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GPCR Regulation of Secretion

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Abstract

Modulation of neurotransmitter exocytosis by activated G_{i/o} coupled G-protein coupled receptors (GPCRs) is a universal regulatory mechanism used both to avoid overstimulation and to influence circuitry. One of the known modulation mechanisms is the interaction between Gβ and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs). There are 5 Gβ and 12 Gγ subunits, but specific Gβγs activated by a given GPCR and the specificity to effectors, such as SNARE, *in vivo* are not known. Although less studied, Gβγ binding to the exocytic fusion machinery (i.e. SNARE) provides a more direct regulatory mechanism for neurotransmitter release. Here, we review some recent insights in the architecture of the synaptic terminal, modulation of synaptic transmission, and implications of G protein modulation of synaptic transmission in diseases. Numerous presynaptic proteins are involved in the architecture of synaptic terminals, particularly the active zone, and their importance in the regulation of exocytosis is still not completely understood. Further understanding of the Gβγ-SNARE interaction and the architecture and mechanisms of exocytosis may lead to the discovery of novel therapeutic targets to help patients with various disorders such as hypertension, attention-deficit/hyperactivity disorder, post-traumatic stress disorder, and acute/chronic pain.

Keywords

GPCR; secretion; exocytosis; G protein; SNARE; calcium

1. Introduction

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, F. A., et al., 2009; Napper, R. M. & Harvey, R. J., 1988). These neurons communicate by the regulated fusion of neurotransmitter-containing vesicles. Presynaptic vesicles undergo a sequential series of maturation steps known as docking and priming (Fig. 1). Docked vesicles are vesicles that are in close proximity to the plasma me

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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membrane in direct contact with the active zone (Fernandez- Busnadiego, R., et al., 2010; Verhage, M. & Sorensen, J. B., 2008; Weimer, R. M., et al., 2006). Once docked vesicles are primed by the action of synaptic proteins (see the architecture of the synaptic terminal section for more details), they are distributed into one of several vesicle pools, including the readily releasable pool and the slowly releasable pool (Becherer, U. & Rettig, J., 2006), which can then fuse with the plasma membrane in either a calcium (Ca^{2+}) dependent or independent manner (Becherer, U. & Rettig, J., 2006; Malsam, J., et al., 2008; Weimer, R. M., et al., 2006). While Ca^{2+} independent exocytosis remains less clear, 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)(Purves D, A. G., Fitzpatrick D, Hall WC, LaMantia AS, McNamara JO, White LE, 2008; Schoch, S. & Gundelfinger, E. D., 2006; Südhof, T. C., 2004; Zhai, R. G. & Bellen, H. J., 2004). This increases the Ca^{2+} concentration at the presynaptic terminal of neurons and activates Ca^{2+} - dependent exocytosis (Fig. 1). 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 and evoked asynchronous release, or in the spontaneous mini release (Pang, Z. P. & Südhof, T. C., 2010; Park, Y. & Kim, K. T., 2009). In an evoked release, vesicles fuse within a millisecond of action potential arrival. This is in contrast to evoked asynchronous release, where vesicles fuse in a delayed manner over a markedly slower time scale, on the order of seconds (Pang, Z. P. & Südhof, T. C., 2010). Moreover, a single vesicle fuses with the plasma membrane in absence of a depolarizing stimulus for spontaneous mini release (Pang, Z. P. & Südhof, T. C., 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 (Dresbach, T., et al., 2001; Südhof, T. C., 2004; Südhof, Thomas C., 2013). This results in the activation of 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) (Dresbach, T., et al., 2001; Südhof, T. C., 2004; Südhof, Thomas C., 2013).

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 utilized. Synaptosomes are a cellular fragment composed of several structures, including the presynaptic nerve terminal, postsynaptic density, synaptic vesicles, and mitochondria (Evans, G. J., 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 synaptosome (Wilhelm, B. G., et al., 2014). These various presynaptic proteins, such as the Sec-1/ Munc-18 (SM) proteins, Munc-18 and Munc-13, and the calcium sensor, synaptotagmin, provide critical regulatory steps for exocytosis (Südhof, Thomas C., 2013) (Fig. 1).

2.1 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 fungi to vertebrates (Jahn, R. & Scheller, R. H., 2006; Parpura, V. & Mohideen, U., 2008) and the SNARE hypothesis is a central hypothesis in our current understanding of exocytosis. The formation of a four-helix bundle of SNARE proteins generates force to drive the fusion of amphiphilic vesicles with the cell plasma membrane (Südhof, Thomas C., 2013). The exocytotic canonical SNARE complex is further divided into t-SNARE (target) and v-SNARE (vesicle) in the brain. The t-SNARE is made with syntaxin 1 and synaptosome associated protein of 25 kDa (SNAP-25), while the v-SNARE is synaptobrevin/VAMP (vesicle-associated membrane protein)-2.

Syntaxin 1 is made with three conserved domains: an N-terminal helical H_{abc} domain, which regulates SNARE assembly and priming, the H₃ or SNARE forming motif, which contributes a single helix to the four-helix bundle of canonical SNARE, and a C-terminal hydrophobic transmembrane domain anchored to the presynaptic membrane (Fig. 2A). Syntaxin 1 can exist in a 'closed' or 'opened' conformation depending on the interaction of its H_{abc} domain with SM proteins such as Munc-18 (Südhof, T. C., 2004; Teng, F. Y. H., et al., 2001). Alternative syntaxin isoforms such as syntaxin 2–4 are also known to be located at the plasma membrane and involved in various fusion events such as Glut4 translocation and AMPA receptor trafficking (Soo Hoo, L., et al., 2016; Teng, F. Y. H., et al., 2001). Synaptobrevin, the v-SNARE, such as VAMP2, is also a transmembrane protein consisting of a single α -helical SNARE and a cytoplasmic motif (Fig. 2A). It is localized to and embedded within the synaptic vesicles (Hong, W., 2005; Seagar, M., et al., 1999) and forms a canonical SNARE complex consisting of SNAP25, syntaxin 1, and synaptobrevin in the brain. In addition to VAMP2, low-abundance isoforms such as VAMP4 (Raingo, J., et al., 2012), VAMP7 (Scheuber, A., et al., 2006), and Vps10p tail interactor 1 (Vti1a) (Ramirez, D. M., et al., 2012) form non-canonical SNARE complexes and participate in asynchronous and spontaneous neurotransmitter release (Ramirez, D. M. & Kavalali, E. T., 2012). Lastly, SNAP25 consists of two soluble α -helical SNARE motifs joined by an unstructured linker region, contributing two helices to the four-helix SNARE protein (Matteoli, M., et al., 2009). SNAP25 is alternatively spliced, with two isoforms, SNAP25a and SNAP25b, differing by nine residues. Based on the alternative splicing, SNAP25a and SNAP25b, may exist in the central nervous system (Bark, I. C., 1993; Bark, I. C., et al., 1995; Bark, I. C. & Wilson, M. C., 1994). SNAP25a expression predominates in prenatal and early postnatal life while SNAP25b is predominantly in adult brain (Bark, C., et al., 2004; Bark, I. C., et al., 1995; Prescott, G. R. & Chamberlain, L. H., 2011). Localized at the presynaptic membrane, SNAP25 is anchored to the membrane via posttranslational palmitoylation of 4 cysteine residues within the linker region (Jahn, R. & Scheller, R. H., 2006) (Fig. 2A). In addition to SNAP25, the closely related isoforms SNAP23 and SNAP29 may form SNARE complex in certain neurons and regulate intracellular trafficking (Mendez, M. & Gaisano, H. Y., 2013; Ravichandran, V., et al., 1996; Su, Q., et al., 2001; Williams, C. M., et al., 2015; Zhu, Q., et al., 2015). The formation of the ternary SNARE complex is initiated by the formation of t-SNARE with syntaxin 1 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).

2.2 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 (Rizo, J. & Xu, J., 2015; Südhof, Thomas C., 2013). Munc-18 initiates the assembly of SNARE protein by interacting with a 'closed' conformation of syntaxin 1 (Dulubova, I., et al., 1999; Misura, K. M. S., et al., 2000; Rizo, J. & Südhof, T. C., 2002) (Fig.3). This interaction causes a conformational change in syntaxin 1 to an 'opened' state allowing SNARE protein formation (Dulubova, I., et al., 1999; Misura, K. M. S., et al., 2000; Rizo, J. & Südhof, T. C., 2002) (Fig.3). Because the number of docked vesicles was reduced in Munc 18–1 knockout mice (Voets, T., et al., 2001), Munc-18 is speculated to 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 at four α -helices and interacting with the N-terminus of syntaxin 1 (Rizo, J. & Rosenmund, C., 2008; Südhof, T. C. & Rothman, J. E., 2009). In addition to syntaxin 1, it is found to directly interact with both SNAP25a and b isoforms but preferentially binds to the SNAP25b isoform (Weber-Boyvat, M., et al., 2016).

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 (Aravamudan, B., et al., 1999; Augustin, I., et al., 1999; Richmond, J. E., et al., 1999). The MUN domain of Munc-13 accelerates the conformational change of syntaxin 1 for the Munc18-ternary SNARE interaction to stabilize the SNARE protein complex (Ma, C., et al., 2011; Rizo, J. & Südhof, T. C., 2012; Rizo, J. & Xu, J., 2015). Recently, Munc-13 was discovered to promote the proper Syntaxin/VAMP sub-configuration during assembly of ternary SNARE from t-SNARE independent of Munc-18 (Lai, Y., et al., 2017). The cooperation of Munc-18 and Munc-13 is necessary for proper assembly of ternary SNARE complex.

2.3 Synaptotagmins

Synaptotagmins (Syts), Ca^{2+} sensors, are also essential for evoked, synchronous vesicle fusion to occur (Brose, N. P., AG. Südhof, TC. Jahn, R., 1992). In humans, over 17 Syt isoforms are found, in addition to structurally related proteins that also act as calcium sensors for exocytosis. (Südhof, T. C. & Rothman, J. E., 2009). However, Syt1 is known as the primary isoform that is implicated in fast, synchronous, Ca^{2+} dependent neuronal exocytosis (Gao, Z., et al., 2000; Geppert, M., et al., 1994; Rizo, J., et al., 2006; Schonn, J.-S., et al., 2008; Tang, J., et al., 2006). Localized at the vesicular surface of synaptic and large dense core vesicles, Syt1 has an N-terminal transmembrane domain and two cytoplasmic, protein kinase C-like C2 domains: C2A and C2B, respectively (Fig.3). These domains can simultaneously bind to the lipid membrane and Ca^{2+} and play an important role in exocytosis (Chapman, E. R., et al., 1995; Perin, M. S., et al., 1990). Syt1 interacts with both syntaxin1A and the C-terminus of SNAP25 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, K. L., et al., 2007). Leu394 and I352 on C2B domain are also identified to form a hydrophobic coiled-coil like interaction with syntaxin-1A residue in SNARE-complexin-synaptotagmin crystal structure (Zhou, Qiangjun, et al., 2017). In addition, anionic phospholipids are important for Syt1's function (Chapman, E. R., et al., 1998; Chapman, E. R. & Jahn, R., 1994; Fernandez, I., et al., 2001). Syt isoforms may also play an inhibitory role in suppressing spontaneous release. When Syt1 was deleted, a 10- fold enhancement of spontaneous release was found (Broadie, K., et al., 1994; Littleton, J. T., et al., 1993). 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, J. M., et al., 2002; Nishiki, T.- i. & Augustine, G. J., 2004; Shin, O.- H., et al., 2009). While Syts are essential for exocytosis, further characterization of their molecular functions in the 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, slow release that occurs after action potentials (Sun, J., et al., 2007). Doc2 has been implicated as the Ca^{2+} sensor for asynchronous release in cerebellar slices (Groffen, A. J., et al., 2010) and cultured hippocampal neurons (Groffen, A. J., et al., 2010; Yao, J., et al.). Although further studies are needed, calcium sensors are important in both asynchronous and synchronous release.

Recently, it was reported that synaptotagmin did not bind SNAREs at “physiological” concentrations of Mg^{2+} , K^{+} , and ATP (Park, Y., et al., 2015). The authors postulated that synaptotagmin- lipid interactions and not synaptotagmin-SNARE interactions trigger exocytosis. This hypothesis does not seem to be compatible with our current understanding of the action of $\text{G}\beta\gamma$ on exocytosis downstream of calcium channels. Moreover, it is at odds with mutagenesis studies conducted by several groups. First, charge-reversal mutations of the C-terminal portion of the second helix of SNAP25 were shown to attenuate Syt1 binding, and were inhibitory to exocytosis (Zhang, X., et al., 2002; Zurawski, Z., et al., 2016), presumably at the presynaptic terminal, containing physiological concentrations of K^{+} and Mg^{2+} . Studies with residue R398 further link SNARE binding to syt's stimulatory action on lipid mixing. Mutations of R398 to Ala inhibited SNARE binding (both in the presence and absence of Ca^{2+}) and lipid mixing (Gaffaney, J. D., et al., 2008), but not PS binding (Gaffaney, J. D., et al., 2008). Mutation of R398 to Gln had a similar effect on lipid mixing and exocytosis in overexpression studies in Syt1 KO neurons (Xue, M., et al., 2008). It is noted that the neighboring residue R399 has been shown to affect Syt1- lipid binding (Gaffaney, J. D., et al., 2008; Xue, M., et al., 2008), rendering studies conducted with double mutants unsuitable for distinguishing between the SNARE and lipid-binding profiles of Syts.

Aside from mutagenesis studies, Park et al. postulated that Mg^{2+} exhibited a charge-shielding effect on PIP_2 , blocking its ability to interact with Syts (Park, Y., et al., 2015). However, their data do not support this conclusion. Physiological levels of Mg^{2+} in mammalian neurons are estimated at sub- millimolar levels (Li-Smerin, Y., et al., 2001; Romani, A. & Scarpa, A., 1992), while their own data show that no significant charge shielding or inhibition of lipid mixing occurs at 1mM Mg^{2+} (Park, Y., et al., 2015).

Furthermore, Syt-SNARE interactions have been observed to occur at physiological concentrations of K^+ (Brewer, K. D., et al., 2015; Tucker, W. C., et al., 2004; Zhang, X., et al., 2002; Zurawski, Z., et al., 2016), in both the presence and absence of lipid membranes. More recent studies, performed in the presence of 3mM ATP and 1mM Mg^{2+} , still show that synaptotagmin 1 C2B still interacts with SNARE (Wang, S., et al., 2016), with 3mM ATP being weakly inhibitory. The authors attributed this to Park et al.'s selective single-Cys labeling of residue S342, which results in FRET being measured between K326–327 and the SNARE complex instead of the full synaptotagmin 1-SNARE interaction (Wang, S., et al., 2016). Labeling of residues T285 or C277 permit the observation of Ca^{2+} -dependent synaptotagmin-SNARE interactions (Wang, S., et al., 2016; Zurawski, Z., et al., 2017). Taken in tandem with the mutagenesis data, these studies favor the current consensus of Syt1-SNARE interactions being crucial for exocytosis.

2.4 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 (Fig.4). These proteins interact with individual SNARE proteins, the t-SNARE dimer, or the ternary SNARE in different ways to serve critical exocytic regulatory functions.

Complexin is a regulatory protein involved in the priming of vesicles and exocytosis (Chicka, M. C. & Chapman, E. R., 2009; Hobson, R. J., et al., 2011; McMahon, H. T., et al., 1995; Tang, J., et al., 2006). Made with the N-terminus accessory helix and a central helix (Bracher, A., et al., 2002), it interacts with the ternary SNARE by binding as a fifth α -helix to the SNARE bundle of ternary SNARE (Bracher, A., et al., 2002; Chen, X., et al., 2002; Zhou, Q., 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, A., et al., 2002; Chen, X., et al., 2002; Giraudo, C. G., et al., 2006; Maximov, A., et al., 2009). In Munc13–1 knockout mice, complexin levels were also decreased, suggesting it acts in tandem with Munc 13–1 in addition to Syt1 to regulate exocytosis (Augustin, I., 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, J., et al., 2012; Lai, Y., et al., 2014). SNARE-complexin-Syt1 complex locks the complex in the pre- fusion state where SNARE is half- zippered and the membrane apart (Zhou, Qiangjun, et al., 2017). The cooperation between these proteins may be critical for the synchronizing and activating evoked released.

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, U., et al., 2009; Fujita, Y., et al., 1998; Hatsuzawa, K., et al., 2003). It is thought of as a negative regulator of exocytosis for this reason. It has three distinct domains: two sets of 7 WD40 repeats at the N-terminus domain, a C-terminal VAMP like SNARE binding motif, and a hypervariable linker domain (Ashery, U., et al., 2009; Pobbati, A. V., et al., 2004). Both the N- and C- termini are important to tomosyn's *in vivo* regulation of exocytosis (Burdina, A. O., et al., 2011). The C-terminal VAMP- like SNARE motif binds to t-SNARE

and inhibits the priming step in both neuroendocrine cells and neurons (Baba, T., et al., 2005; Gracheva, E. O., et al., 2006; McEwen, J. M., et al., 2006; Yizhar, O., et al., 2004). Interestingly, tomosyn and Munc-13 may act through syntaxin in an antagonistic manner, (Ashery, U., et al., 2009) regulating vesicle docking and priming by the formation of “zippered” SNARE. The N-terminal domain of tomosyn is known to interact with and arrest syntaxin 1 in a fusion-incompetent complex (Yu, H., et al., 2014). This tomosyn–syntaxin 1 interaction is suggested to be required for the recruitment of tomosyn to vesicle fusion sites. Moreover, this tomosyn-syntaxin1 interaction is important in the coordination with Munc-18 and Munc-13 to precisely regulate SNARE complex formation (Li, Y., et al., 2018). The physiological effects of tomosyn are not fully understood yet, as knockdown of tomosyn in SCG neurons reduced synaptic transmission instead of enhancing synaptic transmission (Ashery, U., et al., 2009). Additional studies upon tomosyn and its exact molecular mechanisms and 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 a fusion machinery, binds to Munc13, VDCC, and the vesicle bound GTPase Rab 3/27 in a GTP dependent manner (Südhof, Thomas C., 2013). It is made up of 5 domains: an N-terminal domain with zinc finger, a central PDZ domain, two C-terminal C2 domains, interestingly, without Ca^{2+} binding function, and a conserved proline-rich linker between two C2 domains (Südhof, T. C., 2012). The N-terminal domain is known to interact with C2A domain of Munc13–1 (Betz, A., et al., 2001; Lu, J., et al., 2006) while the central PDZ domain interacts with N- and P/Q type of Ca^{2+} channels (Kaeser, P. S., et al., 2011). The interactions between RIM and Munc13 and VDCC allow vesicles to be close to the plasma membrane for the tight spatial regulation of calcium entry in the active zone (Südhof, Thomas C., 2013). Although further studies are needed to understand the function of C2 domains, C2A domain may interact with SNARE (Coppola, T., et al., 2001) while C2B domain interacts with Syt1 (Schoch, S., et al., 2002).

Cysteine string protein (CSP) is a member of the Hsp40 family of co-chaperones, occurring as a heterotrimer of CSP, Hsc70, and SGT. It is localized in the active zone for synaptic vesicles (Burgoyne, R. D. & Morgan, A., 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 (Chamberlain, L. H., et al., 2001; Nie, Z., et al., 1999; Wu, M. N., et al., 1999), VAMP (Boal, F., et al., 2004; Seagar, M., et al., 1999), Syt (Boal, F., et al., 2011; Evans, G. J. & Morgan, A., 2002), dynamin 1 (Rozas, J. L., et al., 2012), and G protein subunits (Magga, J. M., et al., 2000; Natochin, M., et al., 2005). In CSP α knockout mice, SNAP25 is reduced through increased ubiquitination and degradation of SNAP25, suggesting the role of CSP α as a co-chaperone of SNAP25 folding (Sharma, M., et al., 2011). Despite this, the interaction between CSP and SNAP25 is indirect: binding occurs through Hsc70 (Sharma, M., et al., 2011). Moreover, overexpression of CSP increases the abundance of SNAP25 (Graham, M. E. & Burgoyne, R. D., 2000). Although only 2.8 CSP molecules per synaptic vesicles and 941 CSP molecules per synapse on average were found (Burgoyne, R. D. & Morgan, A., 2015), CSP may be a neuroprotective chaperone of SNAP25 by its interaction with Hsc70 (Johnson, J. N., et al., 2010).

Another SNARE- interacting protein, G $\beta\gamma$ dimers, consisting of G protein β and γ subunits as an obligate heterodimer, interact with ternary SNAREs, t-SNAREs, and the SNARE proteins SNAP25, syntaxin, and VAMP2 to mediate inhibition of exocytosis by G $_{i/o}$ -coupled GPCRs (Blackmer, T., et al., 2005; Blackmer, T., et al., 2001; Gerachshenko, T., et al., 2005a; Hamid, E., et al., 2014; Photowala, H., et al., 2006; Wells, C. A., et al., 2012; Yoon, E.J., et al., 2007; Yoon, E. J., et al., 2008). G $\beta\gamma$ interaction and specificity to each effector and its importance in synaptic transmission are under active study both *in vitro* and *in vivo* (Blackmer, T., et al., 2001; Currie, K. P., 2010; Gerachshenko, T., et al., 2005a; Herlitze, S., et al., 1996; Huang, C. L., et al., 1995; Sadjia, R. & Re uveny, E., 2009; Wells, C. A., et al., 2012; Yoon, E. J., et al., 2007). This review will mainly focus on the G $\beta\gamma$ -SNARE interaction, a major mechanism of presynaptic modulation downstream of Ca $^{2+}$ entry, mediated by the activation of Gi/o inhibitory GPCRs. Further understanding of this mechanism will be necessary to fully understand the architecture and mechanism of exocytosis.

3. G protein-coupled Receptors (GPCRs)

G protein-coupled receptors (GPCRs) make up 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 (Eglen, R. M. & Reisine, T., 2009; Hamm, H. E., 1998; Millar, R. P. & Newton, C. L., 2010; Oldham, W. M. & Hamm, H. E., 2008; Yoon, E. J., et al., 2008). Encoded by approximately 800 genes in the human genome, GPCRs are frequently utilized as drug targets, and a large number of GPCR ligands such as neurotransmitters and peptide hormones have been identified (Fredriksson, R., et al., 2003; Venkatakrishnan, A. J., et al., 2013). However, many GPCRs still remain as orphan receptors with no identifiable ligands (Laschet, C., et al., 2018; Millar, R. P. & Newton, C. L., 2010; Oldham, W. M. & Hamm, H. E., 2008).

Upon ligand binding, GPCRs go through a conformational change to increase the receptors' affinity for the G protein heterotrimer (G $\alpha\beta\gamma$), resulting in the formation of a transient ligand-GPCR-G protein ternary complex (Oldham, W. M. & Hamm, H. E., 2008). GPCRs work as catalytic activators of G $\alpha\beta\gamma$ to transduce extracellular signals into intracellular responses by the release of GTP-bound G α (GTP-G α) and G $\beta\gamma$ (Buranda, T., et al., 2007) which activate downstream effectors (Fig. 5) (Blackmer, T., et al., 2005; Cabrera-Vera, T. M., et al., 2003; Clapham, D. E. & Neer, E. J., 1997a; Gautam, N., et al., 1998a; Gerachshenko, T., et al., 2005a; Smrcka, A. V., 2008a; Vanderbeld, B. & Kelly, G. M., 2000a).

4. G $\beta\gamma$

There are 5 different G β and 12 different G γ subunits (Downes, G. B. & Gautam, N., 1999; Hildebrandt, J. D., 1997; Khan, S. M., et al., 2013; Simon, M. I., et al., 1991). G β subunits are made up of an N-terminal α helix of approximately 20 amino acids and 7 blades of four anti-parallel β strands forming a propeller like WD repeat (Clapham, D. E. & Neer, E. J., 1997b; Gautam, N., et al., 1998b; Smrcka, A. V., 2008a; Sondek, J., et al., 1996) (Fig. 6). G β_{1-4} share up to 90% amino acid sequence identity whereas G β_5 is only 50% identical

(Betty, M., et al., 1998; Smrcka, A. V., 2008a). Interestingly, G β_5 , unlike other G β subunits, interacts with RGS proteins and don't or weakly interact with G γ subunits (Dingus, J. & Hildebrandt, J. D., 2012; Khan, S. M., et al., 2013; McIntire, W. E., 2009). In contrast, G γ subunits are very divergent, sharing only 30–70% sequence identity (Betty, M., et al., 1998; Smrcka, A. V., 2008a). 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 G β while the N-terminal α helix forms a coiled-coil interaction with the N-terminal helix of the β subunit (Clapham, D. E. & Neer, E. J., 1997b) (Fig. 6). G γ subunits can be post--translationally modified at the C-terminal cysteine which is carboxymethylated and modified with a farnesyl or geranylgeranyl moiety via a thioether bond. These modifications enable the membrane localization of G $\beta\gamma$ (Clarke, S., 1992; Cox, A. D., 1995). Together, G β and G γ subunits form G $\beta\gamma$ dimers with the help of chaperonins including TCP-1 (CCT), phosducin- like protein 1 (PhLP1), and dopamine receptor- interacting protein 78 (DRip78) in a stepwise manner, (Dupre, D. J., et al., 2009; Khan, S. M., 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, A. V., 2008a).

Although there are 60 (5 G β x 12 G γ subunits) different theoretical combinations of G $\beta\gamma$ dimers (Dingus, Jane & Hildebrandt, John D., 2012; Dingus, J., et al., 2005; Khan, S. M., et al., 2013), numerous *in vitro* assays and yeast-two hybrid analyses have indicated that not all theoretical G $\beta\gamma$ dimers exist, are equally expressed, or interact with G α subunits, receptors, effectors, and downstream signaling factors (Hildebrandt, J. D., 1997; Khan, S. M., et al., 2013; Pronin, A. N. & Gautam, N., 1992a; Robishaw, J. D. & Berlot, C. H., 2004; Schwindinger, W. F., et al., 2003; Schwindinger, W. F., et al., 2004; Schwindinger, W. F., et al., 2010; Schwindinger, W. F., et al., 2011b; Smrcka, A. V., 2008b; Yan, K., et al., 1996). Some G β and G γ subunits are ubiquitously expressed, while others are localized in specific brain regions and cell types (Betty, M., et al., 1998; Cali, J. J., et al., 1992; Jones, P. G., et al., 1998; Largent, B. L., et al., 1988; Liang, J. J., et al., 1998; Zhang, J. H., et al., 2000). Because most cell types express multiple G β and G γ subunits, specific expression levels and localization may influence intracellular signaling cascades through the formation of specific G $\beta\gamma$ dimers. Recently, we have identified a regional and subcellular expression pattern of four G β (G β_1 , G β_2 , G β_4 , and G β_5) and six G γ (G γ_2 , G γ_3 , G γ_4 , G γ_7 , G γ_{12} , and G γ_{13}) subunits in mouse synaptosomes of cortex, cerebellum, hippocampus, and striatum (Betke, K. M., et al., 2014). In addition, we have quantified and compared the protein level and subcellular localization of each neuronal G β and G γ subunit in whole and fractionated synaptosomes of mouse whole brain, generating a comprehensive, quantitative brain map of neuronal G β and G γ subunits (Yim, Y. Y., et al., 2017). In this study, G β_1 and G γ_2 were the most abundant subunits, while G β_4 , G β_5 , and G γ_{13} were the least abundant subunits, respectively (Yim, Y. Y., et al., 2017). In addition, G β_1 and G γ_{12} were localized at both pre- and post-synaptic membranes while G β_4 and G β_5 were preferentially localized at the postsynaptic membrane (Yim, Y. Y., et al., 2017). Moreover, each G β and G γ subunit exhibited widely varying affinities for each other (Hildebrandt, J. D., 1997; Smrcka, A. V., 2008a; Stephens, G. J., 2009). While G β_1 and G β_4 dimerize with all G γ subunits, G β_2 and G β_3 are unable to dimerize with G γ_1 and G γ_{11} (Dingus, J. & Hildebrandt, J. D., 2012; Khan, S. M., et al., 2013). In addition, G β_5 has low affinity interaction with G γ subunits and

prefer to interact with RGS (Liang, J. J., et al., 1998). $G\beta_2\gamma_1$ shows a stronger association than $G\beta_2\gamma_4$ (Pronin, A. N. & Gautam, N., 1992b; Smrcka, A. V., 2008a; Zhang, H., et al., 2009). Different affinities between $G\beta$ and $G\gamma$ subunits, in combination with the expression and localization of individual $G\beta$ and $G\gamma$ subunit, may determine which $G\beta\gamma$ dimers are active in a given cell (Betty, M., et al., 1998; Khan, S. M., et al., 2013). Despite many attempts to understand the specificity of $G\beta\gamma$ dimers and physiological function of each $G\beta\gamma$ dimer (Betke, K. M., et al., 2014; Cali, J. J., et al., 1992; Liang, J. J., et al., 1998; Morishita, R., et al., 1998; Robishaw, J. D., et al., 1989; Zhang, J. H., et al., 2000), these questions still remained unanswered due to the high sequence homology between subunits and lack of subunit-specific antibodies (Bigler, T. L., et al., 1993). However, with the development of a quantitative targeted multiple reaction monitoring (MRM) method and various genetic approaches, such as ablation (knockout) for each subunit, we may now be able to predict the *in vivo* expression level of $G\beta\gamma$ dimers, supporting the hypothesis of $G\beta\gamma$ specificity to particular GPCRs and effector proteins to maintain normal brain function.

To date, numerous genetic deletion studies and knockout animal studies have suggested specific roles for different $G\beta$ and $G\gamma$ subunits in intact cells and mice (Gautam, N., et al., 1998b; Khan, S. M., et al., 2013). $G\beta_1$ is involved in neuronal development; its knockout leads to perinatal lethality with reduced cortical thickness, brain volume, and impaired neuronal progenitor cell proliferation (Okabe, H. & Iwakura, Y., 2010). $G\beta_2$ is involved in neuronal excitability by the modulation of Ca^{2+} channels (DePuy, S. D., et al., 2006; Wolfe, J. T., et al., 2003). In contrast, $G\beta_5$ specifically interacts with the $G\gamma$ -like domain of the regulator of G protein signaling (RGS) proteins (RGS 6, 7, 9 and 11) in addition to $G\gamma$ subunits (Hillenbrand, M., et al., 2015; Liang, J. J., et al., 1998). $G\beta_5$ knockout mice show impaired development, motor learning, and hyperactivity (Chen, C. K., et al., 2003; Zhang, J. H., et al., 2011). $G\gamma_3$ 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, A. N. & Gautam, N., 1992b; Schwindinger, W. F., et al., 2003). Unique physiological phenotypes of each $G\beta$ and $G\gamma$ subunit suggest a great deal of specificity in $G\beta\gamma$ dimerization and signaling (Albert, P. R. & Robillard, L., 2002; Lim, W. K., et al., 2001; Lindorfer, M. A., et al., 1998) despite the high degree of homology between them.

Interestingly, various $G\beta\gamma$ dimers have been reported to have differential affinities to each $G\beta\gamma$ effectors. $G\beta_1\gamma_2$ has a 40 order of magnitude higher affinity for SNARE and a 20 order of magnitude higher inhibition of exocytosis than $G\beta_1\gamma_1$ (Blackmer, T., et al., 2005). We speculate that the difference in the primary structure of each $G\gamma$ subunit, per se, or in the post-translation modification of $G\gamma_1$ and $G\gamma_2$ may mediate the change in affinity. Peptides consisting of the N-terminus of $G\gamma_2$ inhibit the $G\beta\gamma$ -SNARE interaction more potently than that of $G\gamma_1$ (Zurawski, Z., et al., 2017). Moreover, various studies 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 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 $G\beta_3\gamma_4$, preferentially couple with somatostatin and muscarinic M4 GPCRs (Asano, T., et al., 1999; Hosohata, K., et al., 2000; Kleuss, C., et al., 1992). The expression and subcellular localization of $G\beta$ and $G\gamma$ subunits in the CNS may also determine which $G\beta\gamma$ dimers exist,

their roles in regulating signaling cascades, and their impact in neurological diseases and GPCR targeted drug mechanisms (Betke, K. M., 2014; Yim, Y. Y., et al., 2017).

In addition to SNAREs, G $\beta\gamma$ dimers interact with a wide variety of effectors such as adenylyl cyclases, phospholipase C β , PI3 kinase, calmodulin, and components of the mitogen-activated protein kinase cascade (Fig.5) (Cabrera-Vera, T. M., et al., 2004; Clapham, D. E. & Neer, E. J., 1997b; Goldsmith, Z. G. & Dhanasekaran, D. N., 2007; Myung, C. S., et al., 1999; Tang, W. J. & Gilman, A. G., 1991; Vanderbel, B. & Kelly, G. M., 2000b). In the central nervous system (CNS), G $\beta\gamma$ dimers also interact with voltage-dependent calcium (VDCC) (Currie, K. P., 2010; Herlitze, S., et al., 1996) and inward-rectifying potassium (GIRK) channels (Huang, C. L., et al., 1995; Sadj, R. & Reuveny, E., 2009), and soluble NSF attachment protein receptors (SNARE) (Blackmer, T., et al., 2001; Gerachshenko, T., et al., 2005b; Wells, Christopher A., et al., 2012; Yoon, E. J., et al., 2007) to regulate neurotransmitter release at the synapse (Fig.5). The G $\beta\gamma$ -effector interaction sites were hypothesized to be localized at the G α interface (Clapham, D. E. & Neer, E. J., 1997b; Ford, C. E., et al., 1998). While the G α subunit's interface likely represents a core site of G $\beta\gamma$ and its effector binding, other regions may also play important roles in facilitating G $\beta\gamma$ mediated downstream signaling. Mutagenesis studies revealed the binding regions of effectors within the blades of the G β propeller and its N-terminus coiled-coil, the G α subunit interface, and regions on G γ subunit (Clapham, D. E. & Neer, E. J., 1997b; Myung, C. S., et al., 2006; Panchenko, M. P., et al., 1998; Peng, L., et al., 2003; Smrcka, A. V., 2008a). For example, GIRK channels were found to interact just outside of the G α binding site. Residues Thr 86, Thr87, and Gly131 on G β_1 have been implicated through the structure and functional studies the G $\beta\gamma$ -GIRK interaction (Zhao, Q., et al., 2003). The x-ray structure of the mammalian GIRK2-G $\beta\gamma$ complex suggests that G β residues Leu 55, Lys 78, Ser 98 and Trp 99 are also important in G $\beta\gamma$ -GIRK interaction (Whorton, M. R. & MacKinnon, R., 2013). Although many of these G $\beta\gamma$ -effector interactions and downstream signaling cascades are well understood, it is still unclear which combination of G $\beta\gamma$ dimers are present *in vivo* and what factors control the specificity of G $\beta\gamma$ dimers to their effectors.

5. GPCR-mediated modulation of synaptic transmission

GPCRs are key regulators of synaptic transmission by the actions of GTP-G α and G $\beta\gamma$ (Betke, K. M., et al., 2012). G α_s and G α_q coupled GPCRs mainly regulate synaptic transmission through the actions of GTP-G α subunits. GTP-G α_s and G α_q stimulate AC and phospholipase C β 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, D. A. & Sihra, T. S., 2008). For example, the phosphorylation of K⁺ channels by PKA perturbs cell voltage and activates VDCC (Brunelli M, C. V., Kandel ER, 1976; Castellucci, V. F., et al., 1980). Residue S10 of CSP α is also phosphorylated by PKA, reducing its binding to syntaxin and Syt to prolong the opening of the fusion pore (Evans, G. J. O. & Morgan, A., 2002; Evans, G. J. O., et al., 2001). Lastly, Syt12 is phosphorylated at S97 by PKA (Maximov, A., et al., 2007). This is essential for normal presynaptic mossy fiber LTP in the CA3 region of the hippocampus and long-term enhancement of mossy fiber synaptic transmission (Kaesler-Woo, Y. J., et al., 2013). Moreover, cAMP elevation in the presynaptic

terminal increases transmitter release (Fernandes, H. B., et al., 2015). For example, Epac2, the guanine nucleotide exchange factor, is directly activated by PKA and promote transmitter release (Ozaki, N., et al., 2000). It is required to maintain the readily releasable pool at mossy fiber synapses in the hippocampus (Fernandes, H. B., et al., 2015). Similar to PKA, PKC phosphorylates Munc-18 at S313 (Morgan, A., et al., 2005), the I-II linker of VDCC (Zamponi, G. W., et al., 1997), SNAP25 SN2 helix at S187 (Shimazaki, Y., et al., 1996), and Syt1 at Thr112 (de Jong, A. P., et al., 2016; Hilfiker, S., et al., 1999). In addition, activated GPCRs recruit arrestins and MAP kinase signaling (DeFea, K. A., 2011) and stimulate PLC β to generate diacylglycerol (DAG) (Panchenko, M. P., et al., 1998), a critical second messenger for exocytic regulation. DAG direct interaction with the C₁ (Rhee, J. S., et al., 2002) and Ca²⁺ binding of C₂B domains (Shin, O.-H., et al., 2010) as well as the calmodulin-binding region of Munc13 (Junge, H. J., et al., 2004) directly affect vesicle release and presynaptic plasticity (Rosenmund, C., et al., 2002). The PKC phosphorylated Munc18 and Munc13 pathways may be two incompatible pathways to maintain normal synaptic release rate during repetitive stimulation and in presynaptic plasticity in general (Wierda, K. D., et al., 2007).

Unlike G α_s and G α_q coupled GPCRs, G $\alpha_{i/o}$ coupled GPCRs (G $\alpha_{i/o}$ coupled GPCRS), such as D₂ dopamine, M₄ muscarinic, α_{2a} adrenergic, and opioid receptors, are known to inhibit evoked exocytosis by the actions of both GTP-G α and G $\beta\gamma$ (Betke, K. M., et al., 2012; Stephens, G. J., 2009). G $\beta\gamma$ modulates exocytosis through interactions with VDCC and GIRK channels and SNARE on a fast timescale (Betke, K. M., et al., 2012; Blackmer, T., et al., 2005; Fernández-Alacid, L., et al., 2009; Gerachshenko, T., et al., 2005a; Hamid, E., et al., 2014; Miller, R. J., 1998) while GTP-G α_i inhibits AC and reduces cAMP and the activity of PKA on a slower timescale. G $\alpha_{i/o}$ coupled GPCRs modulate synaptic transmission through the liberation of G $\beta\gamma$ from both autoreceptors activated by the neurotransmitter released from the activated synapse, and heteroreceptors activated by neurotransmitters from adjacent nerve terminals. (Table. 1)(Betke, K. M., et al., 2012; Forray, M. I., et al., 1999; Millan, M. J., et al., 2000; Mongeau, R., et al., 1998).

Given that most cell types express multiple G β and G γ subunits, a greater understanding of G $\beta\gamma$ -effector regulation is necessary to fully elucidate the physiological importance of G $\beta\gamma$ in regulating synaptic transmission and how specificity is achieved for each mechanism at each synapse. Despite many attempts to understand the specificity both *in vitro* and *in vivo*, we do not fully understand G $\beta\gamma$ specificity. The *in vitro* G $\beta\gamma$ specificity is difficult to determine (Butkerait, P., et al., 1995) although the identity of G β and γ subunits was suggested to influence the activation of receptors and downstream signaling in various studies (Hou, Y., et al., 2000; Hou, Y., et al., 2001; McIntire, W. E., et al., 2001; Richardson, M. & Robishaw, J. D., 1999a). *In vivo* studies, authors identified the G $\beta\gamma$ specificity and suggested that unique G $\beta\gamma$ combinations may play specific roles in mediating interactions with both receptors and effectors (Albert, P. R. & Robillard, L., 2002; Kleuss, C., et al., 1991; Kleuss, C., et al., 1992, 1993; Robishaw, J. D. & Berlot, C. H., 2004) and that isoforms may show tissue-dependent specificity for an individual effector (Robishaw, J. D. & Berlot, C. H., 2004; Schwindinger, W. F., et al., 2003; Schwindinger, W. F., et al., 2004; Schwindinger, W. F., et al., 2010; Schwindinger, W. F., et al., 2011a). For example, activated alpha-2a adrenergic receptors (α_{2a} -ARs) are found to interact with G α_{i1} , G $\beta1$, G $\beta2$, G $\gamma2$,

G γ_3 , G γ_4 , and G γ_7 as shown by a fluorescence resonance energy transfer (FRET) assay (Gibson, S. K. & Gilman, A. G., 2006; Richardson, M. & Robishaw, J. D., 1999b). Moreover, M $_4$ muscarinic receptor was found to inhibit L-type Ca $^{2+}$ channel by G α_o , G β_3 , and G γ_4 (Krumins, A. M. & Gilman, A. G., 2006). However, these studies monitor second messenger productions or other downstream responses which are not a direct assessment of G protein activation (Gibson, S. K. & Gilman, A. G., 2006). With a recent development of quantitative multiple reactions monitoring mass spectrometry (Yim, Y. Y., et al., 2017), we now can examine the specificity of G $\beta\gamma$ further and determine how G $\beta\gamma$ specificity may affect the inhibition of exocytosis.

5.1 The regulation of G protein-activated Inward Rectifying Potassium (GIRK) channels by G $\beta\gamma$.

Expressed in neurons, atrial cells, and ventricle, activation of GIRK channels reduces membrane excitability by hyperpolarizing the neurons with positive K $^{+}$ current with inward K $^{+}$ flow (Clapham, D. E. & Neer, E. J., 1997b; Fernández- Alacid, L., et al., 2009; Kulik, A., et al., 2006; Liang, B., et al., 2014; Lüscher, C., et al., 1997; Lüscher, C. & Slesinger, P. A., 2010; Yokogawa, M., et al., 2011). Today, 4 mammalian GIRK isoforms (GIRK 1–4) have been identified to form various functional heterotetramers in the brain (Dascal, N., 1997; Finley, M., et al., 2004; Peng, L., et al., 2003; Sadjia, R. & Reuveny, E., 2009). The activity of GIRK channels is regulated by activated Gi/o coupled GPCRs, such as opioid, 5-HT $_{1a}$ serotonin, and GABA $_B$ receptors (Ikeda, K., et al., 2003; Ponce, A., et al., 1996; Sadjia, R., et al., 2003) to dampen postsynaptic responses. G $\beta\gamma$ 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 (Huang, C. L., et al., 1995; Logothetis, D. E., et al., 1987; Sadjia, R. & Reuveny, E., 2009; Whorton, M. R. & MacKinnon, R., 2013). As with many G $\beta\gamma$ effectors, GIRK channels interact with the G α -binding interface of G $\beta\gamma$, including Leu55, Lys78, Ile80, Lys89, and Trp99 of G β (Albsoul- Younes, A. M., et al., 2001; Ford, C. E., et al., 1998). The co-crystal structure shows these residues interact with Gln248, Thr249, Glu251, Gly252, Glu253, and Leu344 of GIRK's cytoplasmic channel domain (Finley, M., et al., 2004; He, C., et al., 1999; Whorton, M. R. & MacKinnon, R., 2013; Yokogawa, M., et al., 2011). In addition to residues on G β subunits, the residues on G γ subunits are important for the G $\beta\gamma$ modulation of GIRK channels. G γ_2 residues 35–71 are required for the full stimulation of GIRK4 currents by G $\beta_1\gamma_2$ (Peng, L., et al., 2003).

Specificity is mediated by G $\beta\gamma$ within the regulation of GIRK channel by GPCRs. G $\beta\gamma$ dimers containing G β_{1-4} enhance GIRK channel activity, while those containing G β_5 , although the physiological relevance of the interaction of G β_5 with G γ subunits is not clear, suppress it (Lei, Q., et al., 2000; Lei, Q., et al., 2001; Reuveny, E., et al., 1994; Takao, K., et al., 1994; Wickman, K. D., et al., 1994; Yamada, M., et al., 1994). While G β_5 containing G $\beta\gamma$ dimers are less likely to be available relative to other G $\beta\gamma$ dimers due to its preferential tendency to form heterodimers with RGS proteins, G β_5 containing dimers have been shown to compete with other G β_{1-4} containing G $\beta\gamma$ for binding sites localized on the cytoplasmic domains of the GIRK channels, with a functional consequence of prevention of channel activation (Lei, Q., et al., 2003). Despite this, G β_5 in complex with RGS has been also

shown to interact with the cytoplasmic domains of GIRK channels to facilitate their coupling to GABA_B receptors (Xie, K., et al., 2010).

In addition, D₂ receptor mediated activation of presynaptic GIRK channels are found in ventral tegmental neurons (Michaeli, A. & Yaka, R., 2010). Presynaptic GIRK may inhibit exocytosis by decreasing the amplitude and duration of action potentials. However, it is unclear whether presynaptic GIRK effects are mediated through Gβγ in a similar manner to postsynaptic GIRK. Given the diversity of binding sites on both the channel and the Gβγ, the crystal structure suggests multiple Gβγ binding to GIRK at the same time to elicit the conformational change of GIRK for its activation (Sadjia, R. & Reuveny, E., 2009; Whorton, M. R. & MacKinnon, R., 2013).

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

The most well understood mechanism of fast, inhibitory, GPCR-mediated modulation of exocytosis is the Gβγ-VDCC interaction (Dolphin, A. C. & Scott, R. H., 1987; Hille, B., 1994; Ikeda, S. R., 1996; Zamponi, G. W. & Currie, K. P. M., 2013). Composed of α1, β, α2δ, and γ subunits, VDCCs control the calcium concentration at the presynaptic terminal and are critical in the regulation of vesicle exocytosis (Catterall, W. A., et al., 2005). As stated previously, VDCC are activated upon presynaptic terminal depolarization and mediate increased Ca²⁺ concentration at the terminal, activating Ca²⁺-dependent processes, such as the association of synaptotagmin with lipid members and SNAREs, triggering exocytosis. For example, α₂ adrenergic, GABA, somatostatin, and opioid receptors are shown to function via this mechanism (Dolphin, A. C. & Scott, R. H., 1987; Womack, M. D. & McCleskey, E. W., 1995). At present, five classes of VDCC have been identified in mammals based on their pharmacological and electrophysiological properties, with low voltage (T type) (Perez-Reyes, E. & Lory, P., 2006) and high voltage (L, N, P/Q, and R types) subtypes (Catterall, W. A., et al., 2005). Out of these VDCC, Gβγ directly interacts with N or P/Q type VDCC (Ca_v2) to inhibit Ca²⁺ influx into presynaptic cells and inhibit Ca²⁺ dependent exocytosis (Catterall, W. A., et al., 2005; Currie, K. P., 2010; Herlitze, S., et al., 1996; Ikeda, S. R., 1996; Tedford, H. W., et al., 2006; Tedford, H. W. & Zamponi, G. W., 2006). More recently, R-type VDCC has been shown to be a target for opioid receptor-mediated Gβγ modulation of synaptic transmission (Berecki, G., et al., 2016). Although further studies are needed, these studies suggest that specificity within Gβγ subunits may affect how each Gβγ interacts with various type of VDCC. The Gβγ-VDCC interaction involves the N-terminal loop (Canti, C., et al., 1999), the I–II loop (Herlitze, S., et al., 1996; Zamponi, G. W., et al., 1997), and the C-terminal loop (Furukawa, T., et al., 1998; Li, B., et al., 2004; Qin, N., et al., 1997) 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 (De Waard, M., et al., 1997; Herlitze, S., et al., 1996; Zamponi, G. W., et al., 1997). Although two binding sites were found, the kinetic data suggests that Gβγ and channel interact in a 1:1 ratio (Zamponi, G. W. & Snutch, T. P., 1998). On the Gβ subunit, Lys78, Met101, Asn119, Thr43, Asp186, and Trp322 Gα binding residues are important for the Gβγ-VDCC interaction (Ford, C. E., et al., 1998). Interestingly, Gβγ binding on a QXXER motif of I–II loop of VDCC, similar to the Gβγ binding site on AC2 (Chen, J., et al., 1995), involves PKC as a modulator. The phosphorylation of G protein interaction

domain on a Thr by PKC prevents the binding of G $\beta\gamma$ (Zamponi, G. W., et al., 1997). For example, PKC reduces the somatostatin receptors` mediated inhibition of Ca²⁺ currents (Swartz, K. J., 1993) while phosphorylated Ser and Thr in $\alpha 1$ 416–434 reduce G $\beta\gamma$ binding of VDCC (Zamponi, G. W., et al., 1997).

Additionally, syntaxin 1A may regulate VDCC and the interaction between VDCC, syntaxin, and G $\beta\gamma$ (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, M. K., et al., 1992; Jarvis, S. E. & Zamponi, G. W., 2001; Leveque, C., et al., 1994; O'Connor, V. M., et al., 1993). Upon syntaxin binding, VDCCs undergo a hyperpolarizing change in the voltage dependence of inactivation (Bezprozvanny, I., et al., 1995; Rettig, J., et al., 1996). A ternary complex formed between syntaxin1A, VDCC, and G $\beta\gamma$ may promote the inhibition of VDCC as G $\beta\gamma$ and VDCC bind different domains of syntaxin 1A (Jarvis, S. E., et al., 2000). Overall, the regulation of VDCC by G $\beta\gamma$ of inhibitory GPCRs is widespread and an important regulatory factor controlling neurotransmitter release.

5.3 The inhibition of synaptic transmission via the G $\beta\gamma$ -SNARE interaction.

Upon Ca²⁺ influx, Syt1 binds to the lipid membrane simultaneously with the SNARE complex, and promotes both vesicle priming and fusion of the vesicle by disordering the lipid bilayer in a Ca²⁺ dependent manner (Blackmer, T., et al., 2005; Blackmer, T., et al., 2001; Gerachshenko, T., et al., 2005a) (Fig.7). However, G $\beta\gamma$ subunits are known to compete with Syt1 downstream of calcium entry through the direct binding to SNARE (Blackmer, T., et al., 2005; Yoon, E. J., T. Gerachshenko, B.D. Spiegelberg, S. Alford, and H.E. Hamm, 2007; Zurawski, Z., et al., 2017). This observation was first obtained using GST-pull downs, in which G $\beta\gamma$ and Syt1 C2AB domains (residues 96–421) were shown to competitively displace each other in a concentration-dependent manner on GST- fusions of the cytosolic domains of ternary SNARE complexes (Blackmer, T., et al., 2005). More recently, this observation has been extended to full- length t-SNARE complexes embedded in a lipid environment utilizing fluorescently labeled Syt1 C2AB and TIRF microscopy (Zurawski, Z., et al., 2017). Given that Syt1 contains both lipid-binding and SNARE-binding motifs (Chapman, E. R. & Davis, A. F., 1998; Davis, A. F., et al.; Davletov, B. A. & Südhof, T. C., 1993; Rizo, J. & Xu, J., 2015; Zhang, X., et al., 1998), observing this competition in the environment of a lipid bilayer harboring anionic phospholipids enhances its construct validity.

Synaptotagmins are C2-domain calcium binding proteins that exhibit an enhancement of their ability to bind lipid membranes and SNAREs when Ca²⁺ is present (Chapman, E. R. & Davis, A. F., 1998; Zhang, X., et al., 2002). Predictably, competition with G $\beta\gamma$ is also calcium-dependent: the formation of the holo-Syt1 complex via the addition of 1mM Ca²⁺ increases the amount of G $\beta\gamma$ displaced (Blackmer, T., et al., 2005) relative to an equivalent quantity of apo-Syt1. From this, we infer that the displacement of G $\beta\gamma$ from the SNARE complex occurs by Syt1 binding to it. The specific binding site of Syt1 upon SNARE has been elucidated by crystal structures, with two synaptotagmin C2AB domains binding to one SNARE complex (Zhou, Q., et al., 2015; Zhou, Qiangjun, et al., 2017). The major binding site is a tripartite interface in which the two helices of C2B bind to complexin, VAMP,

syntaxin, and the first helix of SNAP25 (Zhou, Qiangjun, et al., 2017), while a second binding site occurs midway between the two helices of SNAP25 (Zhou, Qiangjun, et al., 2017). It is known that synaptotagmin can compete with Gβγ for binding to syntaxin and SNAP25, but not VAMP (Yoon, E. J., T. Gerachshenko, B.D. Spiegelberg, S. Alford, and H.E. Hamm, 2007). The N-terminal Gβγ binding site (Wells, Christopher A., et al., 2012) is covered by the C2B primary binding interface (Zhou, Qiangjun, et al., 2017). It is less clear how the C-terminal binding site can be displaced by synaptotagmin, although it would seemingly involve the C2A domain of the primary interface. Together, the data suggest that competitive displacement of Gβγ from SNARE may occur at both the N-terminal and C-terminal Gβγ binding sites. To date, biochemical competition studies have utilized only the C2AB domain of synaptotagmin: it is currently unknown if the full-length protein behaves similarly to the isolated domain, although this is inferred from electrophysiological studies with G_{i/o}-coupled GPCRs. It is known that the function of the full-length synaptotagmin is predicated on the prevalence of phospholipids such as PIP2 (Wang, Z., et al., 2011).

The molecular requirements for SNARE to bind Gβγ have progressively become more identified. The first insight into the direct interaction between Gβγ and SNARE proteins was derived from studies utilizing botulinum toxin A (BoNT/A), which cleaves the C-terminal 9 amino acids from SNAP-25 (Binz, T., et al., 1994; Schiavo, G., et al., 1993). Treatment of presynaptic lamprey reticulospinal axons with BoNT/A disrupted the ability of lamprey serotonin receptors (equivalent in function to the human 5-HT_{1B} receptor) to inhibit excitatory postsynaptic action potentials (Gerachshenko, T., et al., 2005a). Furthermore, a 14-amino acid peptide based on the C-terminus of SNAP25 was able to block Gβγ-mediated inhibition (Blackmer, T., et al., 2005; Gerachshenko, T., et al., 2005a). 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 SNAP25 with Gβγ (Yoon, E. J., et al., 2007; Zhao, Y., et al., 2010). Both isoforms of SNAP25, SNAP25a and SNAP25b, are identical at the C-terminal where Gβγ interacts to prevent Syt1-SNARE interaction (Bark, I. C., 1993; Bark, I. C., et al., 1995; Bark, I. C. & Wilson, M. C., 1994). However, Gβ₂ was identified to preferentially interact with SNAP25b isoform in mouse hippocampus (Daraio, T., et al., 2018). Interestingly, SNAP25a isoform has two-fold less interaction with Munc18-1 compared to SNAP25b isoform (Daraio, T., et al., 2018).

In addition to binding to SNAP25, Gβγ also binds individually to the other SNARE proteins, syntaxin 1A and synaptobrevin, as well as to the t-SNARE dimer (SNAP25 with syntaxin 1A) and to the ternary SNARE complex (Yoon, E. J., et al., 2007). Moreover, the presynaptic transmitter inhibition by Gβγ-SNARE interaction have been identified in neurons (Blackmer, T., et al., 2001; Delaney, Andrew J., et al., 2007; Zhang, X.-l., et al., 2011), chromaffin cells (Chen, X. K., et al., 2005; Yoon, E. J., et al., 2008), and pancreatic β cells (Zhao, Y., et al., 2010).

Subsequent *in vitro* binding experiments showed that Gβγ bound to syntaxin1A and synaptobrevin in addition to SNAP25 (Blackmer, T., et al., 2005; Yoon, E. J., T. Gerachshenko, B.D. Spiegelberg, S. Alford, and H.E. Hamm, 2007), with a 2-fold loss in binding affinity for a SNAP25 9 mutant that phenocopies the effect of BoNT/A (Yoon, E. J., T. Gerachshenko, B.D. Spiegelberg, S. Alford, and H.E. Hamm, 2007). The SNARE-

forming (H3) motif of syntaxin1A was highlighted as a critical area of interaction in these studies (Yoon, E. J., T. Gerachshenko, B.D. Spiegelberg, S. Alford, and H.E. Hamm, 2007), with minimal interaction found in Habc in the absence of H3. Efforts at elucidating the individual Gβγ-binding residues upon SNAP25 utilized a peptide array in which individual peptides corresponding to the human SNAP25 primary sequence were screened for their ability to bind Gβγ (Wells, Christopher A., et al., 2012). This approach highlighted eight residues spread throughout seven exons of SNAP25: subsequent Ala mutagenesis of these residues produced a mutant SNAP25 termed SNAP258A that was almost completely unable to support modulation of exocytosis by the lamprey serotonin receptor or bind Gβγ in solution-based binding studies (Wells, Christopher A., et al., 2012). These eight residues were found in two clusters: a cluster of three residues (R135, R136, and R142 on the second helix) adjacent to the N-terminal Habc domain of syntaxin, and a C-terminal second cluster consisting of five residues (E62 on the first helix, D99 and K102 in the linker region, and R198 and K201 on the second helix) (Wells, Christopher A., et al., 2012). The distance between these clusters appears to be greater than the diameter of a single Gβγ subunit, potentially implying that multiple Gβγs may bind to a single SNARE complex. This observation is supported by Hill coefficients >1 for the saturation binding isotherms produced from the interaction of Gβγ with t-SNAREs or WT SNAP25 (Wells, Christopher A., et al., 2012; Zurawski, Z., et al., 2017; Zurawski, Z., et al., 2016), indicating potential cooperativity in Gβγ binding. Selective mutation of the two residues R198 and K201 within the C-terminus to Ala was insufficient to abolish the inhibitory effect of the lamprey serotonin receptor (Zurawski, Z., et al., 2016), indicating that other residues within the C-terminus of SNAP25 were implicated in Gβγ binding. The extreme C-terminus of SNAP25, containing the residues G204, S205, and G206, emerged as an area of focus for the interaction of Gβγ with SNARE. A truncation mutant, SNAP25^{Δ3}, in which the three C-terminal residues were deleted, provided an intermediate state between SNAP25^{Δ9} and wild-type SNAP25 in which G_{i/o}-coupled GPCRs were partially inhibited and Gβγ binding was reduced twofold (Zurawski, Z., et al., 2016), but vesicle release and synaptotagmin I binding were not different from wild-type. This mutant is compelling for further investigation, since the introduction of it into transgenic organisms with CRISPR/Cas9 is simple, and the resulting organism is viable, unlike SNAP25^{Δ9}. A transgenic mouse has been prepared in this manner, and characterization of it is ongoing (Zurawski, Z., et al., 2018).

Currently, little is known about the *in vivo* physiological importance and regulation of Gβγ-SNARE interaction. Only few G_{i/o} coupled GPCRs have been examined and found to modulate synaptic transmission using the Gβγ-SNARE interaction (Table. 1). 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, A. J., et al., 2007) and vasopressin-expressing magnocellular neurosecretory cells (Iremonger, K. J. & Bains, J. S., 2009). Recently, 5HT_{1B} and GABA_B receptors localized to the same presynaptic terminal of CA1 pyramidal neurons in the hippocampus inhibit the evoked release through Gβγ, but via two different Gβγ-mediated pathways. GABA_B receptors use VDCC while 5-HT_{1B} receptors use SNARE, implying a selectivity of Gβγ to its effectors (Hamid, E., 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 $G\alpha_i$, $G\beta\gamma$ 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, X.-l., et al., 2011). The C-terminus of SNAP25 is critical for mGluR mediated presynaptic depression by the $G\beta\gamma$ -SNARE interaction and stimulus-evoked LTD, but not LTP, and cleavage of residues 198–206 of SNAP25 completely prevented the induction of stimulus-evoked LTD (Zhang, X.-l., et al., 2011). In a preliminary study, we have also found a subset of $G\beta$ and $G\gamma$ subunits specifically interacting with auto- and hetero- α_{2A} adrenergic receptors upon the epinephrine treatment. Interestingly, a subset of activated $G\beta$ and $G\gamma$ subunits from receptor activation was identified to directly interact with SNARE. Taken together, $G\beta\gamma$ -SNARE interaction mediated modulation of synaptic transmission may affect not only normal neural processes but also various neurological and neuropsychiatric diseases.

6. 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, Katherine M., et al., 2012; Bruns, D. & Jahn, R., 2002; Fasshauer, D., 2003; Mahmoud, S., et al., 2012; Stephens, G. J., 2009; Sudhof, T. C., 2004; Tjolsen, A., et al., 1992; Ungar, D. & Hughson, F. M., 2003; Verhage, M. & Toonen, R. F., 2007). However, gaps in our understanding of the physiological implications of $G\beta\gamma$ mediated modulation of synaptic transmission in various neurological and neuropsychiatric diseases remain.

Various mutations and dysregulation of $G_{i/o}$ coupled GPCRs with $G\beta\gamma$ mediated synaptic transmission mechanisms are identified and used as drugs targets in several 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, C., et al., 1999). Similar to this, D_2 autoreceptor mediated inhibition of dopamine release by dopamine agonists is suggested to contribute to the deterioration of cognitive function in Parkinson's diseases (Arnsten, A., et al., 1994; Breitenstein, C., et al., 2006). Moreover, risperidone targeting the D_2 dopamine, $5HT_{1A}$ serotonin, and α_{2A} adrenergic receptors is often used as a treatment of schizophrenia, anxiety disorders, and attention deficit hyperactivity disorder, respectively (Hunter, T., 1995; Janssen, P. A., et al., 1988; Taylor, D. P., et al., 1985). $G_{i/o}$ coupled GPCRs, such as M_4 muscarinic and mGluR2, 3, and 4 metabotropic glutamate receptors, are being 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 $G\beta\gamma$ specificity to each receptor and effector. A better understanding of the $G\beta\gamma$ specificity and the $G\beta\gamma$ -SNARE interaction may provide insight into disease progression and reveal more disease selective targets for therapeutic intervention (Fig.8).

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Figure 1. Calcium dependent exocytosis at the presynaptic terminal.

In neurons, synaptic vesicles are loaded with neurotransmitters and docked to the plasma membrane via the interaction of VAMP2 and t-SNARE (SNAP25 and syntaxin 1). Further zippering of SNARE by synaptotagmin (often Syt1, although other isoforms are present in certain neurons), Munc-13 and Munc-18 (not shown) 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 the release of neurotransmitter into the synaptic cleft to activate a specific receptor or population of receptors on postsynaptic terminals.

Figure 2. The ternary SNARE.

Domain of synaptobrevin (VAMP), syntaxin, and SNAP25. Cartoon showing the crystal structure of the SNARE complex (PBD1SFC) with the NMR structure of syntaxin Habc domain (PBD1BR0). Gray represents undetermined regions.

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 conformation via the N-peptide of syntaxin1. Once the full SNARE is assembled, the fusion pore opens and neurotransmitters are released.

Figure 4.
Domain diagrams of accessory proteins required for the fusion.

Figure 5. The effectors of G prote ins.

Upon the activation of GPCRs, G α and G $\beta\gamma$ subunits are released and interact with numerous effectors to mediate downstream signaling cascades.

Figure 6. The structure of G $\beta\gamma$.

The structure of the G $\beta\gamma$ (PDB ID: 1TBG) (Sondek, J., et al., 1996). The structure of the G β subunit (green) - is an N-terminus α -helix followed by a 7 bladed propeller β sheets. A single blade is a WD-40 repeat. The structure of the G γ subunit (red) is two tandem α -helices that form interactions with the N-terminus α helix of G β and a surface of the G β propeller, opposite of the G α interaction site.

Figure 7. Gβγ regulation of the presynaptic vesicle release.

Synaptic vesicles are primed by a tethering interaction between VAMP2 on the vesicle and the SNAP25/syntaxin 1 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 c-terminal of SNARE proteins and displace synaptotagmin in a competitive manner. In addition to c-terminal of SNARE, we found residues at N-terminal of SNARE complex which may affect the Gβγ-SNARE interaction (orange circle). At 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 8. Therapeutic implications of Gβγ-SNARE interaction in diseases.

A compound targeting Gβγ-SNARE interaction to perturb this interaction may synergize with the presynaptic GPCR agonists and positive allosteric modulators (PAMs), often used as a treatment of neuropsychiatric disorders such as bipolar, schizophrenia, and depression. The combination of these two drugs may lead to the achievement of same therapeutic effect at lower doses which might reduce off-target effects and adverse consequences.

Table 1.
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 based on localization to the synapse or soma of neurons in the central nervous system. Table adapted from Betke, KM. *et al.* 2012 *Prog. Neurobiol.*96:304–21.

GPCRs	Synapse		Soma
	VDCC	SNARE	VDCC
M2/M4 Ach	Higley <i>et al.</i> , 2009	Bellingham and Berger, 1996, Scanziani <i>et al.</i> , 1995	Allen and Brown, 1993, Delmas <i>et al.</i> , 1998
Group II mGlu		Capogna, 2004, Giltch, 2006, Scanziani <i>et al.</i> , 1995	
Group III mGlu	Glustizieri <i>et al.</i> , 2005	Van Hook <i>et al.</i> 2017	
GABA _B	Dittman and Regehr, 1996, Isaacson, 1998, Wu and Saggau, 1995	Dittman and Regehr, 1996, Harvey and Stephens, 2004, Wu and Saggau, 1995	
μ opioid	Heinke <i>et al.</i> , 2011	Heinke <i>et al.</i> , 2011	Wilding <i>et al.</i> , 1995
CB1 cannabinoid	Brown <i>et al.</i> , 2004		
A1 adenosine	Dittman and Regehr, 1996, Scholz and Miller, 1992, Umemiya and Berger, 1994	Scholz and Miller, 1992	Umemiya and Berger, 1994
D2 dopamine	Hamilton and Smith, 1991, Higley and Sabatini, 2010	Nicola and Malenka, 1997	Cabrera-Vera <i>et al.</i> , 2004
α_2 adrenergic	Boehm, 1999	Delaney <i>et al.</i> , 2007	Boehm, 1999
5HT _{1B}		Hamid <i>et al.</i> , 2014	