ subunits. Gβγ dimers, other than $G\beta_1\gamma_2$, are involved in α_{2a}AR-mediated signaling cascades. 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 types of neurons. Further efforts will be needed to determine the specificity and roles of each unique Gβγ dimer in regulating various α_{2a}-AR signaling cascades, and their impacts in neurological diseases and GPCR targeted drug mechanisms.

CHAPTER 4

α_{2a} ADRENERGIC RECEPTOR-MEDIATED Gβ AND Gγ SUBUNIT SPECIFICITY TO SNARE

4.1 Introduction

Synaptic transmission is an essential method of interneuronal communication in both the central and peripheral nervous systems, as described in Chapter 1. Briefly, the arrival of an action potential activates presynaptic voltage-gated calcium channels, and increases the intracellular calcium concentration at the presynaptic terminal. Synaptotagmin 1 (Syt1), a calcium sensor, senses the increase in Ca^{2+} concentration and interacts with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), comprised of SNAP25, syntaxin 1A, and synaptobrevin (VAMP) (Zhai and Bellen 2004, Schoch and Gundelfinger 2006, Sudhof 2004, Purves D 2008, Sudhof 2012). As SNARE complexes zip up mediated by the Syt1 interaction, vesicle membrane and plasma membrane come into proximity leading to vesicle fuseion (Brose 1992). Neuronal vesicles are loaded with neurotransmitters, which are released into synaptic junctions during membrane fusion. Neurotransmitters are the chemical communicators between neurons which convey both external and internal sensory inputs, and promote responsive behaviors in postsynaptic cells (Sudhof 2012). It is important to understand how synaptic transmission is regulated in healthy subjects, and mechanisms of dysfunction in neurological diseases.

Inhibitory GPCRs, which are $G\alpha_{i/o}$ coupled, play an important role in regulating synaptic transmission by acting as auto- and hetero-receptors in feedback mechanisms

limiting neurotransmitter release (Hein, Altman, and Kobilka 1999, Gilsbach et al. 2009b, Richter et al. 2012, Norenberg et al. 1997, Mateo and Meana 1999). The α_{2a}ARs is one of the best studied inhibitory GPCRs, with excellent availability of knock out and transgenic mice with tagged receptors (Lu et al. 2009, Gilsbach and Hein 2012, Mizobe 2001, Lahdesmaki et al. 2002, Bucheler, Hadamek, and Hein 2002). The activation of α_{2a}ARs inhibits synaptic transmission by Gβγ in both adrenergic and non-adrenergic neurons such as glutamatergic, serotonergic and dopaminergic neurons (Hein 2006, Forray, Bustos, and Gysling 1999, Millan, Lejeune, and Gobert 2000, Mongeau et al. 1998). In particular, numerous studies have reported that Gβγ subunits released from activated α_{2a}ARs reduce presynaptic Ca^{2+} influx through the inhibition of VDCC (Dolphin 2003, Ikeda 1996b, Ikeda and Dunlap 1999) and inhibit exocytosis through direct interaction with the SNARE (Jahn and Scheller 2006a, Malsam, Kreye, and Sollner 2008, Delaney, Crane, and Sah 2007a) complex .

The inhibition of presynaptic noradrenaline release via direct interaction between Gβγ and SNARE has been determined as a primary mechanism inhibiting exocytosis by α_{2a}ARs in the central amygdala (Delaney, Crane, and Sah 2007a). These authors show that inhibition of noradrenaline release, where high levels of presynaptic α_{2a}ARs are present, is mediated by a $G\alpha_{i/o}$-coupled receptor, and that Gβγ dimers released from active α_{2a}ARs directly interact with SNARE downstream of calcium entry and inhibit noradrenaline release (Delaney, Crane, and Sah 2007a). Although the Gβγ–SNARE interaction has been well-characterized in α_{2a}AR-mediated inhibition of presynaptic transmission, the specificity of Gβγ dimer isoforms to the SNARE complex is not known. Here, we evaluated whether Gβγ dimers released from epinephrine (epi)-activated α_{2a}ARs, as

measured in Chapter 3, interact with SNARE to regulate synaptic transmission. Interestingly, we discovered that only a subset of Gβ and Gγ subunits released from activated α_{2a}ARs interact with SNARE. Further understanding of α_{2a}AR-mediated Gβγ specificity to SNARE may lead to the development of new therapeutic targets that may complement or contrast with currently available α_{2a}AR-targeted drugs.

4.2 Experimental Procedures

Animals. Adult, male FLAG-tagged alpha2a adrenergic receptors (α_{2a}-ARs), α_{2a}-AR knockout (KO), and wildtype mice (Gilsbach et al. 2009a) were decapitated and brain tissues were immediately homogenized to produce crude synaptosomes as described below. To block α_{2a}-ARs, some mice were treated with intraperitoneal (i.p.) injection of saline or 10mg/kg BRL44408, a selective α_{2a}-AR antagonist, and 30 minutes prior to sacrifice (Cleary, Vandeputte, and Docherty 2003, Dwyer et al. 2010). 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. All drugs used were those used in chapter 3 used. In addition, BRL44408 maleate (catalog 1133) was purchased from Tocris.

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. As described in chapter 3, all crude synaptosomes were prepared, stimulated, and lysed.

Co-immunoprecipitation. Similar to the co-immunoprecipitation (coIP) of α_{2a}ARs and Gβγ in chapter 3, we coIPed SNARE and Gβγ from the precleared lysates (Fig.22A). Precleared lysates were aliquoted 400ul per tube. They were incubated with an anti-SNAP25 coIP antibody. We used rabbit IgG as a control, because homozygous SNAP25 knockout mouse exhibits neonatal lethality (Washbourne et al. 2002).

Elution and trichloracetic acid (TCA) precipitation of coIPs. Samples were eluted from antibody-beads using 100mM glycine (pH 2.5). 40µL of it was added to coIP beads and placed on a room temperature vortex shaker at medium speed for 8 mins and centrifuged at 5,000 x g for 2mins to pellet beads. Elutants were transferred to clean 1.5mL eppendorf tubes. The elution was repeated a second time, and the elutants were pooled together with the first elution. For western blot analysis, one tube per condition per genotype was saved. Remaining elutants were pooled per genotype per condition and TCA precipitated to concentrate the Gβ and Gγ subunits for MRM analysis as stated in chapter 3. All samples were stored at -80°C for western blot or MRM analysis.

Immunoblot Analysis. The analysis was done similar to chapter 3. We used 15%, instead of 10% SDS-PAGE gel.

Heavy labeled peptide cocktail. As described in chapter 2, the heavy labeled peptide cocktail was made. According to the mass spectrometry signal strength of coIP samples,

the amount of peptides was further adjusted to create a "heavy labeled peptide cocktail."

Quantitative MRM of Gβ and Gγ subunits. Similar to chapter 3, coIP samples containing Gβ and Gγ subunits were separated, digested, and analyzed by a TSQ vantage triple quadrupole mass spectrometer (Thermo Scientific).

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

4.3 Results

Interactions between Gβγ and SNARE complex. To study the specificity of Gβ and Gγ subunits to SNARE complexes following α_{2a}AR activation, we used synaptosomes from wildtype, α_{2a}AR KO, and FLAG-α_{2a}AR mice. Using a SNAP25 antibody, Gβγ and SNARE coIPs were visualized by Western blot (Fig. 22A). To further validate these interactions, in separate samples we used the SNAP25 antibody to pull down the SNARE complex, and detected the presence of VAMP2 and Syntaxin1 in the IPs (data not shown). 'Input' represents precleared lysates from each genotype, while 'supernatant' represents proteins remaining in lysates after immunoprecipitation. Interestingly, we detected Gβγ in the presence and absence of epi, evidence of Gβγ-SNARE interactions in both unstimulated and stimulated conditions (Fig 22B and C). To ensure that this result wasn't an artifact of crosslinker treatment, coIP studies were repeated in the presence and absence of both crosslinker and epi in wildtype mice (Fig 22D). Western blots revealed that without

the crosslinker, the interaction between Gβγ and SNARE was detected, albeit weakly. While DSP crosslinker is used to increase the detection of Gβγ-SNARE interaction, we believe the interaction is specific, as little non-specific binding was observed in IgG controls. Given that the interaction between Gβγ and SNARE is low affinity, the crosslinker is necessary to sustain the intact complex for accurate comparisons. Quantitative western blot analysis estimated approximately 1000ng Gβγ was pulled down with SNAP25 per half brain (data not shown).

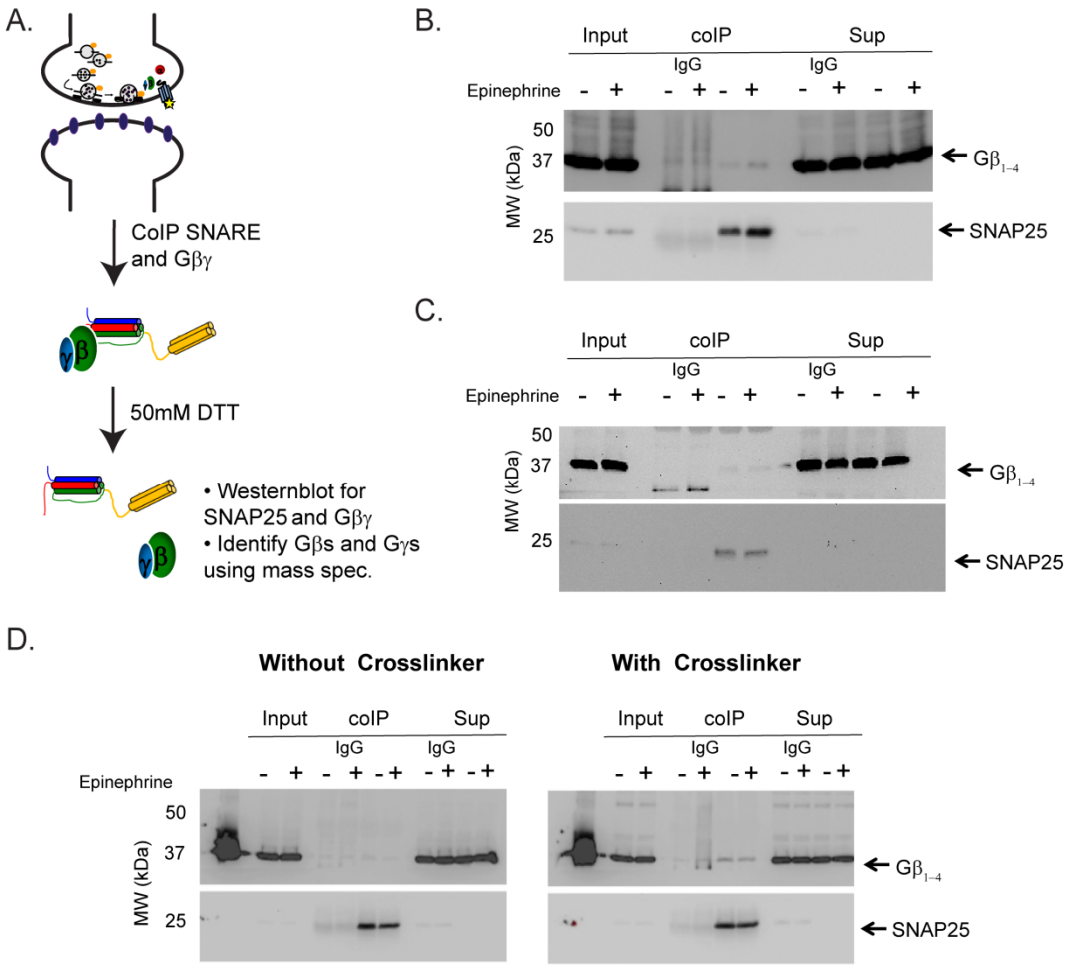

Figure 22. Co-immunoprecipitation of SNARE and Gβγ.

The workflow of co-immunoprecipitation (coIP) experimental protocol (**A**), and representative western blot of coimmunopreciptiation of SNAP25 and Gβγ in the wildtype (**B**) or FLAG-α_{2a}ARs (**C**) mice following the stimulation of synaptosomes with resuspension (unstimulated/-) or stimulation buffers (stimulated/+, 100μM epi). We can detect the Gβγ and SNARE interaction regardless of receptor stimulation (**D**). This interaction is not due to the DSP crosslinker. coIP: co-immunoprecipitated fraction; Sup: depleted supernatant

$G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ may specifically interact with SNARE upon α_{2a}AR activation. To examine which neuronal Gβ and Gγ subunits interact with SNARE upon the activation of adrenergic α_{2a}ARs, we applied the quantitative MRM method (described in Chapter 3) to TCA precipitated and trypsin digested SNAP25 coIP samples (approximately 11 coIPs per condition per genotype) of wildtype (WT), α_{2a}AR KO, and FLAG-α_{2a}AR mouse synaptosomes (see Experimental Procedures for details). WT samples were used to detect Gβγ–SNARE interactions mediated by both auto- and hetero-α_{2a}ARs, while α_{2a}AR KO samples were used to detect non α_{2a}AR-mediated interactions. Lastly, FLAG-α_{2a}AR samples were used to detect only auto-α_{2a}AR-mediated Gβγ-SNARE interactions. We used unstimulated WT (WT no epi), α_{2a}AR KO (α_{2a}AR KO no epi), and FLAG-α_{2a}AR (FLAG-α_{2a}ARs no epi) samples as controls, as described in chapter 3, to understand basal Gβγ–SNARE interactions. By comparing the amount of Gβ and Gγ subunits detected per mg of lysate between stimulated WT (WT + epi), α_{2a}AR KO (α_{2a}AR KO +epi), and FLAG-α_{2a}AR (FLAG-α_{2a}AR +epi), we can detect not only auto-receptor mediated Gβγ-SNARE interactions, but also quantify those mediated by heteroreceptor interactions. Here, we present preliminary data from WT, α_{2a}AR KO, and FLAG-α_{2a}AR mice (N=4, otherwise noted in graphs). Although we cannot make any conclusive statements, we are able to detect the trends of Gβ and Gγ subunit specificities to SNARE upon α_{2a}AR activation.

We used both unstimulated (WT IgG no epi) and stimulated (WT IgG +epi) WT IgG samples as controls to identify nonspecific interactions of Gβ and Gγ subunits. Comparing the amount of Gβ subunit detected per mg of lysate between WT IgG +epi and

WT +epi samples, $G\beta_1$, $G\beta_2$, and $G\beta_4$ seem to significantly interact with SNARE upon α_{2a}AR stimulation (Fig. 23 A, B and C). More $G\beta_1$ was detected than $G\beta_2$ and $G\beta_4$. In contrast, $G\beta_5$ did not interact with SNARE following α_{2a}AR activation.

Next, we further identified the specificity of Gγ subunits to α_{2a}ARs to determine the possible Gβγ dimers that interact with SNARE following receptor activation. Here, we found trends of enrichment of the $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ subunits with SNARE upon epi stimulation (Fig.24A, B, and C). We detected $G\gamma_3 > G\gamma_2 \approx G\gamma_4$. Interestingly, we were not able to detect an interaction between $G\gamma_{12}$ and SNARE (Fig. 24E). Although $G\gamma_{12}$ may be specifically associated to α_{2a}ARs (Fig. 19E), it may not interact with SNARE but interact with other Gβγ effectors such as VDCCs (see Discussion section for more details). From the subunits we have detected, we postulate that there are 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 may interact with SNARE upon α_{2a}ARs activation. Based on the detected abundances of $G\beta_1$ and $G\gamma_3$, $G\beta_1\gamma_3$ may be the most likely dimer to interact with SNARE following receptor stimulation. Further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities to SNARE upon α_{2a}AR activation in both adrenergic and non-adrenergic neurons.

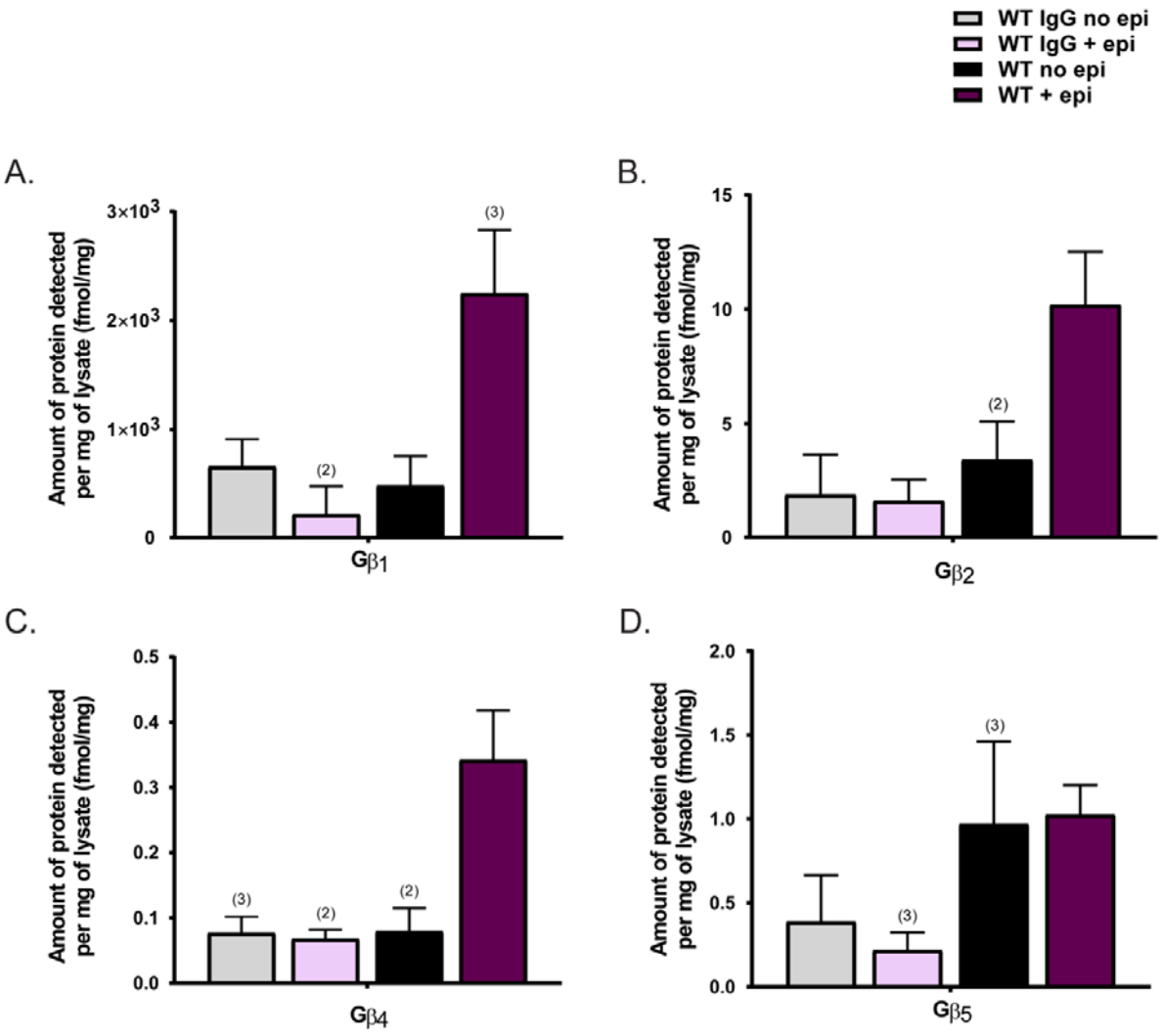

Figure 23. Gβ specificity to SNARE upon α_{2a}AR activation.

Quantification of Gβ subunits interacting with SNARE in wildtype synaptosomes (N=4, otherwise noted). $G\beta_1$, $G\beta_2$, and $G\beta_4$ may specifically interact with SNARE upon α_{2a}AR activation with epi. Data are presented as mean ± SEM.

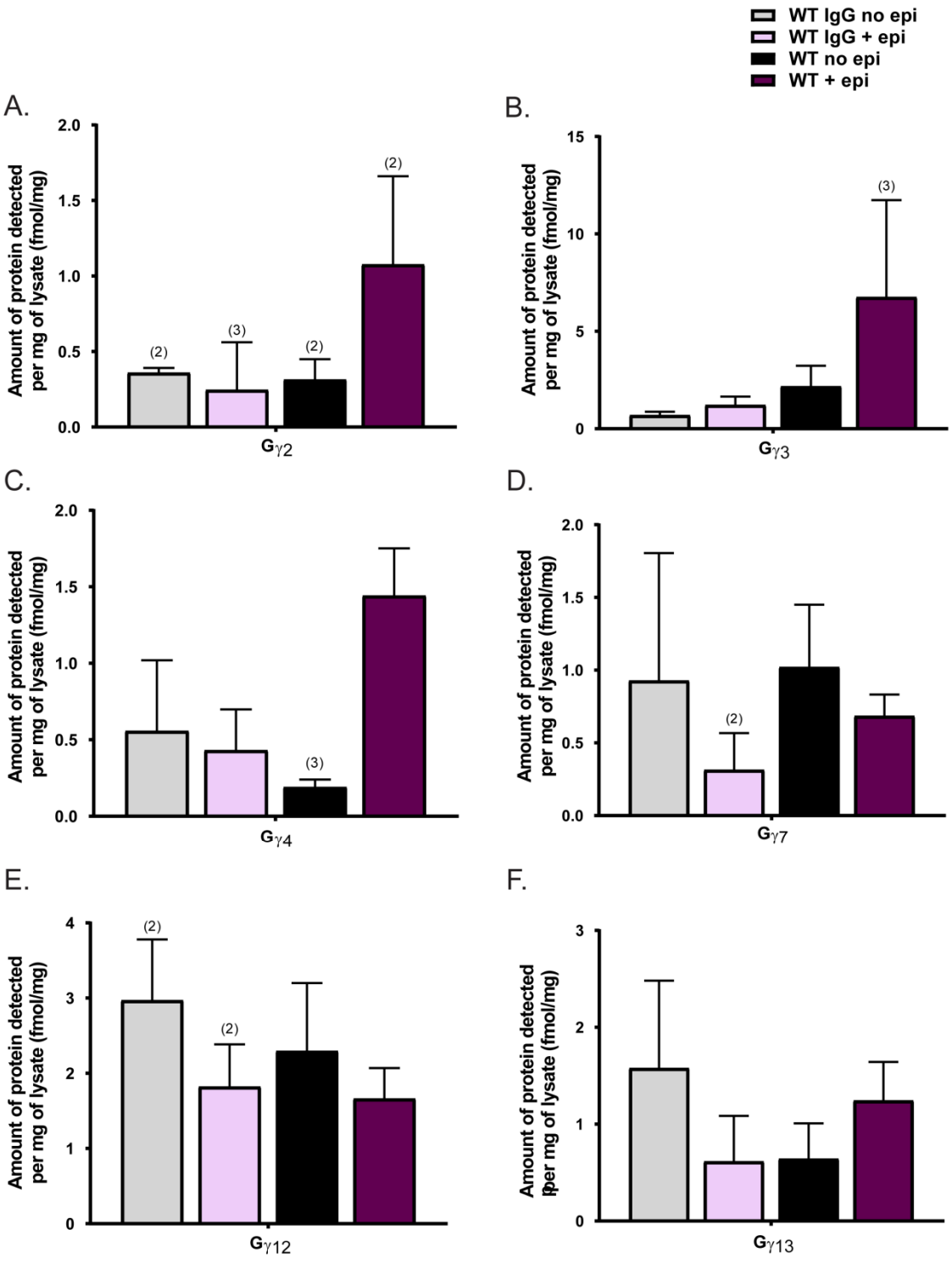

Figure 24. Gγ specificity to SNARE upon α_{2a}AR activation.

Quantification of Gγ subunits interacting with SNARE in wildtype synaptosomes (N=4, otherwise noted). $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ may specifically interact with SNARE upon α_{2a}AR activation. Data are presented as mean ± SEM.

$G\beta_1$ and $G\gamma_3$ may specifically interact with SNARE upon auto-α_{2a}ARs activation. One of the important physiological functions mediated by auto-α_{2a}ARs is the inhibition of neurotransmitter release at the presynaptic terminal (Fig. 15). As previous described, auto-α_{2a}ARs inhibit neurotransmitter release through the interaction between $G\beta\gamma$ and SNARE. To test whether auto-α_{2a}ARs and presynaptic hetero-α_{2a}ARs utilize different $G\beta\gamma$ dimers upon activation, we again applied the quantitative MRM method to SNAP25 coIPed samples from α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes (see Experimental Procedures for details). Similarly to the previous experiment, α_{2a}AR KO no epi and FLAG-α_{2a}AR no epi samples were used as controls to identify nonspecific interactions, and α_{2a}AR KO +epi samples were used to detect non-α_{2a}AR-mediated $G\beta\gamma$-SNARE interactions.

Although it is a preliminary study, we were able to detect a subset of $G\beta$ and $G\gamma$ subunit interactions with SNARE upon auto-α_{2a}AR activation. Comparing the results from α_{2a}AR KO +epi and FLAG-α_{2a}AR +epi synaptosomes, $G\beta_1$, but not $G\beta_4$, seems to be enriched with SNARE following receptor activation (Fig.25A and C). With preliminary data, it is difficult to say whether $G\beta_2$ specifically interacts with SNARE upon auto-α_{2a}AR activation (Fig. 25B). It needs to be re-examined with an increase in sample size. As expected from the Western blot analysis, $G\beta$ subunits, especially $G\beta_1$ and $G\beta_2$, were also detected without epi stimulation in both α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes (Fig.25A and B). A comparison between these results and the results from IgG controls will be needed to validate and quantify the basal $G\beta\gamma$–SNARE interaction. Previously, we have shown that this basal interaction is not an artifact from the DSP crosslinker (Fig. 22D). Further studies, such as GPCR antagonist and SNAP25 mutation studies, will be needed to validate and understand the basal interaction.

In contrast to the 3 Gγ subunits enriched with SNARE upon stimulation of both auto- and hetero-α_{2a}ARs, we were only able to detect a trend of enrichment of $G\gamma_3$ with SNARE following auto-α_{2a}AR activation (Fig. 26). Similarly to the Gβ subunits (Fig. 25), we detected $G\gamma_3$ even in the unstimulated conditions of both genotypes. Although the sample size has to be increased, $G\beta_1\gamma_3$ may be one of Gβγ dimers modulating synaptic transmission through the Gβγ-SNARE interaction upon auto-α_{2a}AR stimulation. In addition, further biochemical analysis will be needed to validate the presence of these Gβγ dimers and their specificities with SNARE upon auto-α_{2a}AR activation.

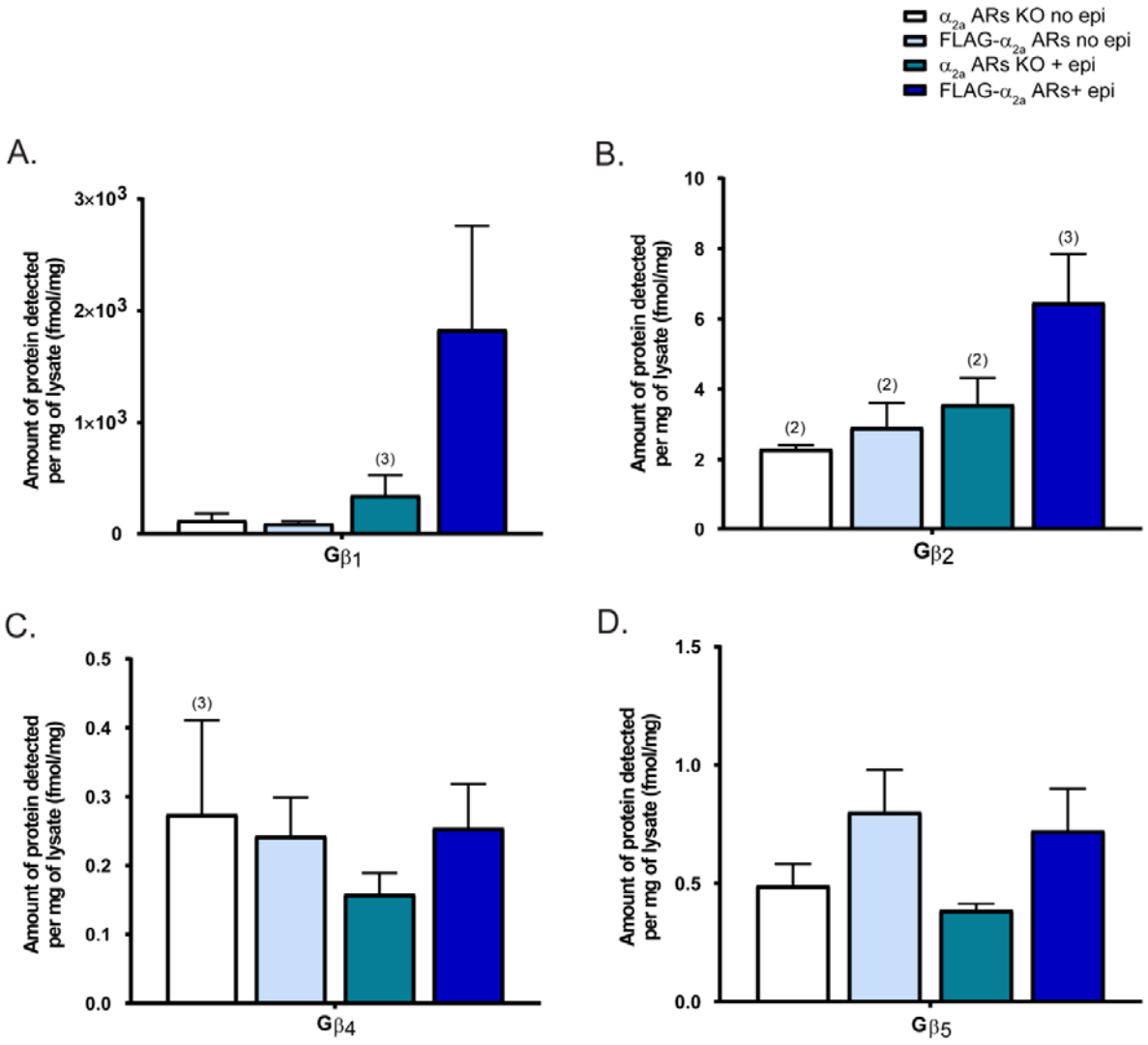

Figure 25. Gβ specificity to SNARE upon auto-α_{2a}ARs activation.

Quantification of Gβ subunits interacting with SNARE in α_{2a}AR KO and FLAG-α_{2a}AR synaptosomes (N=4, otherwise noted). $G\beta_1$ may specifically interact with SNARE with auto-α_{2a}AR activation. Data are presented as mean ± SEM.

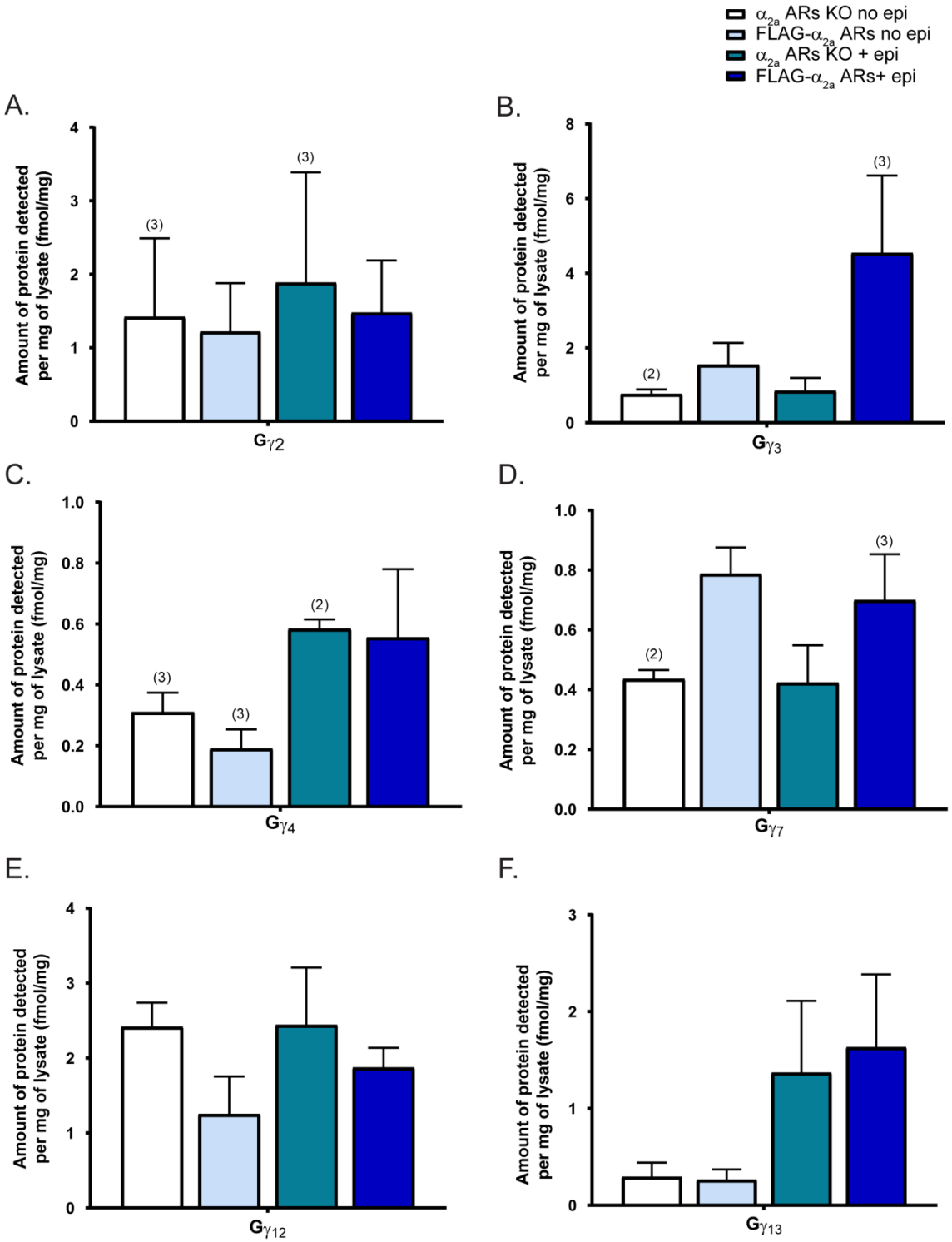

Figure 26. Gγ subunit specificity to auto-α_{2a} adrenergic receptors.

Quantification of Gγ subunits interacting with SNARE in α_{2a}ARs KO and FLAG-α_{2a}AR synaptosomes (N=4, otherwise noted). $G\gamma_3$ specifically interacts with SNARE upon auto-α_{2a}AR activation. Data are presented as mean ± SEM.

4.4 Discussion

Numerous *in vitro* studies have been performed to determine the effectors of Gβγ and their downstream signaling cascades; however, only a few studies have attempted to identify the specificity of Gβγ isoforms to their effectors. Here, we addressed the specificity of Gβγs to SNARE, as a part of the Gβγ-SNARE interaction that modulates synaptic transmission. Preliminary data suggest that Gβγs may interact with SNARE regardless of receptor activation, and only a subset of neuronal Gβγ dimers, which can be formed with $G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$, can interact with SNARE (Fig. 23 and 24) following presynaptic α_{2a}AR activation. Auto-α_{2a}ARs may utilize only $G\beta_1\gamma_3$ to inhibit neurotransmitter release through the Gβγ-SNARE interaction.

Although we cannot be definitive as to which Gβ and Gγ subunits or which Gβγ dimers are physiologically relevant with the preliminary data, we can speculate which possible Gβγ dimers may be important for Gβγ–SNARE interactions. From our results, $G\beta_1\gamma_3$ may be specific for auto-α_{2a}AR-mediated Gβγ–SNARE interactions, while $G\beta_4\gamma_2$ and $G\beta_4\gamma_4$ may be specifically utilized by presynaptic hetero-α_{2a}ARs. In previous biochemical studies, the formation of these dimers was observed *in vitro* (Hildebrandt 1997, Smrcka 2008b, Stephens 2009, Dingus and Hildebrandt 2012a, Pronin and Gautam 1992b). However, further studies are needed to validate their presence in *in vivo*. Interestingly, $G\beta_1$ was determined to specifically interact with SNARE (Fig.23), although it was not selective for auto-α_{2a}ARs (Fig.20.A). Moreover, $G\gamma_2$ and $G\gamma_4$ seem to interact with hetero-α_{2a}ARs, although it was not selective for hetero-α_{2a}ARs (Table 5). We hypothesize that the abundance and affinity of each neuronal Gβ and Gγ subunits, and their localizations at the presynaptic terminal may affect Gβγ subunit specificities to SNARE. Although we were

not able to detect a statistically significant interaction between the receptor and these subunits in previous studies, a small subset of α_{2a}ARs interacting with these dimers may be dominant players in Gβγ-SNARE interaction-mediated modulation of synaptic transmission. Because we were not able to obtain any statistical significance in this preliminary study, it is also difficult to fully understand these phenomena. Once the sample size is increased, and our results achieve statistical significance, we can further discuss these phenomena.

The specificity of Gβγ dimers to the SNARE complex is not fully understood, but its importance and the relationship of GPCRs to this interaction have been well-studied. *In vitro* binding studies of calcium-triggered exocytosis demonstrated that different Gβγ dimers may exhibit functional differences in their ability to modulate SNARE activity (Blackmer et al. 2005). Recently, Hamid et al demonstrated that $5HT_{1b}$ and $GABA_B$ receptors inhibit exocytotic release at the same synaptic terminal through two different mechanisms (Hamid et al. 2014). This study reported that $5HT_{1b}$ receptor-mediated inhibition of exocytosis used the Gβγ-SNARE interaction, while $GABA_B$ receptor-mediated inhibition exclusively used the Gβγ-VDCC interaction. The authors suggested that this difference may depend on the N-terminal SNAP25 residues. The association of specific Gβγ subunits to GPCRs, such as $5HT_{1b}$ and $GABA_{B,}$ and to SNARE may add a further layer of regulating Gβγ-effector selectivity. Although our study focuses on α_{2a}AR-mediated Gβγ-SNARE interactions, it will be interesting to determine if other α_{2a}AR-mediated Gβγ-effector interactions use identical Gβγ dimers. Furthermore, we would like to know whether the same Gβγ dimers modulate Gβγ-SNARE interactions through other $G_{i/o}$-coupled GPCRs. This knowledge would be helpful to determine if Gβγ variants are

important for both receptor and effector binding.

In both Western blot and quantitative MRM studies, we detected Gβγ-SNARE interactions in the basal state, without receptor stimulation. Because the 'no epi' condition was identical to it of previous receptor study, and it was clear that the α_{2a}AR did not basally interact with Gβ (Fig.16B), it is surprising to see this basal interaction of Gβγ and SNARE (Fig.25 and 26). As we eliminated the possibility that this effect was an artifact of the DSP crosslinker, this finding may still be caused by the close apposition between Gβγ and SNARE at the membrane (Fig.22D). The detection of Gβ and Gγ subunits with SNARE prior to receptor activation in FLAG-α_{2a}ARs suggests that Gβγ may be pre-coupled to SNARE before receptor activation. The fast rate of Gβγ-SNARE-mediated inhibition also supports the hypothesis that Gβγ, SNARE, and other synaptic proteins may be scaffolded together prior to receptor activation to facilitate Gβγ-SNARE interaction.

Previously, we identified the Gβγ binding sites on both amino- and carboxy-terminal regions of SNAP25 (Blackmer et al. 2005, Yoon 2007, Wells et al. 2012), far enough apart to not both be occupied by a single Gβγ. So far, the physiological importance of these sites remains unknown (see Chapter 5 for more details). We might speculate that the two sites may have different functions; the amino terminal region may be a Gβγ binding (or "holding") site that is less physiologically important than the carboxy-terminus (Wells et al. 2012). Potentially, activation of α_{2a}ARs could produce a conformational change to promote Gβγ translocation from the amino terminus to the carboxy terminus, to compete with Syt1 to inhibit exocytosis. It will be interesting to determine the stoichiometry of Gβγ binding to SNARE complexes, and whether identical Gβγ dimers bind to both amino- and carboxy-termini of SNARE. Detailed proteomic analysis in

combination with SNAP25 mutagenesis studies will be necessary to answer these questions.

4.5 Conclusions

Here, we report that only a subset of Gβγ dimers made with $G\beta_1$, $G\beta_2$, $G\beta_4$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ may specifically interact with SNARE upon presynaptic α_{2a}AR activation in both adrenergic and non-adrenergic neurons. $G\beta_1$ and $G\gamma_3$ may interact with SNARE upon auto-α_{2a}AR activation. This implies that the specificity of Gβγ subunits in binding their effectors may further mediate the selection of signaling pathways. Moreover, the Gβγ-SNARE interaction may exist basally. Although further studies are needed, this basal interaction may be necessary for the fast inhibition of synaptic transmission.

CHAPTER 5

THE MICROARCHITECTURE OF SYNAPTIC TRANSMISSION: Gβγ, SNARE, AND SYNAPTIC PROTEINS

5.1 Introduction

Exocytosis is a complex, regulated process involving the exocytotic machinery proteins, synaptic proteins that play roles in docking and priming the vesicle, ion channels, calcium sensors, and pre-synaptic inhibitory G-protein coupled receptors (GPCRs). Despite numerous attempts to understand the synaptic molecular composition and function, the microarchitecture of exocytosis with various modulators and synaptic proteins at each step has still remained unclear.

To map and profile the structural and functional dynamics of the presynaptic compartments, proteomic analyses of synaptosomes are widely used as a first step (Witzmann et al. 2005, Schrimpf et al. 2005, Corti et al. 2008, Bai and Witzmann 2007, McClatchy et al. 2007). For example, Wilhelm et al. generated a 3D model of 60 vesicle trafficking proteins, such as SNAP25, VAMP2, syntaxin1, CSP, complexin1/2, and Munc18. In an average synaptosomes from rat`s brain, they estimated the presence of 300,000 proteins per average presynaptic terminal (Wilhelm et al. 2014). These neuroproteomic studies allow a global assessment of the total synaptosome proteome estimated to contain over 1000 different types of proteins (Collins et al. 2006) and serves

as a complementary method to detect and measure the protein abundances in biological systems in addition to western blot analysis. Although it is technically difficult to detect hydrophobic and membrane-bound proteins such as receptors, ion channels, and the molecular machinery for synaptic vesicle cycling (Bai and Witzmann 2007), synaptosomal proteomics, in combination with specific protein enrichment and complexity reduction methods, is by far the best approach to survey various synaptic proteins at once and compare any changes of synaptic proteins` abundance in both normal and neuropsychiatric and neurodegenerative diseases.

At presynaptic terminals, Gβγ is an important regulator of neurotransmission through interactions with calcium channels and the SNARE complex as descried earlier (Blackmer et al. 2005, Blackmer et al. 2001, Gerachshenko et al. 2005, Betke, Wells, and Hamm 2012a). Briefly, it can bind directly to ternary SNARE (a trimer of SNAP25, syntaxin 1A, and synaptobrevin (VAMP) and t-SNARE dimer (SNAP25 with syntaxin 1A) (Blackmer et al. 2005, Yoon, Hamm, and Currie 2008, Delaney, Crane, and Sah 2007b, Zhang, Upreti, and Stanton 2011, Zhao et al. 2010, Blackmer et al. 2001, Gerachshenko et al. 2005, Photowala et al. 2006a, Yoon et al. 2007), and it competes with Syt1 for SNARE binding to inhibit the fusion of synaptic vesicles. In our recent study understanding the specificity of Gβγ to SNARE (see chapter 4 for more details), we detected the basal Gβγ-SNARE interaction and developed a hypothesis that other synaptic proteins may be in complex with Gβγ and SNARE in the "microarchitecture of exocytosis." However, the specific Gβγ binding sites on each SNARE protein, SNAP25, syntaxin 1A, and VAMP, and the interaction of Gβγ and other synaptic proteins are not well understood.

The Gβγ-SNAP25 interaction is the most well documented of the three SNARE

proteins. With botulinum toxin A (BoNT/A) that cleaves the C-terminus of SNAP25 and a 14-amino acid peptide of the C-terminus of SNAP25, the carboxy(C)- terminus of SNAP25 was found to be important for its exocytotic function and its interaction with Gβγ (Yoon et al. 2007, Gerachshenko et al. 2005, Zhao et al. 2010, Blackmer et al. 2005, Binz et al. 1994, Schiavo et al. 1993). Molecular studies showed that a 1.8 fold decrease in affinity between $G\beta_1\gamma_1$ and SNAP25 when its C- terminus 9 amino acids were removed as compared to the wild type SNAP25, but a complete loss of binding when the C-terminus 26 amino acids were removed, suggesting that a major binding site of Gβγ was within those C- terminus 26 amino acids (Yoon et al. 2007). In addition, ternary SNARE made with the loss of 9 C-terminus residues on SNAP25, VAMP and syntaxin1A was more sensitive to syt1 binding than Gβγ compared to the SNARE with wildtype SNAP25 (Yoon et al. 2007). A 14-amino acid peptide (193-206) of human SNAP25 also blocked the Gβγ mediated inhibition of synaptic transmission (Blackmer et al. 2005, Gerachshenko et al. 2005). Although the importance of C- terminus of SNAP25 was found, SNAP25 residues involved in the Gβγ-SNARE interaction still remain unclear.

To further understand the basal Gβγ-SNARE interaction and its microarchitecture with other synaptic proteins with and without α_{2a}ARs activation, here we attempt to map the binding site of Gβγ and SNAP25 interaction and detect other synaptic proteins which may exist as a scaffold protein with Gβγ and SNARE using various transgenic mice, peptide arrays, coIP, and discovery proteomics. Although the Gβγ-SNARE co-crystallization would be the best approach to determine the interaction sites, peptide arrays, followed by the alanine screening and other biochemical methods to test identified residues in the context of the full-length proteins for their functional role in the interaction, will be

used due to the difficulty expressing and purifying neuronal Gβγ, such as $G\beta_1\gamma_2$. Moreover, the discovery proteomics on Gβγ-SNARE coIP samples will use as a screen to scan for synaptic proteins that may be involved in this interaction.

5.2 Experimental Procedures

Animals. Adult, male FLAG-tagged alpah2a adrenergic receptors (α_{2a}ARs)(Gilsbach et al. 2009a), α_{2a}ARs knockout (KO), SNAP25Δ3 transgenic (Zurawski et al. 2017), and wildtype mice 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. All drugs used in chapter 3 were used.

Plasmids. The open reading frame for SNAP25 was subcloned into the glutathione-S-transferase (GST) fusion vector pGEX-6p-1 (GE Healthcare) for expression in bacteria. Mutagenesis of SNAP25 was accomplished via the overlapping primer method. The SNAP25(8A) mutant was subcloned from pGEX-6p-1 into the pRSFDuet-1 plasmid, a dual expression vector that contains cDNAs for both full-length syntaxin 1A and SNAP25 that results in concomitant expression and formation of t-SNARE complexes (kindly provided by E. Chapman) Plasmids were verified to contain desired mutations via Sanger sequencing utilizing BigDye Terminator dyes and resolved on an ABI 3730 DNA Analyzer (Applied Biosystems).

Antibodies. For the immunoprecipitation, rabbit anti-SNAP25 (Sigma, S9684) and rabbit

ChromePure IgG (Jaskson Immuno Research, 011-030-003) were used. For the westernblot analysis, mouse anti-SNAP25 (Santa Cruz, sc-376713, 1:500), rabbit anti-Gβ (Santa Cruz, sc-378, 1:10,000 and 1:5,000), mouse anti-synaptotagmin1(Synaptic Systems, 105-011, 1:1,000), rabbit anti-$G\alpha_o$ (Santa Cruz, K-20, 1:200), and rabbit anti-$G\alpha_i$ (Abcam, ab-3522, 1:1,000) 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). Anti-GST (goat) Antibody DyLight™ 800 Conjugated (-145-200) and the anti-mouse IgG (goat, H&L) Antibody IRDye700DX® Conjugated Pre-adsorbed (-130-121) were both from Rockland Immunochemicals, Inc.

The expression and purification of SNARE proteins. Recombinant bacterially-expressed glutathione-S-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain Rosetta 2 (EMD Biosciences) and lysed by a sonic dismembranator with the lysis buffer (25mM potassium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (HEPES-KOH) pH 8.0, 150mM KCl, 5mM βME, 10.66 μM leupeptin, 1.536 μM aprotenin, 959 nM pepstatin, 200μM phenylmethylsulfonyl fluoride, and 1mM ethylenediaminetetracetic acid (EDTA)) at 4°C for 5 min. Lysates were spun at 26,000 x g for 20 min in a TI-70 rotor (Beckman Coulter) and supernatant was further purified by affinity chromatography by GE Sepharose 4 FastFlow (GE Healthcare). GST-SNAP25 was eluted with the elution buffer (25mM HEPES-KOH pH 8.0, 150mM KCl, 5mM βME, 0.5% n-octylglucoside, 1mM EDTA, 10% glycerol) and GST is cleaved via proteolytic cleavage with a GST-tagged fusion of rhinovirus 3C protease. Protein concentrations were determined with a Bradford assay kit (Pierce) and purity was verified by SDS-PAGE analysis. For the t-SNARE with full-length

syntaxin, this was expressed using the tandem vector (pRSFDuet-1) previously characterized by Chicka et al. (Chicka et al. 2008). Purification of this SNAP25/syntaxin 1A dimer was performed as previously described (Tucker, Weber, and Chapman 2004). For purification, a 6xHis tag is present upon the N-terminus of SNAP25.

Gβγ Purification. $G\beta_1\gamma_1$ was purified from bovine retina as described previously (Mazzoni, Malinski, and Hamm 1991a). Recombinant $G\beta_1\gamma_2$ was expressed in Sf9 cells and purified via a 6xHis tag on $G\gamma_2$ using Talon™ immobilized metal affinity chromatography (Clontech).

Peptide array synthesis. Peptide array synthesis was performed using the Respep SL (Intavis AG) according to standard SPOT synthesis protocols (Frank 2002, Eaton et al. 2008, Yim, Betke, and Hamm 2015). Human SNAP25 sequence, P60880 (UniprotKB/Swiss Protein Database), was used, and peptides of 15 residues in length were synthesized on membranes. Once the synthesis was done, the membranes were stored in 4°C. For the alanine-mutagenesis screening of peptides, the 14 residue peptides were synthesized.

Peptide membrane Far-Western. Membranes were washed with ethanol and rehydrated with water. Then, the membrane was blocked with 5% Milk in TBST (0.1%) for 1 hr at room temperature and washed with TBST (0.1%). To understand the SNAP25 and $G\beta_1\gamma_1$ interaction, the membrane was incubated overnight with 0.44nM $G\beta_1\gamma_1$ in a binding buffer of 20 mM HEPES, pH 7.5, and 5% glycerol at 4°C after the blocking in 5% Milk in TBST (0.1%). Next day, the peptide membranes were treated as if they were nitrocellulose protein transferred membranes for the western blot analysis as described in chapter 3 (Yim, Betke, and Hamm 2015).

GST-pull down Assay. The 5 μg of GST-SNAP25 and GST-SNAP25 mutants

immobilized on GST resin were incubated with 400μM of the C2AB domain of synaptotagmin-1 (residues 96-422) for 1 h at 4°C and washed three times with assay buffer (20 mM HEPES, pH 7.0, 80 mM KCl, 20 mM NaCl, and 0.1% n-octylglucoside) in a 1.5 mL Eppendorf tube. After the 2nd wash, the tube was changed to reduce non-specific binding. After the completion of washes, the complex was eluted with 20 μl of SDS sample buffer followed by separation via SDS-PAGE.

Synaptosome preparation, stimulation, and lysate protocol. As described in chapter 3, all crude synaptosomes were prepared, stimulated, and lysed.

Co-immunoprecipitation, elution, TCA precipitation, and immunoblot analysis. As described in chapter 4, the co-immunoprecipitation (coIP) of Gβγ and SNARE were prepared, eluted, TCA precipitated and western blotted. The western blots of GST-pulldown assays were imaged using the Licor Odyssey imager (Licor Biosciences).

Discovery proteomics The Gβγ-SNARE coIP samples were run on 12.5% SDS-PAGE gel as described in chapter 4, but the whole gels was trypsin digested instead of excising the Gβ and Gγ bands only. Because SNAP25 antibody was not conjugated to the beads, heavy and light IgG chains were cut out and run separately to increase the detection possibility of low abundance proteins. Then, peptides were analyzed by LC-coupled tandem mass spectrometry (LC-MS/MS). An analytical column was packed with 20cm of C18 reverse phase material (Jupiter, 3 μm beads, 300Å, Phenomenox) directly into a laser-pulled emitter tip. Peptides were loaded on the capillary reverse phase analytical column (360 μm O.D. x 100 μm I.D.) using a Dionex Ultimate 3000 nanoLC and autosampler. The mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). Peptides were gradient-eluted at a flow rate of 400

nL/min using either a 95-minute or 120-minute LC gradient. The 95-minute gradient consisted of the following: 1-2min, 2% B (sample loading from autosampler); 2-18 min, 2-10% B; 18-78 min, 10-40% B; 78-80 min, 40-90% B; 80-81 min, 90% B; 81-84 min, 90-2% B; 84-95 min (column re-equilibration), 2% B. The 120-minute consisted of the following: 1-2min, 2% B (sample loading from autosampler); 2-95 min, 2-40% B; 95-105 min, 40-90% B; 105-106 min, 90% B; 106-108 min, 90-2% B; 108-120 min (column re-equilibration), 2% B. A Q Exactive mass spectrometer (Thermo Scientific), equipped with a nanoelectrospray ionization source, was used to mass analyze the eluting peptides using a data-dependent method. The instrument method consisted of MS1 using an MS AGC target value of 1e6, followed by up to 20 MS/MS scans of the most abundant ions detected in the preceding MS scan. A maximum MS/MS ion time of 100 ms was used with a MS2 AGC target of 5e4. Dynamic exclusion was set to 20s, HCD collision energy was set to 26 nce, and peptide match and isotope exclusion were enabled. For identification of peptides, tandem mass spectra were searched with Sequest (Thermo Fisher Scientific) against a *Mus musculus* database created from the UniprotKB protein database (www.uniprot.org). Variable modification of +15.9949 on Met (oxidation) and +57.0214 on Cys (carbamidomethylation) were included for database searching. Search results were assembled using Scaffold 4.3.2. (Proteome Software), where a 2% false discovery rate (FDR) protein threshold and a minimum peptide probability of 95% were required for protein and peptide identifications.

Protein structure visualization. All representatives of protein structure were made using the computer program Pymol (Schrodinger 2010).

Statistical analysis. Images were analyzed for densitometry using ImageJ (available from http://rsbweb.nih.gov/ij/index.html). All statistical tests were performed using GraphPad

Prism v.4.0 for Windows, (GraphPad Software, La Jolla, California, USA, www.graphpad.com) and R, (R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/).

5.3 Results

SNAP25 peptide array to identify the Gβγ-SNAP25 interaction. In previous study, we have found that the removal of varying portions of the C-terminus of SNAP25 diminishes or disrupts binding between Gβγ and SNAP25 (Yoon et al. 2007). However, the interaction residues were not fully determined. To fully investigate the determinants of interaction between Gβγ and SNAP25, we first searched for small linear sequences from SNAP25 that would interact with Gβγ.

With a peptide synthesizer (Respep SL, Intavis), we generated a sequential series of 15-mer peptides shifting 3 amino acids for each successive peptide spot (1-15, 4-18, 7-21, etc.) from the entire sequence of SNAP25 on a membrane. Then, the membrane was exposed to $G\beta_1\gamma_1$, washed, and probed as a western blot with Gβ antibody and a secondary antibody for a chemiluminescent assay (Fig.27A). For positive controls, peptides that were previously reported to bind Gβγ, such as SIRK (SIRKALNILGYPDYD) (Scott et al. 2001), QEHA (QEHAQEPERQYMHIGTMVEFAYALVGK) (Weng et al. 1996), the C-terminus of βARK (WKKELRDAYREAQQLVQRVPKMKNKPRS) (Koch et al. 1993)), the Gβγ-binding sequence from the calcium channel Cav2.2 (GID site: KSPLDAVLKRAATKKSRNDLI) (De Waard et al. 2005) and the epitope of Gβ antibody, were used. In addition, negative control were determined by understand the non-specific

binding of $G\beta_1\gamma_1$ or the Gβ antibody with the membrane alone (Fig. 27B). We found several clusters of consecutive peptides of SNAP25 interacting with $G\beta_1\gamma_1$ (Fig. 27C). Across 65 peptides, SNAP25 residues 49-75, 82-108, 121-144, 145-168, and 184-206 showed the interaction with $G\beta_1\gamma_1$ (Red circle, Fig.27C).

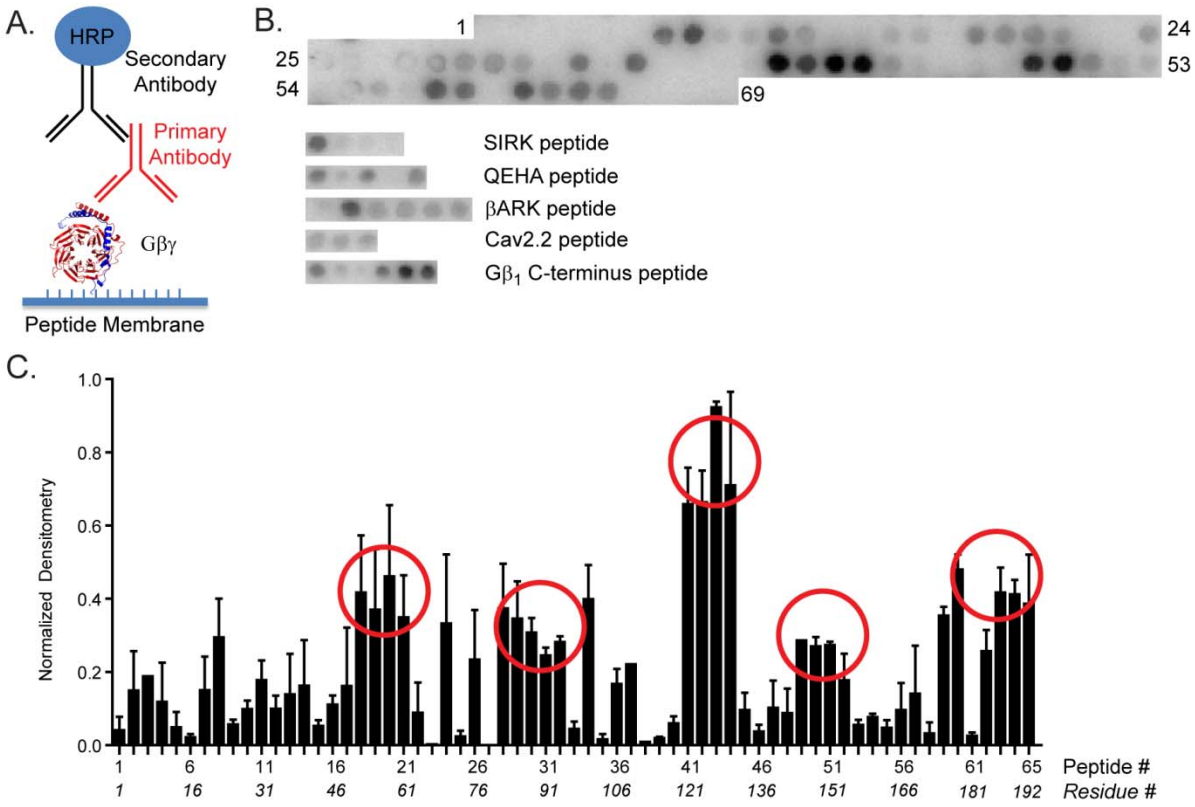

Figure 27. Screen of the $G\beta_1\gamma_1$-SNAP25 interaction.

Peptide array membrane was incubated with $G\beta_1\gamma_1$, then primary and secondary antibodies just like the western blot to understand the interaction (**A**) (see experimental procedures for more details). Representative peptide array of SNAP25 was shown (**B**). Numbering reflects spots with successive peptides 1-65. The sequences for the SIRK peptide, QEHA peptide, βARK peptide, the Gβγ binding domain of the calcium channel CaV2.2, and the C-terminus of $G\beta_1$ were used as positive controls. No peptides were made on spot 66-69 and used as negative controls. Densitometry of these peptide arrays suggested 5 clusters of SNAP25 regions that may be important for the $G\beta_1\gamma_1$-SNAP25 interaction (N=3) (**C**). Figure adapted from Wells, C.A.et al. 2012 *Mol. Pharm*. 82:1136-1149.

Based on these results, the five peptides that interacted with $G\beta_1\gamma_1$ (Fig. 27) were then subjected to an alanine mutagenesis. In this screen, we compared each non-mutated, wildtype SNAP25 peptide with an alanine mutated peptide. For each peptide, 14 mutated peptides were made. Each residue of 14-mer peptide was mutated to alanine one by one individually creating 14 spots on a membrane (Fig. 28A). Comparing to the wildtype peptides, we identified the nine amino acids that show the loss of binding in the $G\beta_1\gamma_1$-SNAP25 interaction (Fig. 28B). The identified nine amino acids were mapped to the crystal structure of the ternary SNARE, complex of SNAP25, syntaxin 1A, and VAMP(Sutton et al. 1998a) (Fig. 28C). The ternary SNARE complex is depicted in a "primed" state with a docked vesicle that has not yet fused, which is the proposed location and state that would bind Gβγ (Blackmer et al. 2005, Yoon et al. 2007). These amino acids were located at both N- and C-termini of ternary SNARE (Fig. 28C). Identified C-terminus residues well correlated with the previous finding of Gβγ interaction site on the C-terminus of SNAP25 (Yoon et al. 2007) including two residues within the extreme C-terminus of SNAP25: Arg198 and Lys201. In addition, Asp99 and Lys102 in the linker region between the two helices of SNAP25 in close proximity to the palmitoylation sites of SNAP25 (Cys 85, 88, 90, and 92) may be important for this interaction. Interestingly, Gly63 and Met64 that had significant reduction in binding (Fig. 28B) actually are buried in the interface with syntaxin 1A and VAMP. These two residues were omitted from further mutagenesis studies with full-length SNAP25 protein.

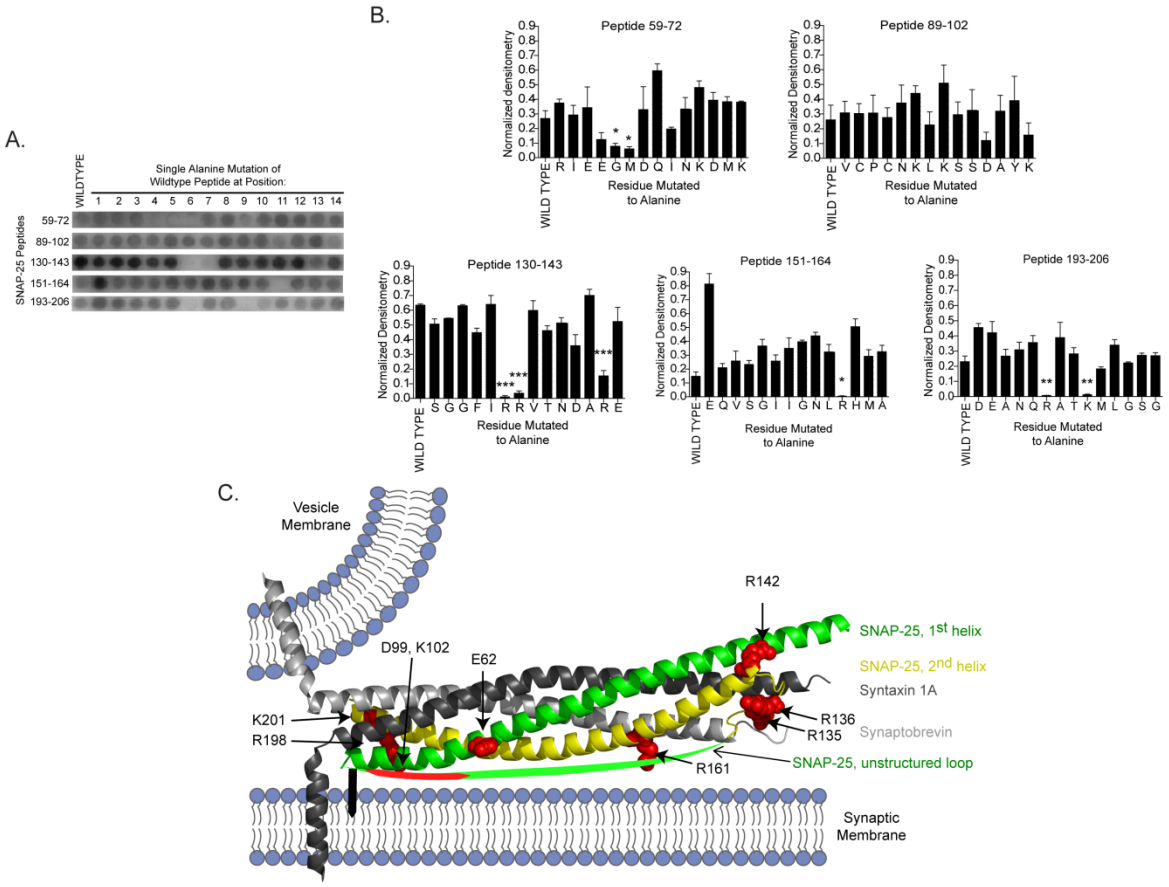

Figure 28. Alanine mutagenesis screening of SNAP25 peptides interacting with $G\beta_1\gamma_1$.

Representative image of the alanine screening for five identified 14-mer SNAP25 peptides synthesized on a membrane (**A**). 14 peptides were made for each peptide by a single alanine replacement of the residue at position 1, 2, 3…14 for each wild type peptide. Densitometry was performed for each respective peptide and its series of mutants (N=3) (**B**). The residues (spheres, red) important for the $G\beta_1\gamma_1$-SNAP25 binding were mapped onto the x-ray crystal structure of ternary SNARE (PDB ID: 1sfc) made with syntaxin 1A (dark gray), VAMP (light gray), first SNAP25 helix (green), second SNAP25 helix (yellow), and unstructured domain between the two SNAP25 α-helices (green cartoon arc) (**C**). Data were presented as mean ± SEM and compared by a Student's t-test, *P<0.05, **P<0.01, and ***P<0.01. Figure adapted from Wells, C.A.et al. 2012 *Mol. Pharm.* 82:1136-1149.

The importance of C- and N-termini SNAP25 in the SNAP25–$G\beta_1\gamma_1$ interaction. We examined the nine residues of SNAP25 determined from the peptide array by expressing and purifying nine different, full-length, mutated SNAP25 proteins (Table 6). We made a c-terminus mutant first as it was implicated to be important in the Gβγ and t-SNARE interaction (Yoon et al. 2007). Then, these mutants were tested with a sensitive, quantitative fluorescence assay with MIANS, an environmentally sensitive fluorescent probe, labeled $G\beta_1\gamma_1$. SNAP25 2A-5A showed a decrease in affinity of $G\beta_1\gamma_1$ while SNAP25 6A-9A had a limited enhancement of affinity (data not shown). Mutations of full-length SNAP25 suggest a complex binding mechanism between SNAP25 and Gβγ that extends beyond the C-terminus of SNAP25. Moreover, only the C- terminus peptide ($SNAP25_{193-206}$) was able to inhibit the $G\beta_1\gamma_1$-SNAP25 interaction (data not shown). This is the same peptide that blocked the serotonin-mediated inhibition (via Gβγ) in lamprey central synapses (Gerachshenko et al. 2005). Interestingly, a modified $SNAP25_{193-206}$ peptide with R198A and K201A was unable to inhibit the SNAP25-mediated fluorescent enhancement of MIANS-$G\beta_1\gamma_1$ in the same fashion as the wild-type $SNAP25_{193-206}$ peptide. Lastly, t-SNARE made with SNAP25 8A and syntaxin 1A significantly reduced an affinity of $G\beta_1\gamma_1$ (data not shown).

Table 6. Mutagenesis of full-length SNAP25

Name	Residue of SNAP 25 mutated
WT	N/A
2A	R198A, K201A
3A	**E62A**, R198A, K201A
4A	E62A, **D99A**, R198A, K201A
5A	E62A, D99A, **K102A**, R198A, K201A
6A	E62A, D99A, K102A, **R135A**, R198A, K201A
7A	E62A, D99A, K102A, R135A, **R136A**, R198A, K201A
8A	E62A, D99A, K102A, R135A, R136A, **R142A**, R198A, K201A
9A	E62A, D99A, K102A, R135A, R136A, R142A, **R161A**, R198A, K201A

Effect of SNAP25 mutations on synaptotagmin1 binding. To test whether N-terminus of SNAP25 residues are important in exocytosis by interacting with Synaptotagmin1 (Syt1), the GST-fusion proteins of wild-type SNAP25 or the mutants 6A-9A immobilized to glutathione-sepharose beads were exposed to Syt1, either in the presence of the calcium chelator 2mM EGTA (calcium independent condition) or 1mM $CaCl_2$ (calcium dependent condition). Wild type SNAP25 bound to Syt1 in the absence of calcium and showed an increase of binding in the presence of 1mM calcium as expected (Fig. 29B). Previously, Syt1 was found to interact with SNAP25 and syntaxin 1A calcium independently and with t-SNARE calcium dependently (Brose et al. 1992, Chapman and Jahn 1994b, Geppert, Goda, Hammer, Li, Rosahl, Stevens, and Sudhof 1994, Chapman et al. 1995, Mehta, Battenberg, and Wilson 1996).

In calcium dependent conditions, no statistically significant decrease in Syt1 binding to SNAP25 mutants compared to wild type SNAP25 (Student's t-test, $p>0.05$) were found (Fig. 29B). However, we saw a decreasing trend of Syt1-SNAP25 interaction in calcium independent conditions. Specifically, mutant 9A had a significant reduction of Syt1 binding compared to the wild type SNAP25 ($p<0.01$) (Fig. 29B). This suggests that R161 may be important in calcium independent binding of Syt1-SNAP25. To test this, the single mutation of R161A was made in wildtype SNAP25, and pull down experiments were performed with Syt1 (Fig. 29A). Mutation of the R161 residue resulted in a significant decrease in binding of Syt1 in the absence of calcium compared to the wildtype SNAP25 ($p<0.01$); however, there was no difference between SNAP25 wildtype and SNAP25 (R161A) in the calcium dependent condition (Fig. 29C) similar to the result of SNAP25 9A.

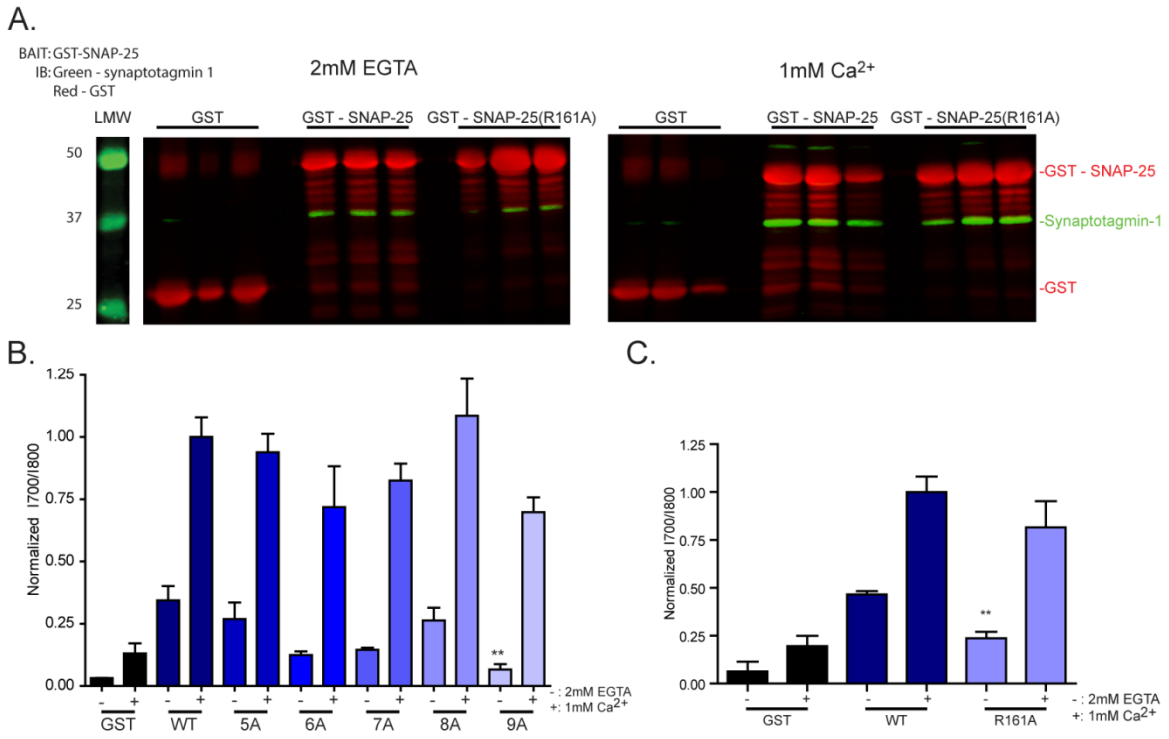

Figure 29. The interaction of SNAP25 mutants and synaptotagmin 1.

GST, GST-SNAP25 wildtype, GST-fused mutants SNAP25 6A through 9A, and GST-fused SNAP25 R161A were glutathione purified (N=3) (see experimental procedures for more details). Shown are representative blots imaged with Odyssey for simultaneous quantitation of Syt1 (green) and GST (red) signal intensity (**A**). The ratio of normalized Syt1 and GST signals for SNAP25 6A-9A (**B**) and R161A (**C**) were plotted in the bar graph. 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. Figure adapted from Wells, C.A.et al. 2012 *Mol. Pharm*. 82:1136-1149.

Gβγ, not Gαβγ heterotrimer, interacts with SNAP25. Using the peptide array and various biochemical assays, we were able to identify important C- and N- termini residues on SNAP25 for the Gβγ-SNAP25 interaction. However, C-terminus, not N-terminus, SNAP25 was involved in the Gβγ mediated inhibition of synaptic transmission. Moreover, we found the basal interaction of Gβγ-SNAP25 without the α_{2a}-ARs activation (see Chapter 4 for more details). Overall, our findings imply either a complex network or multiplicity in binding between the Gβγ, SNARE, and other synaptic proteins. One possibility is that Gβγ-SNARE may be pre-coupled and that other synaptic proteins are involved in this "microarchitecture of exocytosis."

To determine whether Gβγ and SNARE are pre-coupled prior to the receptor activation, we first examined the presence of Gα subunit to determine whether Gαβγ heterotrimer is interacting with SNARE. From FLAG-α_{2a}ARs and α_{2a}ARs KO mice, we coimunoprecipitated (coIPed) Gβγ and SNARE as described in chapter 4 and western blot for the $G\alpha_i$ and $G\alpha_o$ subunits. In both unstimulated and stimulated samples, we were unable to detect $G\alpha_i$ and $G\alpha_o$ subunits (Fig. 30). Gβγ, not Gαβγ, interacts with SNARE regardless of α_{2a}-ARs activation. Furthermore, FLAG-α_{2a}ARs was not present in the coIP samples (Fig. 30). This suggests that there may be an active Gβγ dimer interacting with SNARE without the α_{2a}ARs stimulation. Because the Gβγ mediated inhibition of neurotransmitter only happens with α_{2a}ARs stimulation, and the C-terminus of SNAP25 is important for this mechanism (Yoon et al. 2007), this active Gβγ found in the unstimulated condition may be bound at the N-terminus SNAP25. Further experiments will be needed to determine whether Gβγ is indeed bound at the N-terminus of SNAP25 in unstimulated conditions and which receptor releases this Gβγ.

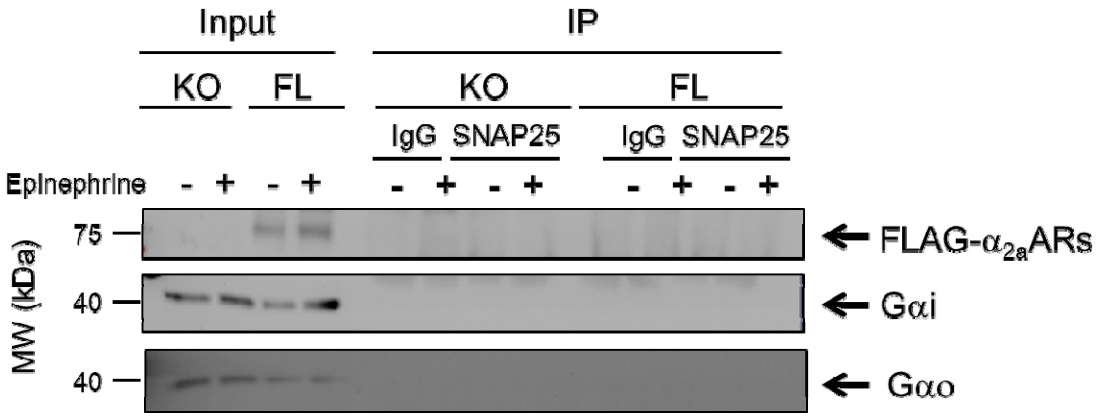

Figure 30. Gα subunits are not involved in the Gβγ-SNARE interaction.

Representative western blot of coimmunopreciptiation of SNAP25 and Gβγ in the FLAG-α_{2a}ARs (FL) and α_{2a}ARs KO (KO) mice following the stimulation of synaptosomes with resuspension (unstimulated/-) or stimulation buffers (stimulated/+, 100μM epinephrine). No Gα subunits and FLAG-α_{2a}ARs were in complex with the Gβγ and SNARE regardless of the receptor stimulation.

SNAP25 Δ3 mice exhibit changes in the level of synaptic protein. In numerous attempts, we understood the importance of SNAP25 and its C- terminus for the Gβγ mediated exocytosis inhibition (Yoon et al. 2007, Blackmer et al. 2005). However, we were limited to examine this interaction *in vitro* due to the neonatal lethality of SNAP25 knockout mice. To overcome this problem, we created a mouse partially deficient in Gβγ-SNARE interaction, the SNAP25Δ3 mouse that lacks 3 amino acids at its C-terminus using CRISPER/Cas9 interaction (Zurawski et al. 2017). In this mouse, tSNARE with SNAP25Δ3 still bound to Syt1 is similar to wildtype mice but has lost the competition of Gβγ with Syt1 for SNARE. In lipid assay, it had a decreased inhibition of fusion by Gβγ. The SNAP25Δ3 homozygote mouse showed a normal inhibitory postsynaptic response to the $G_{i/o}$-coupled $GABA_B$ receptor activation by baclofen, but inhibitory responses to α_{2A}AR or $5HT_{1b}$ agonists were no longer present. Moreover, it exhibits a number of perturbations of normal behavior upon challenges, including elevated stress-induced hyperthermia, impaired sensation of thermogenic pain, and locomotor impairment. Together, this mouse can be used to study the α_{2A}-ARs mediated Gβγ-SNARE interaction specifically and once again suggests the importance of $G_{i/o}$-GPCR mediated inhibition of exocytosis through the Gβγ-SNARE interaction in numerous neurological processes.

The expression levels of synaptic proteins were examined in wildtype and SNAP25Δ3 mice to evaluate whether the deletion of 3 residues on SNAP25 caused any changes in other synaptic proteins in synaptosomes and presynaptic terminal. Synaptosomes were made and fractionated as described in chapter 2. In whole synaptosomes, we did not see any changes in syt1, syt7, syntaxin1, VAMP2, and complexin1/2 (Fig. 31A). However, we detected a significant reduction of cysteine string

protein (CSP) and a trend of reduction in complexin 1/2 (Fig. 31B). Because the Gβγ-SNARE interaction was lost in SNA25Δ3 mice, and both CSP and complexin1/2 were found to associate with SNARE by either binding or competing with each protein of SNARE to modulate the exocytosis (Evans, Morgan, and Burgoyne 2003, Burgoyne and Morgan 2015, Nie et al. 1999, Maximov et al. 2009, Giraudo et al. 2009), we speculate that these proteins may be in complex with Gβγ and SNARE.

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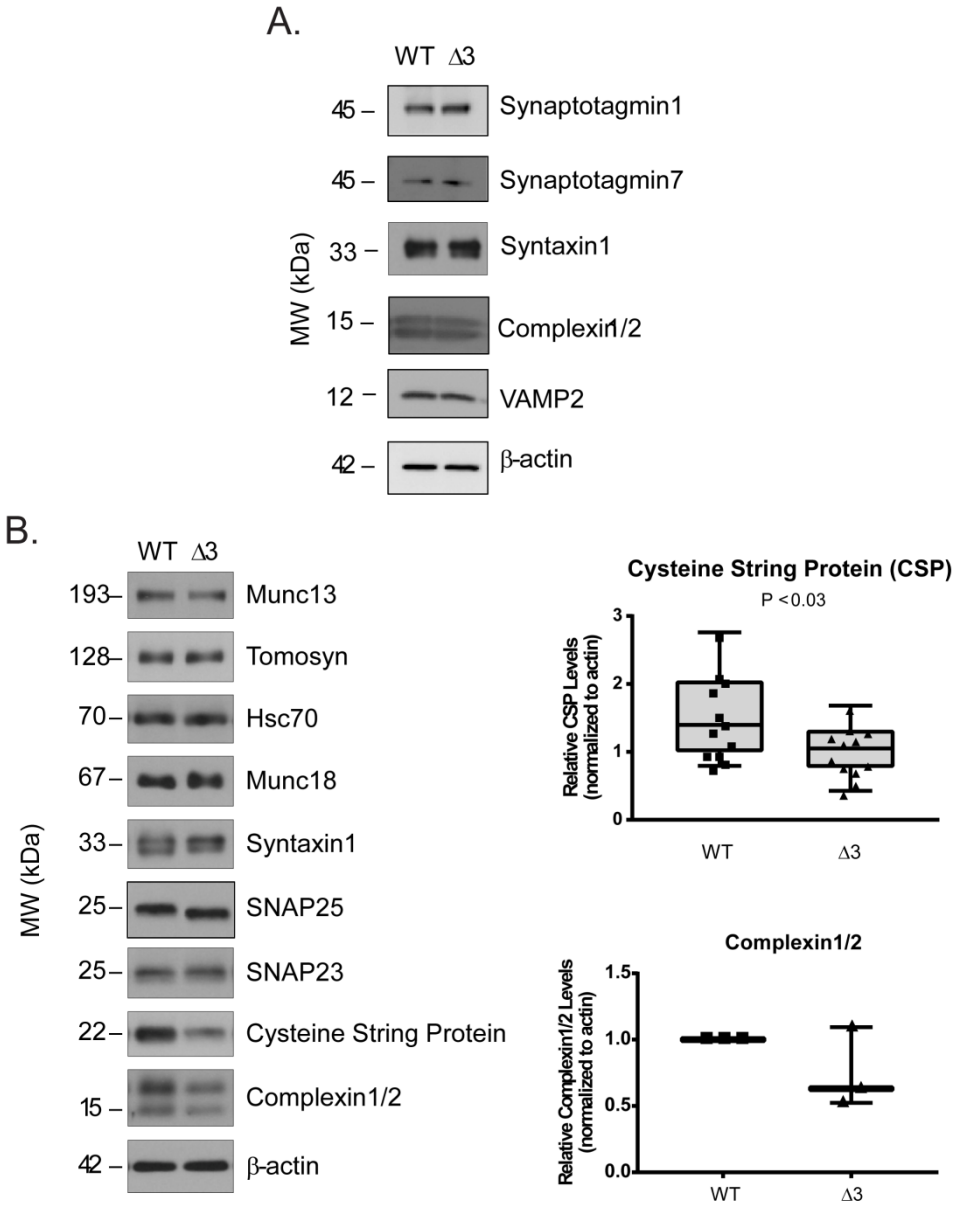

Figure 31. **Protein level of various synaptic proteins in wildtype and SNAP25Δ3`s synaptosomes.**

In whole synaptosomes, no change was detected as shown in representative western blots (N=4) (**A**). In presynaptic fraction, we also didn`t see the changes except cysteine string protein (CSP) (N=12) and compelxin1/2 (N=3) (**B**). For complexin 1/2, we detected a trend of a reduction but no significant was found. Data were presented as Min to Max showing all data points and compared by a Student T-test, P<0.03.

The detection of various synaptic proteins using the discovery based mass spectrometry. Using the western blot analysis comparing SNAP25 coIP from WT and SNAP25Δ3 mice, we were able to test proteins that may be in complex with Gβγ and SNARE to understand the microarchitecture of exocytosis. However, it is a very low throughput method compared to discovery based mass spectrometry. To screen for the changes in abundance of various synaptic proteins, we first prepared unstimulated presynaptic fraction of wildtype and SNAP25Δ3`s synaptosomes and coIPed Gβγ and SNARE using SNAP25 antibody as described in chapter 4. Then, the 11 coIPed samples per genotype were pooled and in-gel digested based on the molecular weight to reduce IgG bands perturbing the signals of low abundance proteins (see Experimental Procedures for more details). The result was analyzed by the fold change of each detected protein between SNAP25Δ3 and wildtype. Its statistical significance was then analyzed by Fisher`s exact test (Fig. 32). From the analysis, we identified 46 proteins that may be in complex with Gβγ-SNARE in the absence of receptor stimulation (Table 7). Various cytoskeletal, vesicle, and scaffold proteins, such as DmX-like protein 2 and WD repeat-containing protein 7, were found on this list. Interestingly, we detected 31 proteins with increases in abundance (log2 fold change>0) and 15 proteins with the decreases in abundance (log2 fold change <0). The proteins with increase in abundance may interact with SNARE in a competitive manner with Gβγ compared to those that show a decrease in abundance (see Discussion section for more details).

A reduction in dmx-like protein 2 (gene name Q8BPN8), also known as rabconnectin-3, was detected (Fig. 32). Enriched in synaptic vesicles, rabconnectin-3 has been identified to serve as a scaffold protein for Rab3 small G proteins, although the exact

mechanism of Rab3 action in exocytosis is not known (Nagano et al. 2002). As a known scaffold protein that forms a complex to modulate synaptic transmission, it may interact with either Gβγ or SNARE. Moreover, a reduction of spectrin was also found. Spectrin, also known as fodrin, is 240kDa and has an internal repeat of 106 amino acids with highly conserved amino acids at the certain sites(Zhang et al. 2013). Although an exact role of spectrin is not well defined, it interacts with calmodulin in a calcium dependent manner and actin (Perrin, Langley, and Aunis 1987, Glenney and Glenney 1983). Its relationship with actin implies a role in the structure of the cytoskeleton. It also is suggested to play a role in the calcium regulation of cytoskeletal dynamics(Sobue et al. 1983). Because the Gβγ-SNARE interaction is highly impacted by the concentration of calcium, it is interacting to detect spectrin.

Although the function is not well understood, we found an enhancement of the neuronal calcium binding protein 2 (NECAB2, Q91ZP9) interaction with the Gβγ and SNARE (Fig. 32). NECAB1 and 2 are expressed in brain while NECAB3 is in muscle(Sugita, Ho, and Sudhof 2002). In brain, both isoforms of NECAB2 exist, 39kDa and 43kDa, depending on the presence of an extrapolative translation initiation codon (Sugita, Ho, and Sudhof 2002, Canela et al. 2007). Interestingly, NECAB2 was detected to directly interact with adenosine A_{2a} receptor (A_{2a}ARs), and mGlu5 receptors which are known to heterodimerize with A_{2a}ARs (Ferre et al. 2002). Although these receptors are not $G_{i/o}$-coupled GPCRs, the direct interaction with receptors suggests that NECAB2 may be in complex with Gβγ and SNARE to modulate this interaction and that it may involve other GPCRs or G proteins.

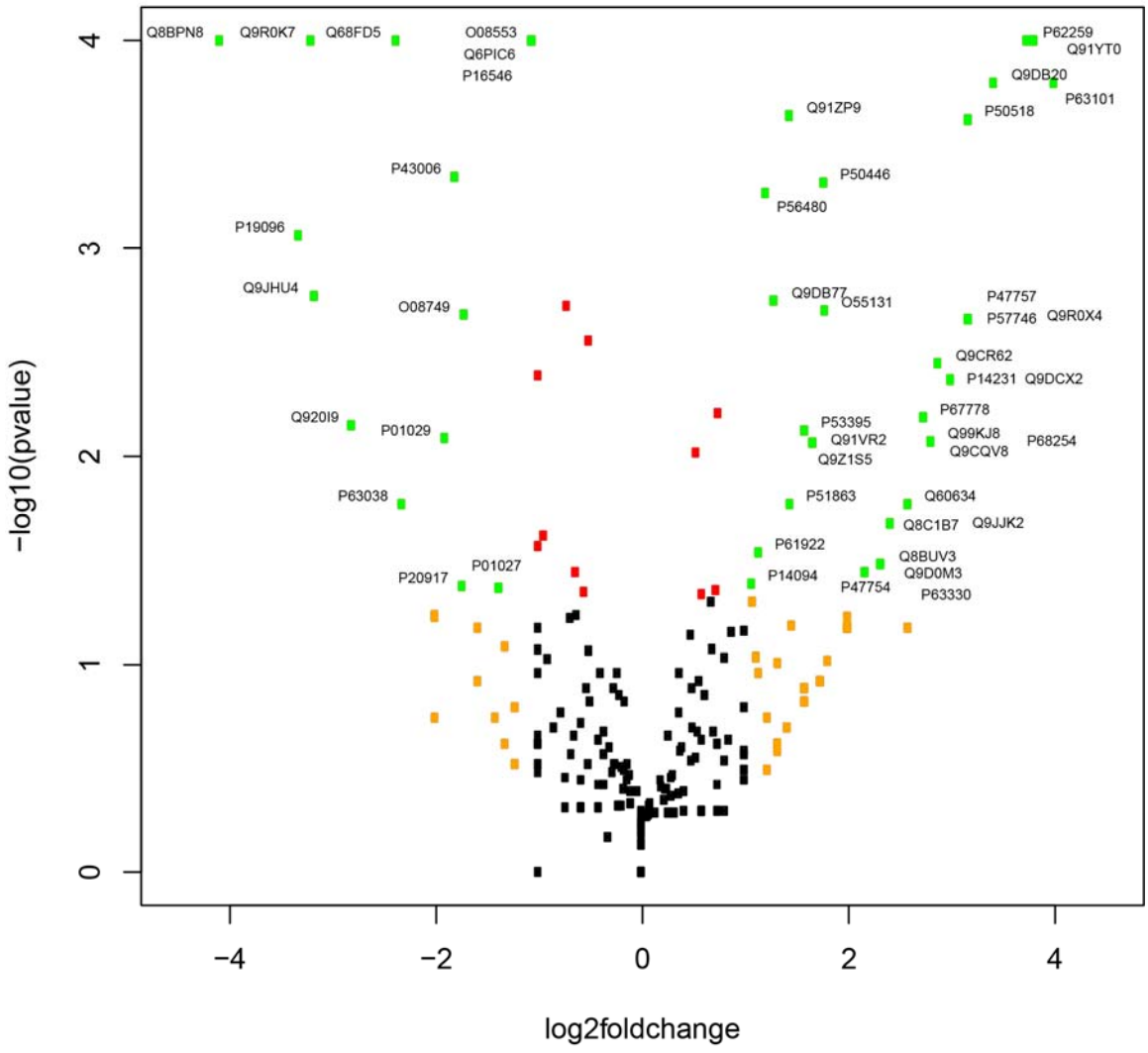

Figure 32. Volcano plot of synaptic proteins` abundances.

From the unstimulated presynaptic fractions of wildtype (WT) and SNAP25Δ3synaptosomes, we coIPed Gβγ, SNARE, and synaptic proteins that may be in complex. All proteins detected were plotted here (square), p <0.05 (red square), log 2 fold change >1 (orange square), p <0.05 and log2 fold change >1 (green square and gene name), and otherwise (black square) (Technical replicates N=2). Data were calculated for the fold change (SNAP25Δ3/WT) and analyzed by fisher exact T-test.

Table 7. List of detected proteins.

Identified Proteins	Gene Name	Fisher `s exact test	Fold Change (SNAP25Δ3/WT)
Dihydropyrimidinase-related protein 2	sp|O08553|DPYL2_MOUSE	0.0001	0.4767442
Spectrin alpha chain	sp|P16546|SPTA2_MOUSE	0.0001	0.4794521
Sodium/potassium-transporting ATPase subunit alpha-3	sp|Q6PIC6|AT1A3_MOUSE	0.0001	0.4805195
Clathrin heavy chain 1	sp|Q68FD5|CLH_MOUSE	0.0001	0.1923077
N-terminal EF-hand calcium-binding protein 2	sp|Q91ZP9|NECA2_MOUSE	0.00023	2.7058824
ATP synthase subunit beta	sp|P56480|ATPB_MOUSE	0.00054	2.3043478
Cytoplasmic dynein 1 heavy chain 1	sp|Q9JHU4|DYHC1_MOUSE	0.0017	0.1111111
Dihydrolipoyl dehydrogenase	sp|O08749|DLDH_MOUSE	0.0021	0.3043478
Cytochrome b-c1 complex subunit 2	sp|Q9DB77|QCR2_MOUSE	0.0018	2.4375
Excitatory amino acid transporter 2	sp|P43006|EAA2_MOUSE	0.00045	0.2857143
ATP synthase subunit gamma	sp|Q91VR2|ATPG_MOUSE	0.0086	3.1666667
DmX-like protein 2	sp|Q8BPN8|DMXL2_MOUSE	0.0001	0.0588235
Septin-7	sp|O55131|SEPT7_MOUSE	0.002	3.4285714
14-3-3 protein zeta/delta	sp|P63101|1433Z_MOUSE	0.00016	16
V-type proton ATPase subunit E 1	sp|P50518|VATE1_MOUSE	0.00024	9
Plasma membrane calcium-transporting ATPase 2	sp|Q9R0K7|AT2B2_MOUSE	0.0001	0.1086957
V-type proton ATPase subunit d 1	sp|P51863|VA0D1_MOUSE	0.017	2.7142857
Sodium/potassium-transporting ATPase subunit beta-1	sp|P14094|AT1B1_MOUSE	0.041	2.1
Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex	sp|P53395|ODB2_MOUSE	0.0075	3
60 kDa heat shock protein	sp|P63038|CH60_MOUSE	0.017	0.2
4-aminobutyrate aminotransferase	sp|P61922|GABT_MOUSE	0.029	2.2
Fatty acid synthase	sp|P19096|FAS_MOUSE	0.00086	0
AP-2 complex subunit alpha-2	sp|P17427|AP2A2_MOUSE	0.25	1.3157895
14-3-3 protein epsilon	sp|P62259|1433E_MOUSE	0.0001	13.333333
Mitochondrial 2-oxoglutarate/malate carrier protein	sp|Q9CR62|M2OM_MOUSE	0.0036	7.3333333
WD repeat-containing protein 7	sp|Q920I9|WDR7_MOUSE	0.0071	0.1428571
Prohibitin	sp|P67778|PHB_MOUSE	0.0065	6.6666667
V-type proton ATPase subunit D	sp|P57746|VATD_MOUSE	0.0022	9
Complement C4-B	sp|P01029|CO4B_MOUSE	0.0082	0.2666667
ATP synthase subunit O	sp|Q9DB20|ATPO_MOUSE	0.00016	10.666667
NADH dehydrogenase [ubiquinone] flavoprotein 1	sp|Q91YT0|NDUV1_MOUSE	0.0001	14
F-actin-capping protein subunit alpha-2	sp|P47754|CAZA2_MOUSE	0.036	4.5
Gephyrin	sp|Q8BUV3|GEPH_MOUSE	0.033	5
F-actin-capping protein subunit beta	sp|P47757|CAPZB_MOUSE	0.0022	9
Dynactin subunit 2	sp|Q99KJ8|DCTN2_MOUSE	0.0085	7
Septin-11	sp|Q8C1B7|SEP11_MOUSE	0.021	5.3333333
Keratin, type II cytoskeletal 6A	sp|P50446|K2C6A_MOUSE	0.00048	3.4117647
LanC-like protein 2	sp|Q9JJK2|LANC2_MOUSE	0.021	5.3333333
Flotillin-2	sp|Q60634|FLOT2_MOUSE	0.017	6
Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	sp|P63330|PP2AA_MOUSE	0.033	5
Cytochrome c1, heme protein	sp|Q9D0M3|CY1_MOUSE	0.033	5
Acyl-coenzyme A thioesterase 9	sp|Q9R0X4|ACOT9_MOUSE	0.0022	9
14-3-3 protein beta/alpha	sp|Q9CQV8|1433B_MOUSE	0.0085	7
Sodium/potassium-transporting ATPase subunit beta-2	sp|P14231|AT1B2_MOUSE	0.0043	8
ATP synthase subunit d	sp|Q9DCX2|ATP5H_MOUSE	0.0043	8
14-3-3 protein theta	sp|P68254|1433T_MOUSE	0.0085	7

5.4 Discussion

In this study, we have further identified and characterized the binding site on SNAP25 for Gβγ and attempted to determine other synaptic proteins that may be in complex with this interaction to further understand the microarchitecture of exocytosis. We have confirmed the previously determined a C-terminus binding site (Yoon et al. 2007) as well as identified additional residues on the N-terminus of SNAP25, especially Arg161, that may be important for the calcium-independent binding of Syt1 to SNAP25. Previously, Syt was identified to interact with SNAP25 and Syntaxin 1A in a calcium independent manner (Gerona et al. 2000, Mahal et al. 2002, Rickman and Davletov 2003, Nishiki and Augustine 2004) but the interacting residues were not determined. Moreover, we have identified 46 candidate proteins that may be in complex with the Gβγ-SNARE interaction by comparing the protein level of various synaptic proteins in the lysates and coIPs of wildtype and SNAP25Δ3`s synaptosomes. Overall, these results suggest a more complex interaction in Gβγ's regulation of exocytosis. The C-terminus of SNAP25 is important for the Gβγ mediated inhibition of exocytosis while the N-terminus of SNAP25 may allow Gβγ to be pre-coupled for the fast inhibition of exocytosis upon the $G_{i/o}$-GPCRs`, such as α_{2a}ARs, activation.

Two Gβγ binding sites on SNAP25 are too distant to allow Gβγ to bind both sites simultaneously. Measuring the x-ray structures of ternary SNARE (PDBID#: 1SFC) and $G\beta_1\gamma_1$ (PDBID#:1TBG), respectively, the distance between the most distal mutated SNAP25 residues, 135 and 201, is ~90Å, and the greatest distance across $G\beta_1\gamma_1$ is ~70Å. This suggests two different possibilities: more than one Gβγ is able to bind a single ternary SNARE (greater than 1:1 stoichiometry) or Gβγ bound in N-terminus moves to C-terminus

SNAP25 upon the α_{2a}-ARs activation. So far, we do not know how many Gβγ dimers bind to ternary SNARE. In addition, no co-crystal structure of Gβγ and ternary SNARE is available to determine the interaction of Gβγ dimers and N–terminus ternary SNARE. Further structural studies are necessary to determine the importance of N-terminus SNAP25.

At the synaptic terminal, the microarchitecture of exocytosis involves numerous synaptic proteins such as calcium channels (Davies, Jarvis, and Zamponi 2011), Munc18 (Smyth, Duncan, and Rickman 2010), tomosyn (Hatsuzawa et al. 2003a), and complexin (Tang 2009) (see Chapter 1 for more details). Interaction between these proteins and SNARE and their conformational changes are necessary for regulated exocytosis, endocytosis and the vesicle cycle. For example, calcium channels were found to be in complex with syntaxin 1A and Gβγ (Jarvis et al. 2000b). The conformational change of syntaxin 1A from a closed to open conformation by the relative movement of the H_{abc} region away from the remainder of the complex containing the SNARE motifs is also necessary for exocytosis (Dulubova, Sugita, Hill, Hosaka, Fernandez, Sudhof, et al. 1999, Hammarlund et al. 2007, Gerber et al. 2008). Additionally, SNARE complexes can be in two different states, "unzipped" and "zipped." An "unzipped" state occurs in the docking phase where there is recognition between vesicle and plasma membrane, but the vesicle is not primed and cannot fuse. The fully "zipped" state is when the full association of the SNARE motifs. It is fully primed if not participating in the fusion of the two membranes (Matos et al. 2003, Borisovska et al. 2005, Sorensen 2004, Pobbati, Stein, and Fasshauer 2006). It is yet to be determined the role of identified N-terminal Gβγ-binding residues of SNAP25 on the Gβγ-SNARE interaction and other SNARE-synaptic proteins interactions

that are important for the microarchitecture of exocytosis.

Previous work by Yoon et al. demonstrated that Gβγ is capable of competing with syt1 in the absence of calcium, while with higher calcium, syt1 is an increasingly better competitor and can compete Gβγ off of tSNARE (Yoon et al. 2007). Remarkably, the mutagenesis of residue Arg161 to Ala produced a significant reduction in calcium-independent binding of syt1 C2AB fragments to SNAP25, but left calcium-dependent binding intact (Fig. 29C). The other seven SNAP-25 mutant proteins generated from the peptide array showed no significant difference from wildtype SNAP25 in calcium-dependent or calcium-independent conditions (Fig. 29B). These once again prove the role of Gβγ in the inhibition of exocytosis and its greater ability to inhibit vesicle fusion by syt1 in low calcium. Our result is also complementary with previous studies showing that three Asp residues (Asp179, Asp186, Asp193) at the C-terminus of SNAP25 are required for calcium-dependent binding of Syt1 to SNAP-25 (Zhang et al. 2002). This work reinforces the hypothesis of Gβγ inhibition of syt1 binding of SNAP25.

Based on our work, we generated various SNAP25 mutants and SNAP25Δ3 transgenic mice, containing 3 amino acids truncated at the C-terminus SNAP25, that can be used in many studies to define the role of Gβγ-mediated inhibition of synaptic vesicle release. Using these tools, we now can determine an effect of the loss of Gβγ mediated inhibition of synaptic transmission with various drugs targeting $G_{i/o}$-coupled GPCRs and the importance of Gβγ-SNARE interaction in various neurological functions such as locomotion, depression, and pain processing (Zurawski et al. 2017). Moreover, we can determine which $G_{i/o}$-coupled GPCRs, in addition to α_{2A}ARs and $5HT_{1b}$ (Delaney, Crane, and Sah 2007a, Hamid et al. 2014), use the Gβγ-SNARE interaction to modulate

exocytosis.

Using SNA25Δ3 mice, we attempted to understand the basal interaction of Gβγ-SNARE without α_{2A}ARs stimulation (Fig. 25 and 26, see chapter 4 for more details). Without receptor stimulation, Gβγ bound to SNARE complex, and neither Gα subunits nor the FLAG-α_{2A}ARs were found in the complex (Fig. 30). This suggests that active Gβγ, not Gαβγ heterotrimer, is bound to SNARE without the α_{2A}ARs stimulation. This implies two different scenarios: Gβγ released from Gαβγ heterotrimer associated to other $G_{i/o}$-GPCRs may be pre-bound to SNARE in the basal condition; Gβγ dimers are active after the synthesis and assembly and can bind to effectors without receptor activation. The Gβγ found in unstimulated conditions may be from other $G_{i/o}$-GPCRs with constitutive activity. Because endogenous neurotransmitter may activate other GPCRs, although it didn`t activate FLAG-α_{2A}ARs (Fig.16), future studies with various $G_{i/o}$-GPCR antagonists is needed to check whether other receptors may be involved in the "basal" interaction of Gβγ-SNARE. To date, no experiment was done to examine whether Gβγ can bind to effectors without receptor activation and formation of Gαβγ heterotrimer and mediate any physiological functions. Moreover, the site of basal Gβγ-SNARE interaction was not determined in this study. First, we will need to examine if the basal interaction is happening at the C- or N- terminal region of SNARE. This will determine whether the basal interaction is physiologically important in the α_{2A}ARs mediated regulation of synaptic transmission. If the N-terminal region of SNARE is important for basal Gβγ-SNARE interaction, most likely the release or relocation of basal Gβγ from SNARE is necessary for the modulation of exocytosis upon α_{2A}ARs activation. Further studies will be needed to examine the mechanism of this phenomenon. As we narrowed down to 4

different Gβγ dimers in this study, we can also design knockdown or knockout studies, as well as overexpression of specific Gβ and Gγ to examine if any perturbation in the synthesis and assembly of these Gβγ dimers may affect the basal interaction. Furthermore, it will be interesting to see if other Gα subunits, such as Gαs or Gαq, are involved in this basal interaction of Gβγ-SNARE.

Although we do not fully understand the mechanism of this phenomenon, we were able to find candidates that may be in complex with Gβγ and SNARE. CSP, as a presynaptic vesicle protein, is known to interact with VAMP, syntaxin1A, syt1, Gα, and Gβγ (Evans, Morgan, and Burgoyne 2003, Burgoyne and Morgan 2015, Nie et al. 1999) and form a CSP-HSC70-SGT chaperone complex to maintain SNAP25 and SNARE stability (Tobaben et al. 2003, Roberts et al. 2015, Sharma, Burre, and Sudhof). The decreased amount of in CSP in SNAP25Δ3 may suggest that SNARE with SNAP25Δ3 is less stable than wildtype SNAP25 (Fig 31B). To further understand whether CSP is in complex with Gβγ-SNARE, we will need to do the coIP studies on Gβγ and SNARE from the presynaptic fraction of unstimulated SNAP25Δ3 compared to wildtype as described in chapter 4. Using syntaxin1 or VAMP2 antibodies, we can coIP the Gβγ-SANRE interaction complex and validate the changes in CSP interacting with Gβγ and SNARE in SNAP25Δ3 compared to wildtype mice.

Discovery proteomics determined an additional 46 proteins that may be important in the basal Gβγ-SNARE interaction (Table 7) although we need more biological replicates. By the loss of 3 residues in SNAP25 to reduce the Gβγ-SNARE interaction, we suspected to see a decrease in the abundance of scaffold proteins that may be in complex with this interaction. Further analysis will be necessary to determine which of the 15

proteins with reduction in abundance in SNAP25Δ3 are involved in the Gβγ-SNARE interaction. Moreover, Gβγ may be playing an inhibitory role to prevent interaction of the 31 proteins with enhancement of abundance in SNAP25Δ3 (Table 7) to interact with SNARE in normal physiology. It will be interesting to examine whether these proteins compete with Gβγ for the SNARE binding. We may detect increases in these proteins by the loss of Gβγ-SNARE interaction in SNAP25Δ3 mice.

Interestingly, we detected reductions of both DmX-like protein 2 (rabconnectin-3) and WD repeat containing protein 7 (WD7R, Q920I9), which were known to be expressed in brain and tightly interact (Kawabe et al. 2003). Rabconnectin-3 was reported to associate with synaptic vesicles at the synapse and may serve as a scaffold molecule for Rab3 GEP and GAP on synaptic vesicles (Nagano et al. 2002). In addition, it was implicated as a regulator of Notch signaling via modulation for V-ATPase activity (Yan, Denef, and Schupbach 2009, Sethi et al. 2010). By WDR7 binding to Rab3 GEP, rabconnectin-3 and WDR7 complex may regulate neurotransmission via modulation of Rab3 activity on synaptic vesicles (Kawabe et al. 2003). Our result suggests that the C-terminus of SNAP25 may be important for this complex`s mediated regulation of neurotransmition although the exact mechanism is unclear and will require more study.

Neuronal calcium binding protein 2 (NECAB2, Q91ZP9) may be involved in the Gβγ-SNARE interaction. It is made with an N-terminus EF-hand domain with a single calcium binding site, a highly conserved coiled-coil domain, and a C-terminus containing DUF176 or antibiotic biosynthesis monoxygenase motif, a bacterial domain of unknown function (Canela et al. 2007). Although NECAB2`s interaction site is not defined, NECAB2 directly interacted with Gαs coupled adenosine A_{2a}ARs and $G\alpha_{q/11}$ coupled

mGlu5 receptors, which are known to heterodimerize with adenosine A_{2a}ARs (Ferre et al. 2002). The direct interaction involves the C-terminus domain of adenosine A_{2a}ARs were detected in the yeast two- hybrid system, cultured neurons, and rat striatum studies (Canela et al. 2007). It is known to regulate the cell surface expression of adenosine A_{2a}ARs and its MAPK pathway (Canela et al. 2007). Interestingly, these receptors are coupled with $G\alpha_s$ and $G\alpha_{q/11}$, not $G\alpha_{i/o}$ (Ferre et al. 2002). Gβγ from $G_{i/o}$ GPCRs, but no other G protein coupled receptors, is known to be involved in the Gβγ-SNARE interaction. However, we do not know how the complex of adenosine A_{2a}ARs-Gprotein-NECAB2 might affect the Gβγ-SNARE interaction. Enhancement of NECAB2 implies that NECAB2 and Gβγ may be in competition for SNARE binding. Further studies will be needed to understand how NECAB2 interacts with SNARE or competes with Gβγ.

5.5 Conclusions

In summary, we have established two interaction sites on SNAP25 for Gβγ and hypothesize the involvement of other synaptic proteins such as CSP, rabconnectin-3, and WD7R as scaffold proteins for the basal Gβγ-SNARE interaction (Fig.33). Further studies will be necessary to examine 47 candidate proteins (46 from the discovery proteomics study and CSP) for their interaction with Gβγ and/or SNARE and their roles in the Gβγ-SNARE interaction. .

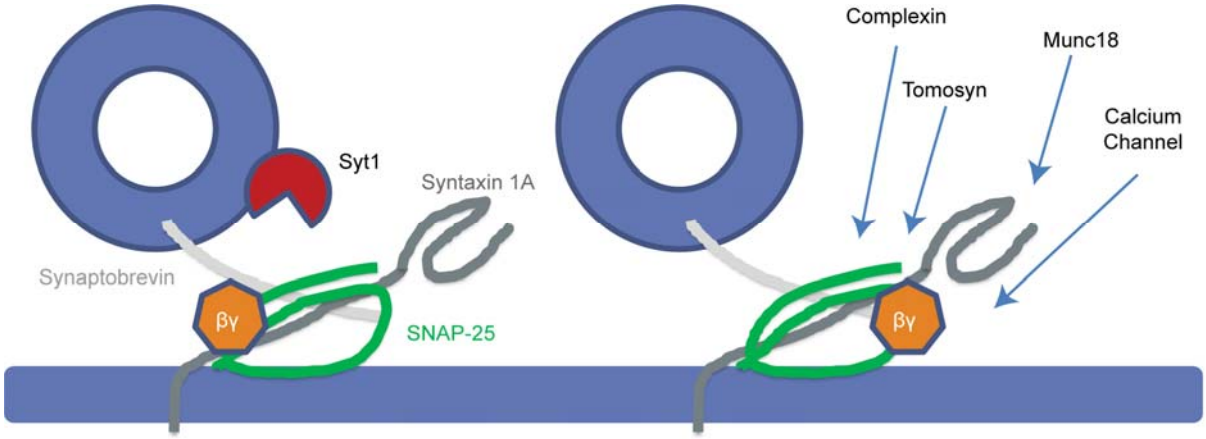

Figure 33. The microarchitecture of exocytosis: Gβγ-SNARE interaction.

Gβγ binds not only C-terminus of SNAP25 but also the N-terminus SNAP25. Taken in the context of ternary SNARE and other synaptic proteins necessary for the exocytosis, a single Gβγ dimer activated by a $G_{i/o}$-coupled GPCR bound to the C-terminus of SNAP25. However, a complex of Gbg, SNARE, and other synaptic proteins such as calcium channels, tomosyn, complexin, Munc18, and CSP may be assembled prior to the $G_{i/o}$-coupled GPCR activation. .

CHAPTER 6

CONCLUSION AND FUTURE DIRECTION

6.1 Conclusion

Much progress was made in the studies described in this dissertation to develop a tool to detect neuronal Gβ and Gγ subunits and understand the molecular interaction of the Gβγ-SNARE complex, as well as the microarchitecture of the Gβγ-SNARE complex at the synapse. First, we developed a quantitative analysis of neuronal Gβ and Gγ subunits. Using this method, we characterized the specificity of Gβ and Gγ subunits to α_{2a} adrenergic receptors and SNARE after activating the receptor with epinephrine. Lastly, we examined residues involved in the Gβγ-SNARE interaction, and other synaptic proteins that may be in complex with Gβγ and SNARE to modulate synaptic transmission. These studies form the basis for further investigations to determine the effect of G protein specificity on its downstream signaling, identify potential pathophysiological states in which the Gβγ-SNARE interaction may be dysregulated, and yield additional insights into $G_{i/o}$-coupled GPCR-mediated regulation of exocytosis.

Heterotrimeric G proteins mediate the actions of many GPCRs to regulate a wide variety of signaling pathways. With such a large number of Gα, Gβ, and Gγ subunits, different GPCRs may activate different heterotrimers and define the effector and its downstream signals (Oldham and Hamm 2008). However, no studies have identified fully which G protein heterotrimers, especially Gβγ, exist *in vivo* and the way in which they

function physiologically. Compared to Gα subunits, Gβγ dimers other than $G\beta_1\gamma_2$ have been studied less often because of the technical difficulty of detecting each Gβ and Gγ subunit. High sequence identity between Gβ and Gγ subunits (Betty et al. 1998c, Smrcka 2008a) has hampered the development of subtype-specific antibodies that would facilitate studies of Gβγ dimers. Genetic deletion or knockdown of Gβ– and Gγ–specific subunits has somewhat countered this difficulty (Chen et al. 2003, Pronin and Gautam 1992b) For example, $G\beta_5$ knockout mice showed impaired development and motor learning, while $G\gamma_3$ knockout mice showed a seizure phenotype. These differences in physiological phenotypes support the concept that Gβγ specificity may exist and control specific physiological processes (Schwindinger et al. 2003b, Schwindinger et al. 2009, Schwindinger et al. 2010a, Zhang et al. 2011). Moreover, various mutations of Gβ and Gγ subunits are found in the lungs, large intestine, and skin cancer. Specifically, $G\beta_2$ and $G\gamma_3$ are found to be mutated in ovarian cancer, while $G\gamma_7$ mutants were found in CNS cancer (O'Hayre et al. 2013). Although the physiological phenotypes of some Gβ and Gγ subunits have been characterized, the protein abundance and localization of each neuronal Gβ and Gγ subunit at the synapse remain unclear, and new experimental tools and approaches are needed to understand Gβγ dimerization and specificity at the synapse further.

We addressed the protein level and subcellular localization of neuronal Gβ and Gγ subunits first using quantitative MRM mass spectrometry in whole and fractionated crude synaptosomes. To overcome the antibody specificity issue, we developed quantitative, targeted mass spectrometry to detect the proteolytic peptides of each neuronal Gβ and Gγ subunit. Despite quantification error, we were able to detect and quantify the abundance of each neuronal Gβ and Gγ subunit. Using a new quantitative tool, we found that $G\beta_1$ and

$G\gamma_2$ were the most abundant Gβ and Gγ subunits in whole crude synaptosomes. $G\beta_1$ was localized primarily at the membrane, while $G\beta_2$ was distributed evenly throughout the membrane and synaptosomal cytosolic fractions. In addition to $G\gamma_2$, $G\gamma_3$ and $G\gamma_{12}$ were abundant in whole crude synaptosomes, and $G\gamma_3$ and $G\gamma_{12}$ also were abundant in membrane fractions. In this study, we highlighted the *in vivo* distribution of neuronal Gβ and Gγ subunits and thus provided insights into the localization and distribution of Gβγ dimers in normal brain function. Although further efforts will be necessary, we were able to discuss the selectivity of Gβγ dimers formation based on the abundance, localization, and affinity of the Gβ and Gγ subunits for a receptor and an effector.

Knowing the abundance and localization of each neuronal Gβ and Gγ subunit, we attempted to understand the way in which the selectivity of Gβγ dimerization may affect Gβγ's specificity to α_{2a}ARs, the therapeutic target of guanfacine and clonidine, and to SNARE complexes (Gribble 2010, Comings et al. 2000, Wakeno et al. 2008). Although α_{2a}ARs exist at both the pre- and post-synaptic terminal, only presynaptic α_{2a}ARs have a negative feedback regulator to modulate neurotransmitters in the CNS (Gilsbach et al. 2009a). Depending on the type of neuron on which α_{2a}ARs are located, they are divided further into auto and heteroreceptors. Autoreceptors are localized on the adrenergic neurons and reduce the release of noradrenaline (Hein, Altman, and Kobilka 1999, Gilsbach et al. 2009a), while heteroreceptors are localized at the non-adrenergic neurons and modulate release of other neurotransmitters, such as 5HT, GABA, and dopamine (Richter et al. 2012) . Although both auto- and heteroreceptors have been found to regulate exocytosis in a similar manner, the mechanistic differences in auto- vs. heteroreceptor-mediated inhibition of exocytosis have not been examined. Using mice that express HA-

and FLAG-tagged α_{2a}ARs and various biochemical approaches, we demonstrated that α_{2a}-ARs exhibit specificity to, and interact only with, a subset of neuronal Gβ and Gγ subunits. Neuronal 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 specifically with $G\beta_2$, $G\gamma_2$, $G\gamma_3$, and $G\gamma_4$ only. Possible Gβγ dimers are $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$, while hetero-α_{2a}ARs may interact with $G\beta_4\gamma_{12}$. Differences in Gβγ dimer association with these receptors suggest that different heterotrimers may be associated with auto- and hetero-α_{2a}ARs. As auto- and hetero-α_{2a}ARs are on different types of neurons, one on noradrenergic neurons, while the other is on non-adrenergic neurons, neuronal type may add another layer of regulation to Gβγ specificity. Although we were unable to characterize each Gβγ dimer's interaction with effectors and involvement in a particular physiological function, these findings imply that the specificity of Gβγ to signaling pathways could be mediated, in part, through the receptors and their locations on different type of neurons. Further studies are needed to determine the way in which these Gβγs affect α_{2a}ARs-mediated effects in various neurological diseases and GPCR-targeted drug mechanisms.

Activated α_{2a}ARs modulate synaptic transmission by Gβγ and one of its effectors, the exocytotic SNARE complex. In amygdala and pancreatic β cells, α_{2a}ARs are found to inhibit neurotransmitter release through direct interaction between Gβγ and SNARE (Delaney, Crane, and Sah 2007a, Zhao et al. 2010). However, little is understood on the specificity of Gβγ to SNARE. Because $G\beta_1\gamma_2$ is the most abundant, it has been used most often to determine interaction with SNARE, and other Gβγ dimers have not been examined to identify their physiological functions and specificity to SNARE. Although we need to repeat the proteomic analysis of the α_{2a}ARs-mediated Gβγ–SNARE interactions, *in*

vitro data and preliminary studies have suggested that only a subset of the Gβγ subunits released from-activated α_{2a}ARs may interact with SNARE; possible candidates are $G\beta_1\gamma_2$, $G\beta_1\gamma_3$, $G\beta_1\gamma_4$, $G\beta_2\gamma_2$, $G\beta_2\gamma_3$, and $G\beta_2\gamma_4$. This implies that Gβγ specificity to its effectors in part mediates further the specificity of signaling pathways. Interestingly, we found a basal interaction of Gβγ-SNARE in the absence of norepinephrine. With the help of other synaptic proteins, the Gβγ-SNARE complex may be pre-coupled and allow the rapid inhibition of synaptic transmission by α_{2a}ARs activation. With the preliminary result, Future studies are necessary to better understand the specificity of Gβγ to SNARE.

To determine the specificities of Gβ and Gγ subunits to α_{2a}ARs and SNARE, and identify the basal Gβγ-SNARE interaction, we examined the molecular interaction between Gβγ and SNARE. Numerous studies have suggested that the C-terminal region of SNAP25 is important in the Gβγ-SNARE interaction (Blackmer et al. 2005, Yoon et al. 2007, Wells et al. 2012). However, the specific residues involved in the Gβγ-SNARE interaction have yet to be determined. In this study, we established two interaction sites on SNAP25 for Gβγ-SNARE interaction. In addition to the C-terminus of SNAP25, the N-terminal region of SNAP25 was identified to be important in Gβγ binding. Although we do not understand the functional importance of these two binding sites fully, we hypothesized that the N-terminus may be involved in basal Gβγ-SNARE interaction, and there may be an additional protein required in the Gβγ-SNARE interaction to use these two binding sites on SNAP25. Interestingly, the basal Gβγ-SNARE interaction identified was not a complex with either α_{2a}ARs or $G\alpha_{i/o}$ subunits. Using transgenic mice with a reduction in the C-terminus Gβγ-SNARE interaction, we determined differential interaction of other proteins, and

speculated that other synaptic proteins, such as CSP, rabconnectin-3, and WD7R, are involved as scaffold proteins in the basal Gβγ-SNARE interaction. To date, we have 47 candidate proteins with which to examine the interactions with Gβγ and/or SNARE and define their roles in the Gβγ-SNARE interaction.

These findings overall elucidate further the complex regulation of synaptic transmission and imply that the abundance, localization, and affinity of each neuronal Gβ and Gγ subunit, and neuronal Gβγ specificity to specific $G_{i/o}$ receptors, α_{2a}ARs, and the effector, SNARE, may affect the regulation of Gβγ–mediated inhibition of synaptic transmission. Although more studies are needed, these studies establish the foundation for future physiological and molecular investigations of the role of each neuronal Gβγ, as well as $G\beta_1\gamma_2$, in the regulation of exocytosis. Furthermore, in-depth understanding of the Gβγ-SNARE interaction may further our understanding of the side effects of many $G_{i/o}$-coupled GPCRs-targeted drugs and suggest this mechanism as a possible drug target to synergize or antagonize other drugs that target $G_{i/o}$-coupled GPCRs to reduce neurotransmitter imbalances.

6.2 Future Directions

Despite countless efforts to understand the specificity of G protein and the Gβγ-SNARE interaction, we have yet to determine fully the *in vivo* preference of Gβγ specificities to various GPCRs and their effectors. To understand Gβγ specificities better, and determine the synergism or antagonism of this mechanism with marketed drugs, the following studies may be of interest.

In a previous study designed to develop quantitative MRM mass spectrometry, we

were unable to select proteotrpic peptides of $G\gamma_5$ and $G\gamma_{11}$ to identify a correct peak of sufficient signal intensity to pass acceptable signal-to-noise criteria (Betke 2014). Because $G\gamma_5$ was observed to be the most abundant Gγ subunit in a neuronal cell line (Kilpatrick and Hildebrandt 2007), further study is needed to identify proteotypic peptides in regions that are not modified post-translationally and characterize the quantity and subcellular localization of $G\gamma_5$ in the brain. In addition, the development of quantitative MRM for non-neuronal Gβ and Gγ subunits will be useful to understand the Gβγ specificities in other physiological systems further.

Currently, we can determine Gβγ specificities by examining Gβ and Gγ subunits separately. Because these subunits do not signal without being in a complex, we need to understand the selectivity of Gβ and Gγ subunits to each other to become Gβγ dimers. Various *in vitro* studies have suggested that each Gβ subunit prefers various Gγ subunits (Hildebrandt 1997, Smrcka 2008a, Stephens 2009). However, no *in vivo* studies have been performed to validate these findings because of their technical difficulty. To detect these dimers, we will need to select the proteolytic peptides in the regions in which Gβ and Gγ interact for each Gβγ dimer. With the help of crosslinker, we can crosslink purified Gβ and Gγ subunits and attempt to identify proteolytic peptides. The method developed to detect Gβγ dimers can be applied to HA or FLAG-α_{2a}ARs and Gβγ coIP samples by running native gels rather than SDS-page gels.

Both Gα and Gβγ subunits mediate various downstream signals upon activation by the α_{2a}ARs (Richardson and Robishaw 1999a, Gibson and Gilman 2006). However, we focused here only on Gβγ specificities to α_{2a}ARs. α_{2a}ARs can be coupled with various

Gα subunits, such as $G\alpha_{o1\text{-}2}$ and $G\alpha_{i1\text{-}3}$ (Straiker, Borden, and Sullivan 2002, Milligan and Kostenis 2006). Others have found that $G\alpha_{i1}$ prefers α_{2a}ARs *in vitro* (Gibson and Gilman 2006, Richardson and Robishaw 1999b). It will be interesting to characterize further which $G\alpha_o$ or $G\alpha_i$ subunits are coupled to auto- and hetero-α_{2a}ARs. Previously, $G\alpha_o$ was found to associate with the presynaptic N type Ca^{2+} channel by interacting with syntaxin1 to affect exocytosis (Li, Lau, et al. 2004). It will be interesting to see the way in which the $G\alpha_o$-syntaxin1-presynaptic N type Ca^{2+} channel mediates the synaptic difference in the Gβγ-SNARE interaction-mediated inhibition of exocytosis.

The G proteins specific to heteroreceptors also requires further understanding. Heteroreceptors are α_{2a}ARs that are located at both the pre- and post-synaptic terminals of non-adrenergic neurons (Gilsbach and Hein 2012). Without further fractionation of synaptosomes, we cannot predict whether the pre- or post-synaptic location and the differences in effectors influence the Gβγ specificities. With fractionated synaptosomes that separate pre- and post-synaptic fractions, we can characterize the difference further, and examine the Gβγ specificities to other effectors, such as GIRK. Moreover, studying the specific brain region in which α_{2a}ARs are known to be involved in ADHD, anxiety, and panic disorder, and comparing the differences in the Gβγ specificity to α_{2a}ARs activated by guanfacine and clonidine versus epinephrine would be an interesting extension of this study (Gribble 2010, Comings et al. 2000, Wakeno et al. 2008, Davies et al. 2003, Marrs et al. 2005). Using synaptosomes from a specific brain region of HA- and FLAG-tagged α_{2a}ARs mice, drugs targeting α_{2a}ARs, and the quantitative MRM method, we can determine whether the activation of α_{2a}ARs would have the same effect with all treatments. This could provide information about the potency and efficacy of drugs.

Although GPCRs represent a major therapeutic target, no specific antibodies were available, which limited our *in vivo* and *ex vivo* studies to a receptor that has a transgenic mouse that expresses a tag, such as HA or FLAG, on a receptor. In addition, the generation of transgenic mice with tagged receptors was difficult before the CRISPR technology became viral, and we were limited to studying only the Gβγ specificities to α_{2a}ARs. With the help of CRISPR and the development of monoclonal GPCR antibodies, we now can determine the way in which other receptors may have different or similar Gβγ specificities compared to α_{2a}ARs.

Nociceptive transmission and pain perception have been found to involve the thalamus, putamen, caudate nucleus, hypothalamus, amygdala, periaqueductal grey (PAG), hippocampus, and cerebellum(Michael J Hudspith 2006). Morphine and opioid derivatives, such as buprenorphine and pethidine, target presynaptic opioid receptors to achieve analgesia by modulating synaptic transmission (Hong 2005). However, the role of each opioid receptor in catecholamine neurons and their mechanism of synaptic transmission inhibition are not fully known. To date, μ- and κ-opioid receptors are known to modulate synaptic transmission by the Gβγ-SNARE interactions (Law, Wong, and Loh 2000, Malsam, Kreye, and Sollner 2008, Südhof and Rothman 2009, Iremonger and Bains 2009). In addition, μ-opioid receptors were found to interact with $G\beta_2$ and $G\beta_4$ *in vitro*, similar to α_{2a}ARs (Mahmoud, Yun, and Ruiz-Velasco 2012). Using a FLAG-tagged μ-opioid, and eGFP-tagged μ– and δ-opioid receptor mice (Arttamangkul et al. 2008, Gendron et al. 2015), we can verify whether α_{2a}ARs and these opioid receptors share Gβ and Gγ subunits for their downstream signals. If they both indeed interact with $G\beta_2$ and $G\beta_4$, Gγ subunits may be more important in determining the specificities to their effectors. Further

understanding may help develop better therapeutic drugs for pain and analgesia to overcome side effects.

Here, we identified the Gβγ specificities of α_{2a}ARs and SNARE; however, no biochemical studies have been conducted to validate the Gβγ specificities determined. Thus, we will have to validate the findings of current studies using various G protein knockout mice, virus to knockdown or overexpressed specific G protein subunits, and drugs that target α_{2a}ARs. For example, $G\gamma_3$ was found to interact specifically with auto-α_{2a}ARs. Fortunately, $G\gamma_3$ knockout mouse are available. Using an α_{2a}ARs agonist, such as guanfacine or clonidine, and antagonist, such as BRL44408, we can determine the physiological function of α_{2a}ARs. In addition, we can examine which Gβ subunits interact with $G\gamma_3$ to mediate auto-α_{2a}ARs downstream signals by crossbreeding HA- and FLAG-tagged α_{2a}ARs mice with $G\gamma_3$ knockout mouse.

Despite numerous attempts to understand the Gβγ-SNARE interaction and its role in various neurological and neuropsychiatric diseases, the implication of this interaction in disease is yet to be determined. We currently are attempting to understand the importance of this interaction in normal and disease-related physiology using SNAP25Δ3 mice (Zurawski et al. 2017). Using various behavior tests, we may be able to identify specific physiological phenotypes associated with the Gβγ-SNARE interaction-mediated inhibition of exocytosis. Comparing the discovery proteomics of coIPs by SNAP25 and other synaptic proteins, such as Munc18, directly interacting with SNARE, we may also determine the scaffold protein important in the microarchitecture of Gβγ-SNARE interaction. Currently, discovery based proteomic study was done only once using SNAP25 coIP samples. This study needs to be repeated with biological replicates and also with other

SNARE and synaptic proteins. The truncation of SNAP25 may have affected the coIP of SNAP25 and SNARE stability.

The development of compounds that modulate the Gβγ-SNARE interaction can be used in combination with current therapies targeting various $G_{i/o}$coupled GPCRs (Albrecht et al. 1999, Le Francois et al. 2008, Rosengren et al. , Devedjian JC 2000) in a synergistic or antagonistic manner (Fig. 34). Application of such compounds could be used in mouse models examining depression, fear conditioning, or working memory to study α_{2a}ARs mediated Gβγ-SNARE interaction. Although early compounds may not have favorable DMPK profiles, cannulation of mice should allow direct application of the compounds to areas of the brain such as the cortex that are known to be important centers for α_{2a}ARs activity. In addition to SNAP25Δ3 mice, compounds can be applied to access whether the Gβγ–SNARE interaction plays a role in normal functioning, such as specific types of learning and memory, using behavior tests. For example, if an inhibitor causes worsening of symptoms, it may indicate that the normal functioning of Gβγ–SNARE interaction was downregulated in some way in the disease state. Conversely, if the inhibitor alleviated the symptoms, the Gβγ–SNARE interaction was upregulated for some reason in the disease state. Modulators of this interaction may maximize efficacy while minimizing potential side effects of marketed, $G_{i/o}$coupled GPCRs-targeted drugs.

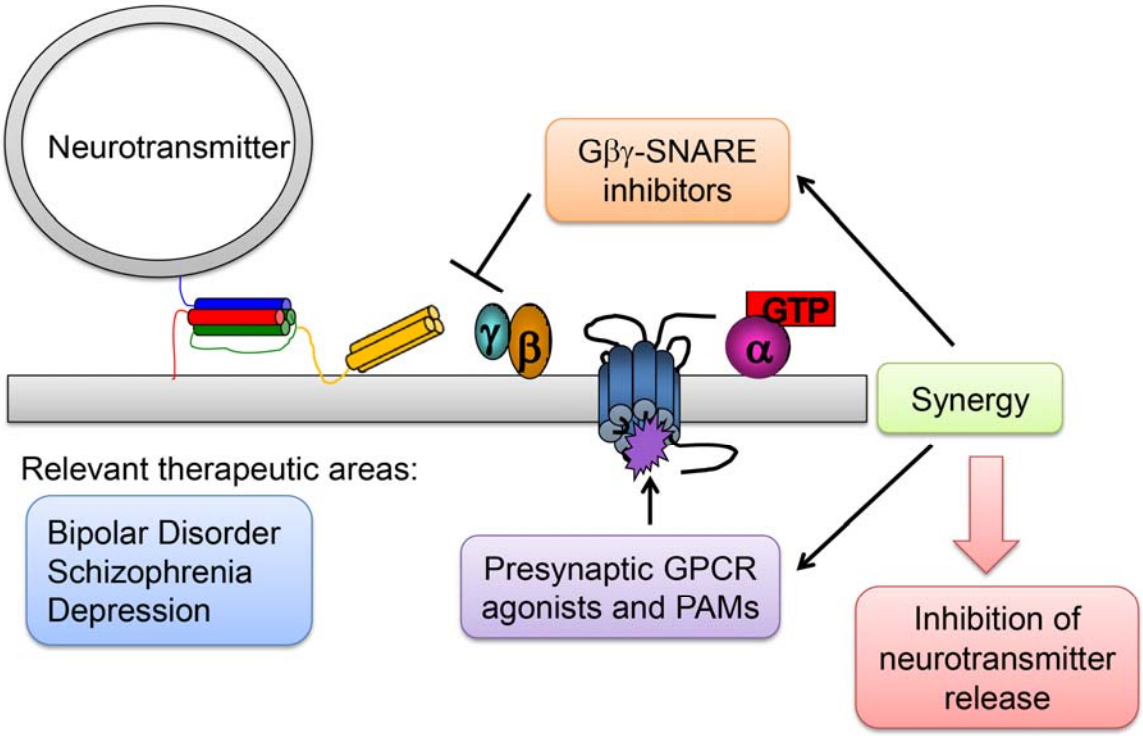

Figure 34. Therapeutic relevance of Gβγ-SNARE interaction

The development of Gβγ-SNARE inhibitors may synergy with the currently marketed presynaptic GPCR agonists and PAMS and lead to the inhibition of neurotransmitter release while reducing off target effects and adverse consequences.

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