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ORIGINAL PAPER

Gβγ SNARE Interactions and Their Behavioral Effects

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Abstract
Presynaptic terminals possess interlocking molecular mechanisms that control exocytosis. An example of such complexity is the modulation of release by presynaptic G Protein Coupled Receptors (GPCRs). GPCR ubiquity at synapses—GPCRs are present at every studied presynaptic terminal—underlies their critical importance in synaptic function. GPCRs mediate presynaptic modulation by mechanisms including via classical Gα effectors, but membrane-delimited actions of Gβγ can also alter probability of release by altering presynaptic ionic conductances. This directly or indirectly modifies action potential-evoked presynaptic Ca^{2+} entry. In addition, Gβγ can interact directly with SNARE complexes responsible for synaptic vesicle fusion to reduce peak cleft neurotransmitter concentrations during evoked release. The interaction of Gβγ with SNARE is displaced via competitive interaction with C2AB-domain containing calcium sensors such as synaptotagmin I in a Ca^{2+}-sensitive manner, restoring exocytosis. Synaptic modulation of this form allows selective inhibition of postsynaptic receptor-mediated responses, and this, in combination with Ca^{2+} sensitivity of Gβγ effects on SNARE complexes allows for specific behavioral outcomes. One such outcome mediated by 5-HT receptors in the spinal cord seen in all vertebrates shows remarkable synergy between presynaptic effects of Gβγ and postsynaptic 5-HT-mediated changes in activation of Ca^{2+}-dependent K^+ channels. While acting through entirely separate cellular compartments and signal transduction pathways, these effects converge on the same effect on locomotion and other critical functions of the central nervous system.

Keywords G proteins · Presynaptic · Serotonin · Locomotion · Presynaptic inhibition · Short term plasticity

Introduction

Synapses are inherently plastic and adaptable, both in the long-term to create memories, but also in the short term as a means for shaping patterns of activity that underlie complex behaviors, and to allow short-term adaptation. While both pre- and postsynaptic terminals may generate this adaptability, much of the focus placed on synaptic plasticity has been on the postsynaptic terminal and modifications to receptor type, numbers and placement. Less emphasis has been placed on the presynaptic terminal and presynaptic mechanisms of plasticity. Nevertheless, one form of presynaptic plasticity is likely ubiquitous at all presynaptic terminals—synaptic modulation mediated by pertussis toxin-sensitive (Gi/o) G proteins [1]. Various neurotransmitters modulate release from presynaptic terminals [2, 3] many mediated by G Protein Coupled Receptors (GPCRs) [4–7] that are activated by many different neurotransmitters. This modulation may occur at axo-axonic synapses [8, 9] or may result from neurotransmitters released at paracrine terminals to diffuse in the tissue [10]. Other GPCRs at presynaptic terminals may be activated by neurotransmitter released in that same terminal, and are referred to as autoreceptors, which may serve as a feedback mechanism at the presynaptic terminal. These have been implicated in both negative [11–13] and positive [6, 14] modulation of neurotransmitter release.

GPCR-mediated short-term synaptic plasticity has been widely considered to result from G protein modulation of the voltage-gated Ca^{2+} channels (VGCCs) responsible for evoking synaptic vesicle fusion [15, 16]. Modulation of VGCCs is now known to be mediated by a direct Gβγ subunit interaction with a number of VGCC subtypes [17]. Although these mechanisms were assumed to take place in synapses, it was not until 1995 that Wu and Saggau directly demonstrated that this occurs in presynaptic terminals [18].

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A number of other researchers had demonstrated that $G_{i/o}$ signaling at presynaptic terminals must involve mechanisms distinct from modulation of VGCCs. The first such indication was that GPCRs inhibit spontaneous release of neurotransmitter independently of presynaptic Ca^{2+} entry was the observation of inhibition of the frequency of spontaneous neurotransmitter release events in the absence of evoked Ca^{2+} entry [19, 20]. It is now clear that membrane-delimited G protein signaling also directly targets the SNARE complex responsible for vesicle fusion. We will discuss this signaling, its complex interaction with GPCR modulation of presynaptic Ca^{2+} channels and consequent effects on presynaptic integration.

Membrane-Delimited Gβγ Targets That Alter Presynaptic Ca^{2+} Entry

GPCRs which inhibit neurotransmitter release have been widely studied. These include both membrane-delimited targets of Gβγ and modulation through activation of downstream second messenger effectors. GPCRs involved in membrane-delimited presynaptic inhibition include $GABA_B$ receptors [21], tachikinin receptors [22], adrenergic receptors [17, 23], metabotropic glutamate receptors (mGluRs) [24], opioid receptors [25], 5-HT receptors [7] and dopamine receptors [26].

Cellular mechanisms involved in neuromodulation at the presynaptic terminal are not particularly well-understood because of the difficulties in recording from vertebrate presynaptic terminals, which tend to be very small. Uncertainty over the mechanisms by which G proteins alter neurotransmitter release also reflects the variety of effector targets of G protein activation by G protein-coupled receptors [27]. Due to a lack of presynaptic access, most molecular studies of the detailed mechanisms come from either transfection of the relevant proteins into cultured cell lines and *Xenopus* oocytes, or from electrophysiological measurements from neuronal cell bodies. However, membrane-delimited inhibition of neurotransmission mediated by G protein βγ subunits at the presynaptic terminal have been hypothesized to be mediated by various mechanisms directed to presynaptic ion channel conductances all of which lead to reduced presynaptic Ca^{2+} entry (Fig. 1A, B).

(A) Gβγ mediated inhibition of synaptic transmission may act by a direct action of G protein βγ subunit on the gating of VGCCs [16, 28–31]. Gβγ-mediated inhibition of VGCCs reduces presynaptic Ca^{2+} entry, causing a reduction in probability of release. This has been shown directly using Ca^{2+} imaging in hippocampal pyramidal neuron terminals [18, 32], and electrophysiologically at the calyx synapse of Held [33].

(B) Membrane-delimited Gβγ might also inhibit neurotransmitter release by an indirect action. For example, GIRKs located at the presynaptic terminal, and activated by Gβγ, could serve to reduce the amplitude of the action potential and repolarize the presynaptic terminal more quickly, allowing fewer Ca^{2+} channels to open. Gβγ activates GIRKs in neuronal cell bodies and in transfected cell lines and *Xenopus* oocytes [34–36], and some studies indicate that this can occur presynaptically [37] although direct evidence of a presynaptic role has not been presented. Gβγ can also directly modulate voltage-gated Na^+ channels [38] to alter persistent

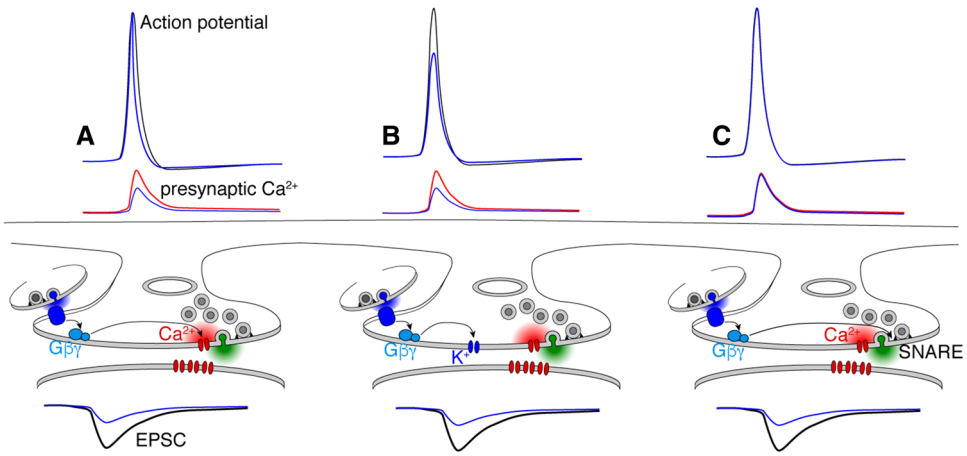

Fig. 1 Schematic showing three possible mechanisms by which G protein $G\alpha_{i/o}$-dependent presynaptic inhibition is mediated by Gβγ. **A** Gβγ (blue) inhibits presynaptic Ca^{2+} entry, shortening the action potential, reducing the Ca^{2+} transient and inhibiting the EPSC. **B** Gβγ activates a presynaptic GIRK channels, reducing action potential amplitude and subsequently presynaptic Ca^{2+} entry and the resultant EPSC. **C** Gβγ directly targets the SNARE complex in the presynaptic terminal, giving no effect on the presynaptic action potential, or Ca^{2+} entry, but still inhibiting the EPSC. (Color figure online)

Na^+ currents, however, no direct evidence for a presynaptic location of this effect has yet been presented.

Membrane-Delimited Targets Downstream of Presynaptic Ca^{2+} Entry

Many G protein effectors have been implicated in modulation of synaptic vesicle fusion. It is possible to postulate multiple sites within the presynaptic terminal at which G proteins may interfere with neurotransmitter release both through membrane-delimited actions of Gβγ, and through second messenger effectors. G proteins may inhibit exocytotic processes in different ways including modulation upstream, distal to, or at the point of Ca^{2+} entry. Exocytotic processes in pancreatic β cells, peritoneal mast cells, chromaffin cells, PC12 cells and secretory granules are directly regulated by G proteins [39–41]. The ability of G proteins to mediate inhibition of neurotransmitter release by directly targeting the release apparatus following evoked Ca^{2+} entry was first demonstrated by Silinsky and Solsona [42] in the neuromuscular junction. Spontaneous exocytotic events, where exocytosis occurs independently of Ca^{2+} entry, can be detected by recording miniature excitatory/inhibitory postsynaptic currents (mE/IPSCs). Measurements of mE/IPSCs allow the exocytotic modulatory processes that occur independently of Ca^{2+} entry to be isolated and studied, unlike evoked EPSCs which require Ca^{2+} entry. These mE/IPSCs have been shown to be regulated by many GPCRs [42–45], including serotonin receptors [1, 46], muscarinic receptors, mGluRs and $GABA_B$ receptors. Evidence that these effects are mediated downstream of Ca^{2+} entry to the presynaptic terminal are supported by experiments in which transmitter release evoked following membrane permeabilization with ionomycin or α-latrotoxin is inhibited by GPCRs [47] as well as by experiments in permeabilized cells in which Gβγ inhibits exocytosis [48]. Indeed, that latter experiment also pointed strongly to a membrane-delimited effect, because the experiments were performed on cell membranes that lacked a cytosolic compartment [48]. These results pointed to a more direct mechanism by which GPCRs might modulate neurotransmitter release (Fig. 1C).

The SNARE Complex as a Target for Signaling

Presynaptic GPCRs, SNARE complexes, and fusogenic Ca^{2+}-sensor proteins, along with other SNARE complex-associated proteins, form molecular machines that evoke exocytosis and shape complex synaptic patterns. Presynaptic modulation requires precise interactions of receptors and effectors with the exocytotic machinery. The resultant short-term plasticity is critical to neural function; it shapes responses governing sensation [49, 50], motor output [9, 51] and oscillations in cognition and neuropsychiatric disorders [52, 53].

There are numerous presynaptic molecular targets that evoke short-term plasticity. Plasticity may be caused by changes in probability [54], vesicle priming [55], the subsynaptic locations of release [56], or the fusion process itself [57, 58]. However, while all of these properties are modified by GPCRs [59, 60] activity leading to synaptic transmission may also modify the properties of GPCR signaling. For example, high frequency stimulation of a presynaptic terminal may alter its cytosolic Ca^{2+} concentration which may modify GPCR targets downstream of Ca^{2+} entry. Multiple GPCRs are present at all synapses, as auto-[61], and heteroreceptors [62], but ultimately their targets are the processes of synaptic vesicle fusion.

Synaptic vesicle fusion is a form of exocytosis and requires protein–protein, protein–lipid and lipid–lipid interactions, each of which provides a possible target for presynaptic modulation. Proteins include the SNARE proteins (Fig. 2A) [63, 64] comprising the *v*esicular—*v*-SNARE, synaptobrevin that contains a SNARE motif that binds to coiled-coil SNARE motifs in the dimer of two *t*arget plasmalemma *t*-SNAREs, syntaxin1A and SNAP25 [63, 64]. Other fusion machinery proteins are VGCCs [65], fusogenic Ca^{2+} sensors [66, 67], and proteins that modify vesicle docking, priming, and fusion (e.g. munc18 [68, 69], munc13 [70, 71], complexin [72] and homomeric [73, 74] and heteromeric G proteins [75–77]). Fusion is triggered by Ca^{2+} sensors—particularly synaptotagmins comprising N-terminal transmembrane domains and tandem Ca^{2+}-binding domains [78, 79]—which cause synchronous [66], and asynchronous synaptic vesicle fusion [80, 81], and frequency-dependent-modulation of synaptic output [82]. In CA1 pyramidal cells, synaptotagmin1 triggers synchronous fusion. It binds SNARE complexes at low affinity in zero or resting Ca^{2+} concentrations [78, 79, 83–85], but binds at higher affinity in elevated Ca^{2+} [86–88] as it simultaneously interacts with lipids [84, 89, 90]. The resultant exocytosis is modulated by $G_{i/o}$-coupled GPCRs, with both the Gα [91–93] and Gβγ G protein subunits activating discrete pathways [59]. In particular, Gβγ injected into presynaptic terminals or released by presynaptic GPCRs can rapidly and reversibly inhibit exocytosis [7, 60] or cause long term plasticity [60, 94, 95]. It is this reversible membrane-delimited Gβγ-mediated inhibition (Fig. 2A) that is discussed in this review and which may act throughout the sequence of events leading to evoked release, from initial Ca^{2+} entry to the SNARE complex-mediated lipid fusion event.

The SNARE Complex as a Gβγ Target

Gβγ interacts directly with the presynaptic SNARE complex to interfere with synaptotagmin1 binding and modify

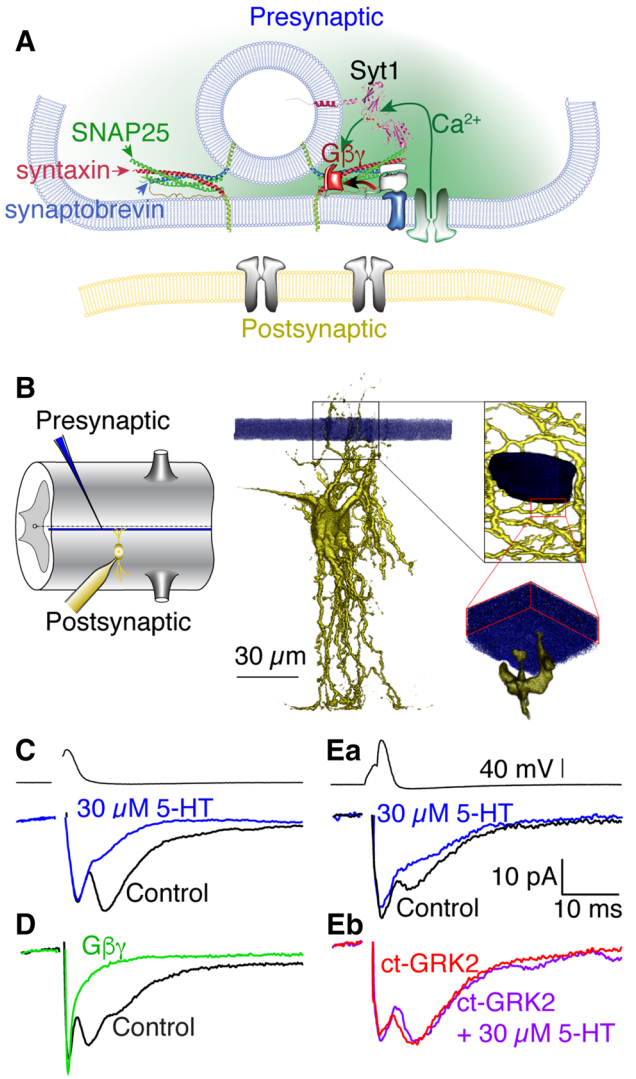

Fig. 2 Paired cell recordings between lamprey giant axons and their post synaptic targets reveal a Gβγ-dependent mechanism of presynaptic inhibition. **A** Schematic showing the competitive interaction between Gβγ and synaptotagmin at the SNARE complex in presynaptic terminals. Gβγ interferes with synaptotagmin binding at the C-terminal region of SNAP25. **B** Arrangement for paired recording between a presynaptic giant axon and its whole cell patch clamped postsynaptic target. Shown also is a confocal reconstruction of a presynaptic axon (blue) making synapses onto its postsynaptic target (yellow). Image shown at 3 levels of magnification. **C** Paired recording—top—presynaptic action potential and bottom postsynaptic EPSC in control (black) and 5-HT (blue; note the chemical component, but not the early electrical component, is inhibited by 5-HT). **D** Similar recording to (**C**) in which Gβγ is injected into the presynaptic terminal (green) to inhibit synaptic responses. **E** The effect of 5-HT is blocked by presynaptic injection of the ct-GRK2 to buffer Gβγ. **Ea** control effect of 5-HT prior to ct-GRK2 injection—5-HT inhibits the chemical component of the EPSC. **Eb** After ct-GRK2 injection, the effect of 5-HT is blocked. (Color figure online)

vesicle fusion. In the experimentally accessible lamprey giant reticulospinal synapse (Fig. 2B), a number of agonists inhibit synaptic release of glutamate, including dopamine receptors, mGluRs and 5-HT receptors. However, agonists to most of these receptors act without altering presynaptic Ca^{2+} entry [6, 96] and neurotransmitter release evoked by presynaptic flash photolysis of Ca^{2+} is inhibited by 5-HT and Gβγ at these synapses to the same extent as for responses elicited by action potential [97] indicating that release is inhibited "downstream" of an effect on VGCCs. Nevertheless, Gβγ released by activation of $5\text{-HT}_{1B/1D}$ like receptors [98] inhibits neurotransmission by a membrane-delimited action [7, 96]. Indeed, direct injection of Gβγ buffering peptide, phosducin, or C-terminal fragments of G protein receptor kinase 2 (ct-GRK2) [7] prevent GPCR-mediated presynaptic inhibition at lamprey giant synapses (Fig. 2E), and a myristoylated G-protein βγ selective binding peptide (mSIRK) [94] prevents GPCR-mediated synaptic modulation in mammalian hippocampal synapses.

This Gβγ-mediated inhibition proved to be mediated by a direct action at the C-terminal region of SNAP-25 and acts late in the synaptic vesicle fusion cycle at primed vesicles after SNAP-25 is incorporated into ternary SNARE complexes. Botulinum toxins were utilized to identify the specific step in vesicle fusion inhibited by Gβγ. These toxins cannot access the ternary SNARE formed as vesicles are primed prior to evoked synaptic transmission [99]. Consequently, Botulinum B toxin (BoNT/B), which cleaves synaptobrevin (Fig. 3A–D), has little immediate effect on neurotransmission immediately after its injection into an axon. However, if after toxin injection into the axons, a period of 1 Hz stimulation is used to deplete primed vesicles, BoNT/B causes a complete loss of synaptic transmission (Fig. 3B). Yet during this period, during which BoNT/B has cleaved unprimed SNAP-25, GPCR activation (using 5-HT as an agonist) still inhibits vesicle fusion and synaptic transmission to the same extent as in controls (Fig. 3C, D). The speed of action of Gβγ-mediated inhibition (< 20 ms after receptor activation [97]) also points to an effect late in the vesicle cycle. Thus, GPCRs can inhibit release late in the exocytotic/endocytotic cycle after vesicle priming.

The target for Gβγ was also identified using botulinum toxin injected into the giant axon. Botulinum A toxin (BoNT/A) cleaves a C-terminal 9 amino acid fragment from SNAP-25. This leaves a truncated SNAP-25 that can still support synaptic transmission [76, 88], albeit at a reduced amplitude. Thus, in a similar experiment to that using BoNT/B, BoNT/A was injected into lamprey reticulospinal synaptic terminals. After injection and subsequent elimination of primed vesicles with 300 stimuli, synaptic responses were reduced in amplitude. However, this application prevented GPCR mediated presynaptic inhibition (Fig. 3E, F) [97]. The BoNT/A sensitivity of this effect of Gβγ provided

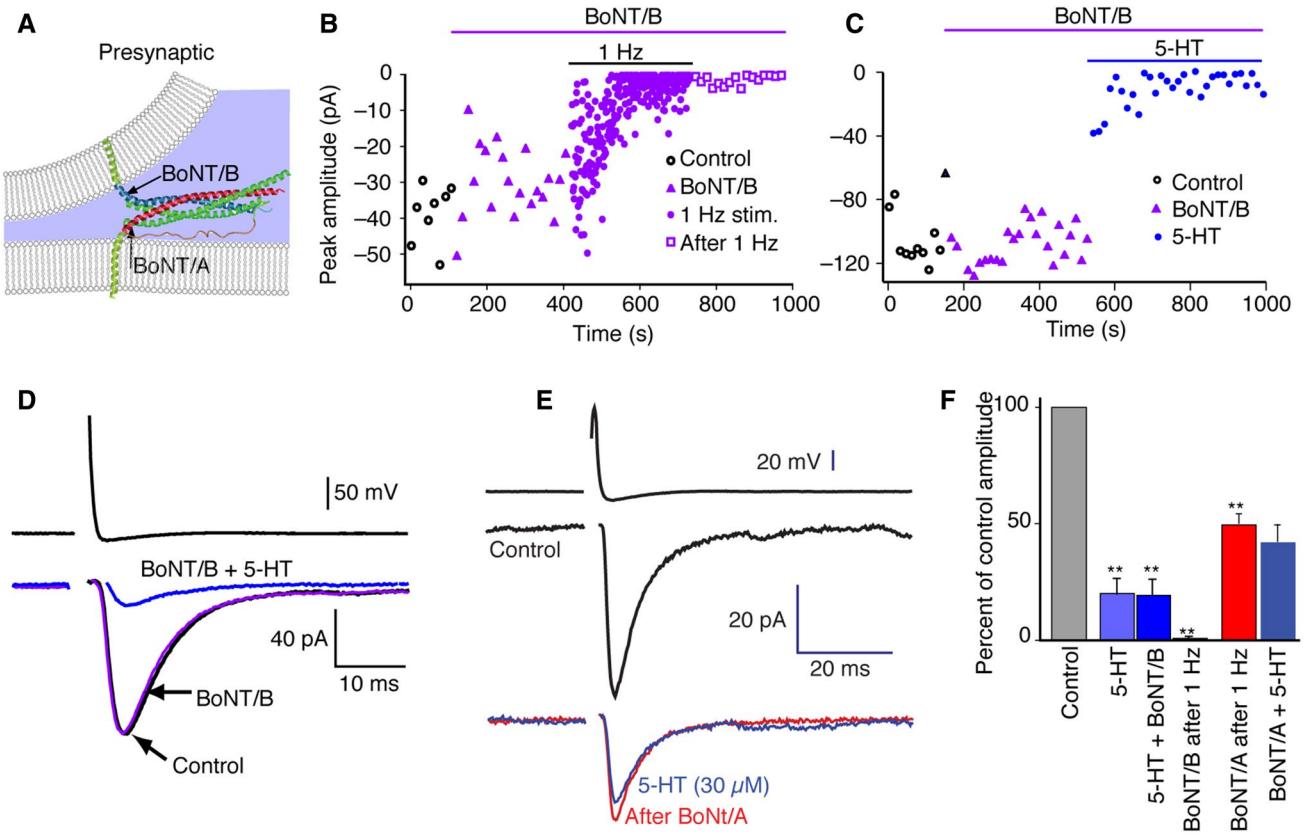

Fig. 3 GPCRs target primed vesicle SNAREs late in the exocytotic/endocytotic cycle. **A** Schematic showing the targets for BoNT/A and B on the SNARE complex adapted from [64]. **B** Graph of the amplitude of synaptic responses before and after presynaptic BoNT/B injection and after depletion of primed vesicles by a period of 1 Hz stimulation. BoNT/B has no immediate effect on EPSCs (purple triangles) after injection because primed vesicle SNARE complexes are inaccessible to the toxin. After loss of these primed vesicles by 1 Hz stimulation, EPSCs are abolished (purple squares). **C** After presynaptic injection of BoNT/B, after a period during which BoNT/B has cleaved unprimed synaptobrevin, 5-HT can still inhibit EPSCs (blue circles), indicating 5-HT acts on primed vesicles. **D** example traces from experiments shown in (C) control, after BoNT/B injection (purple) and after 5-HT (blue). **E** Experiment using BoNT/A as in (**C**) above. BoNT/A injection and subsequent depletion of primed vesicles with 1 Hz stimulation leaves approximately 50% of the EPSC intact. This remaining EPSC can no longer be inhibited by 5-HT indicating its target on the SNAP-25 C-terminus. **F** Histogram summarizing the effects of BoNTs on synaptic transmission and its modulation by 5-HT. (Color figure online)

a starting point to determine how Gβγ mediates presynaptic inhibition of synaptic transmission. It has also enabled this mechanism of presynaptic modulation to be identified in a number of other synapses, and utilizing other GPCRs and agonists [23, 94, 100].

Gβγ Competition with Synaptotagmin1 During Fusion

To determine functional sites of interaction between Gβγ and SNARE complexes, alanine screening mutagenesis was used to identify critical residues. Residues on the C and N-terminal regions of SNAP-25 proved necessary for its interaction with Gβγ, but which after modification, do not interfere with Ca^{2+}-synaptotagmin SNARE interactions [101]. To study the effects of these mutant forms of SNAP-25, the mutations were introduced on a Botulinum E toxin (BoNT/E)-resistant form of SNAP-25 (D179K) and injected into giant axons along with BoNT/E light chain to remove endogenous protein. After 300 stimuli to clear primed vesicles, and subsequent cleavage, SNAP-25 D179K substituted successfully for the endogenous SNAP-25 supporting synaptic transmission that would otherwise have been lost. After this substitution, 5-HT mediated GPCR activation failed to inhibit neurotransmission [101].

The C-terminal region of SNAP-25 cleaved by BoNT/A represents a site for interaction between synaptotagmin1 and the ternary SNARE complex to evoke synaptic transmission [88]. Therefore, it was possible that Gβγ and synaptotagmin compete to interact with SNARE complexes at the C-terminal region of the complex. Indeed, Ca^{2+}-synaptotagmin1 binding to the SNARE complex is reduced by Gβγ, consistent with competition between Ca^{2+}-synaptotagmin1 and Gβγ. Furthermore, removal of the C-terminal 9 amino acids

from SNAP-25 favors synaptotagmin over Gβγ binding to the t-SNARE complex as well as the ternary SNARE complex [76]. This competition can be recorded in a fluorescence assay with the proteins dissolved in aqueous solution and detergent, but also when SNARE complexes are more physiologically localized into lipid membranes. Anisotropy of fluorescence produced via the binding of fluorescent synaptotagmin1 to t-SNAREs was detected in lipid bilayers using TIRF imaging of a glass cover slip *supported lipid bilayer*. Addition of Ca^{2+} increased the anisotropy of the synaptotagmin1 fluorescence. This rise in anisotropy produced via the binding of synaptotagmin1 to t-SNARE complexes could be reversed in a concentration-dependent manner by purified Gβγ applied in the solution (Fig. 4A, B). While the C-terminal 9 residues of SNAP-25 are not highlighted as a critical binding site for synaptotagmin I-SNAP-25 interactions in X-ray crystallographic studies [84, 87], the synaptotagmin molecule's binding site, which is lost after cleavage of the C-terminal 26 residues of SNAP-25 with BoNT/E [88], may overlap with that of Gβγ through a direct steric effect, resulting in competition [101].

If Gβγ competes with Ca^{2+}-synaptotagmin binding at SNARE complexes, then it should be possible to reproduce this mechanism in an artificial fusion assay with only lipid, SNARE complexes, synaptotagmin and Gβγ present. This indeed proved possible (Fig. 4C, D), and under these circumstances Gβγ inhibits Ca^{2+}-synaptotagmin-mediated fusion of t-SNARE and v-SNARE containing vesicles. A reconstituted fusion assay [102–104] was used. Liposomes containing v-SNARE were prepared using recombinant

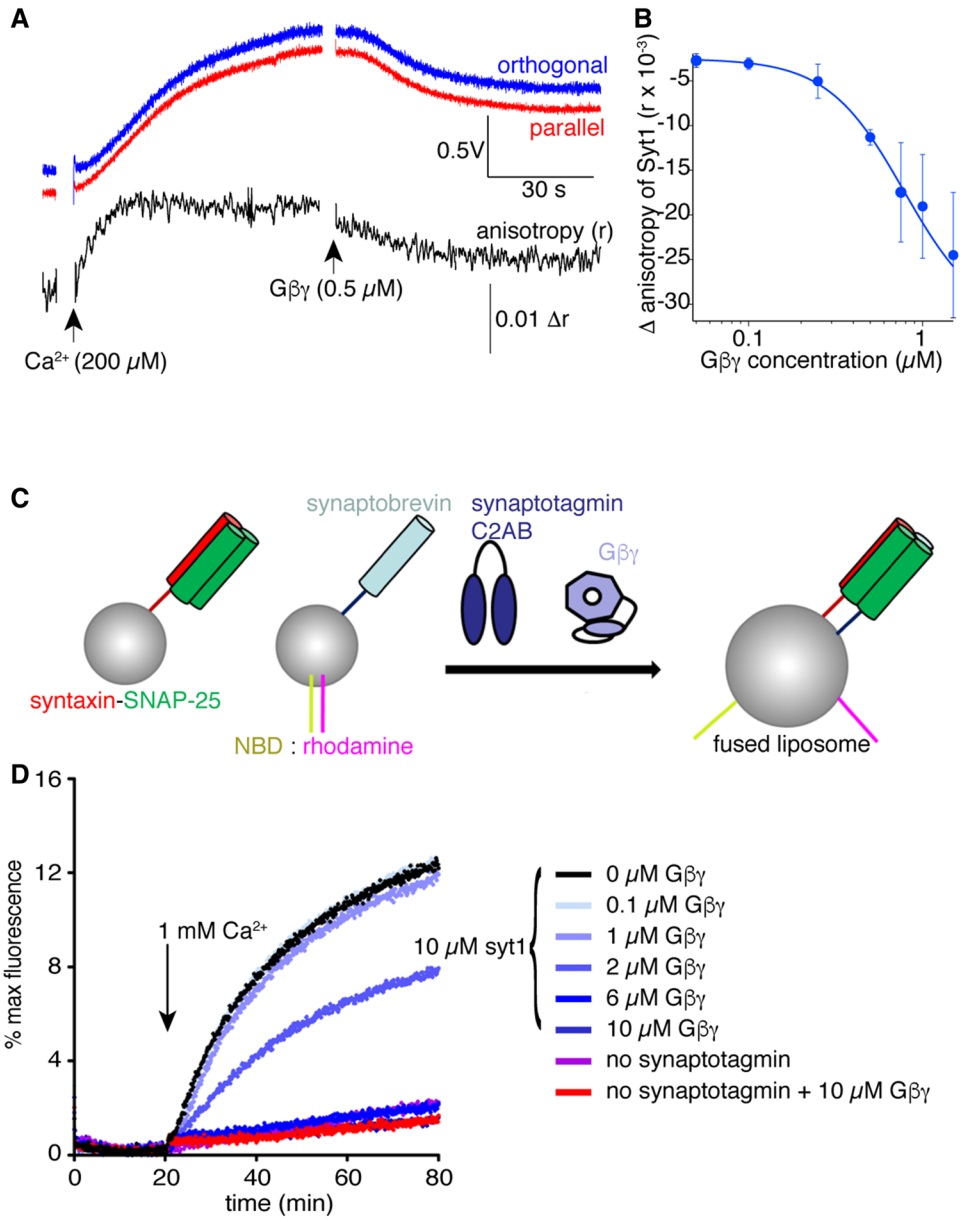

Fig. 4 Gβγ inhibits synaptotagmin–SNARE interactions and SNARE-dependent lipid vesicle fusion. **A** Fluorescently labeled synaptotagmin1 (C2AB) was applied above a t-SNARE containing lipid bilayer and fluorescence emission measured orthogonally and parallel to the excitation polarization angle. Addition of Ca^{2+} (200 μM) caused an increase in fluorescence and anisotropy as the synaptotagmin interacted with the lipid membrane and t-SNAREs. Addition of Gβγ caused a reduction in both fluorescence and anisotropy. **B** Dose response of purified $G\beta_1\gamma_1$ vs synaptotagmin1 anisotropy. **C** Diagram showing assay principle. Synaptobrevin-bearing liposomes containing the FRET pair NBD and rhodamine covalently attached to rhodamine react with unlabeled liposomes containing t-SNARE complexes consisting of syntaxin1A and SNAP25. The increased surface area of the fused liposome reduces the quenching of NBD fluorescence by rhodamine and NBD fluorescence increases as a result. **D** NBD fluorescence traces over time for synaptotagmin1 (10 μM) and Ca^{2+} (1 mM)-dependent liposome fusion in the presence of a series of concentrations of purified bovine $G\beta_1\gamma_1$ (concentrations from 100 nM to 10 μM were tested)

full-length synaptobrevin and a fluorescence resonance energy transfer (FRET) donor–acceptor pair. t-SNARE liposomes contained full-length recombinant rat syntaxin1A and SNAP25B heterodimers. As the quenched FRET-pair containing liposomes fuse with the unlabeled liposomes, the concentration of the FRET acceptor is lowered, to increase the donor fluorescence. Addition of synaptotagmin1 (10 μM) in Ca^{2+} (1 mM) [105] stimulated fusion. Gβγ, inhibited this Ca^{2+}-synaptotagmin-1 triggered fusion in a concentration-dependent manner (Fig. 4D). This Gβγ-mediated inhibition is also Ca^{2+} sensitive. At higher Ca^{2+} concentrations fusion is more rapid, but Gβγ is less effective in preventing this fusion [111].

This same Ca^{2+} sensitivity of inhibition is seen during synaptic transmission. If Ca^{2+}-synaptotagmin interactions with SNARE complexes provide a competitive site for Gβγ to modify synaptic transmission, then it may be hypothesized that GPCR-mediated inhibition is Ca^{2+} sensitive. Indeed, raising the extracellular Ca^{2+} concentration surrounding synapses during evoked synaptic transmission causes a substantially enhanced presynaptic Ca^{2+} transient. At the same time, GPCR mediated presynaptic inhibition is lost [76, 106].

Implications of Presynaptic Targets of Gβγ in Modulating Neurotransmitter Release

GPCRs can liberate Gβγ into presynaptic terminals and this inhibits neurotransmission, either by inhibiting Ca^{2+} entry to the presynaptic terminal, or by competing with Ca^{2+}-synaptotagmin interaction with the t-SNARE. While the effect of inhibiting Ca^{2+} entry on synaptic transmission is predictable and established—a reduction in neurotransmitter release probability [18], the outcome of Gβγ effects on SNARE complexes is hard to predict. Presynaptic effects on release probability can be demonstrated by examining effects of paired-pulses on synaptic transmission. In a pair of pulses if vesicle fusion occurs on the first pulse, this lowers available primed vesicles for the second pulse and thus release probability. Thus, any synaptic manipulation that reduces release probability allows a relative enhancement of the probability of release on the second pulse. This is demonstrated by reducing Ca^{2+} entry to lamprey giant axons by raising the extracellular Mg^{2+} concentration which enhances paired pulse ratios. In contrast, presynaptic GPCR activation by activating 5-HT receptors has no such effect. Thus, 5-HT receptors on lamprey giant axons reduce neurotransmitter release, but not release probability [107].

Analysis of the quantal size of unitary synaptic events between reticulospinal axons and their postsynaptic targets also demonstrated that 5-HT-mediated inhibition is caused by a reduction in size of unitary EPSCs [58]. Furthermore, FM dye destaining experiments indicate that 5-HT causes vesicles to trap FM1-43 within vesicles that still transiently fuse and release neurotransmitter. In intact lamprey spinal cord, destaining of FM1-43 loaded into giant axon synaptic vesicles could be visualized in the intact spinal cord [108]. Synaptic vesicle clusters were labeled with FM1-43. In control conditions stimulation of these axons caused almost complete destaining. However, this destaining was nearly abolished using the same protocol in 600 nM 5-HT, a dose that only halved the amplitude of EPSCs recorded from these axons. These results suggested that after treatment with 5-HT, membrane-bound FM1-43 could not escape vesicles from which, nevertheless, glutamate was released [58].

If, during this incomplete fusion, glutamate can escape from vesicles, because glutamate dissolved in the vesicle interior can escape a transiently formed vesicle pore, but the lipophilic FM1-43 cannot [109], then the vesicle interior must contact the extracellular space to allow glutamate release. Indeed water-soluble molecules in the extracellular space (e.g. sulforhodamine) proved able to permeate these vesicle fusion pores and quench FM1-43 by FRET. Thus, introducing sulforhodamine into the extracellular space during application of 5-HT allowed destaining to recover [58]. This strongly implies that vesicles transiently fuse with a kiss-and-run mechanism in the presence of 5-HT, to allow some release of glutamate but trapping FM1-43 in their interior.

Thus, GPCRs that release Gβγ to directly target the SNARE complex do not modify probability of release. An alternative mechanism might be that these receptors modify the concentration of glutamate in the synaptic cleft. Competitive low affinity AMPA receptor antagonists such as kynurenate can be used to test this [110, 111]. A competitive low affinity antagonist rapidly unbinds from the receptor after binding. If the antagonist affinity is sufficiently low, a portion of an EPSC only partially blocked by such an antagonist will be due to non-equilibrium unbinding of the antagonist from the receptor and its subsequent competitive replacement by synaptically released glutamate [110]. The degree to which antagonist, unbinding from the AMPA receptor, is replaced by glutamate is dependent on the cleft glutamate concentration. It is therefore possible to monitor changes in cleft glutamate concentration by measuring the degree of low affinity antagonist replacement by glutamate. For example, in kynurenate, if the synaptic cleft glutamate concentration is lowered by 5-HT, there will be less replacement of the antagonist by glutamate and the magnitude of inhibition mediated by the same dose of 5-HT on the EPSC will be greater than under control conditions. Likewise, the extent of inhibition mediated by application of kynurenate will be greater in the presence of 5-HT than during application of kynurenate alone.

This is precisely the outcome obtained from sequential application of kynurenate and 5-HT on paired synaptic

responses recorded from stimulation of lamprey giant axons. After recording control responses, an approximately half maximal dose of kynurenate effectively doubled the efficacy of 5-HT to inhibit synaptic transmission [107].

Thus, GPCRs at presynaptic terminals can cause a reduction of the peak concentration of glutamate released into the synaptic cleft during evoked synaptic transmission. This form of synaptic plasticity may differentially modify the activation of different postsynaptic receptors [112, 113]. NMDA receptors with relatively high affinity glutamate interactions and slow unbinding rates at 2 glutamate binding sites are more sensitive to low cleft glutamate concentrations than are AMPA receptors with up to 4 higher affinity glutamate binding sites. Indeed, comparing postsynaptic effects of 5-HT at the lamprey giant synapse demonstrates that 5-HT has both a lower half maximal efficacy and a greater maximal inhibitory effect in inhibiting AMPA receptor-mediated synaptic responses than against NMDA receptor-mediated effects [106, 107] (Fig. 5Ab).

On lamprey reticulospinal axons, 5-HT receptor activation causes Gβγ to compete with Ca^{2+}-synaptotagmin for binding sites upon docked and primed ternary SNARE complexes. While the outcome of this is to reduce the evoked peak synaptic cleft concentration of glutamate during synaptic transmission [107] the efficacy of Gβγ in inhibiting synaptic transmission is reduced by high evoked Ca^{2+} concentrations that can be evoked by trains of stimuli. Physiologically trains of stimuli cause summating Ca^{2+} concentrations in presynaptic terminals which reduces the efficacy 5-HT's inhibitory action at reticulospinal axon terminals [106, 107]. Thus, if short trains of action potentials (eg 10 action potentials at 20 Hz) are applied to reticulospinal giant axons, paired EPSCs recorded in their target neurons show a series of EPSCs. In control conditions, the EPSCs at the start of the train are inhibited, however this inhibition is lost towards the end of the train (Fig. 5Ac).

The Implications of Presynaptic Targets of GPCRs on Behavior

The target for GPCRs and Gβγ on the SNARE complex, thus, opens up a number of complex non-linear effects on synaptic transmission following the activation of presynaptic receptors. The effects of presynaptic inhibition on behavior has not been widely investigated. In the case of 5-HT receptors on reticulospinal and intraspinal glutamatergic terminals in the lamprey spinal cord, this is testable because these terminals form part of an extraordinarily well-understood spinal motor system [114]. Furthermore, the effects of presynaptic $5\text{-}HT_{1B/1D}$ receptors are selective to glutamatergic synapses in the spinal cord, both at reticulospinal synapses and intraspinal excitatory synapse (Fig. 5A) and have definable effects on synaptic transmission. $5\text{-}HT_{1B/1D}$ receptor activation favors NMDA receptor-mediated synaptic transmission which evokes slower locomotion than when AMPA receptors are selectively activated [115–117] and stimulus trains evoke responses that are inhibited at the start but not the end of the train. Computer simulations of the effect of this on locomotion demonstrate a slowing of the locomotor rhythm [118, 119].

To test the effects of presynaptic 5-HT receptors on locomotor behavior, fictive locomotion was evoked by brainstem stimulation of the descending motor command system [120]. This neural correlate of the activity that generates a traveling wave of activity in the lamprey spinal cord and consequently the trunk musculature (Fig. 5B) can be evoked in an isolated brainstem/spinal cord preparation by stimulation of the Mesencephalic Locomotor Region with a pulse of pressure-applied glutamate. This caused short episodes of fictive locomotion (Fig. 5C), representing the neural output from the spinal locomotor central pattern generators responsible for locomotion. Application of a selective agonist of presynaptic $5\text{-}HT_{1B}$ receptors to the spinal cord modified locomotion, to reduce the frequency of left right alternation (Fig. 5Cb, Cc), but without modifying the total output of the spinal cord [106]. This outcome clearly results directly from the cellular effects of 5-HT receptors in the presynaptic terminal, both that the receptor activation favors synaptic activation of NMDA receptors over AMPA which will cause slower fictive locomotion [115, 121], but also because the response to stimulus trains allows recovery from 5-HT-mediated inhibition during the stimulus train. This augmenting synaptic output favors slower locomotor activity in mathematical models of the locomotor pattern generator [118, 119]. From these data, it is apparent that presynaptic 5-HT receptors act as a modulator rather than an inhibitor of activity. This ability to modulate rather than inhibit synaptic output is critical to the function of serotonergic presynaptic modulation. Thus, for example, at a low dose of the glutamate receptor antagonist kynurenate, sufficient to half the amplitude of synaptic responses from giant axons in the spinal cord, locomotion is entirely abolished. Similarly, sparing postsynaptic activation of NMDA receptor-mediated synaptic responses is also critical. Blocking these also prevents fictive locomotion [106]. However, a further striking function of the presynaptic receptors discussed in this review, is that though their site and mechanism of action is entirely distinct from postsynaptic serotonin receptors that respond to the same paracrine release of 5-HT, their effect on behavior is entirely synergistic. Postsynaptic 5-HT receptors also slow the locomotor rhythm, but do so by inhibiting postsynaptic Ca^{2+} activated K^+ channels [122–126].

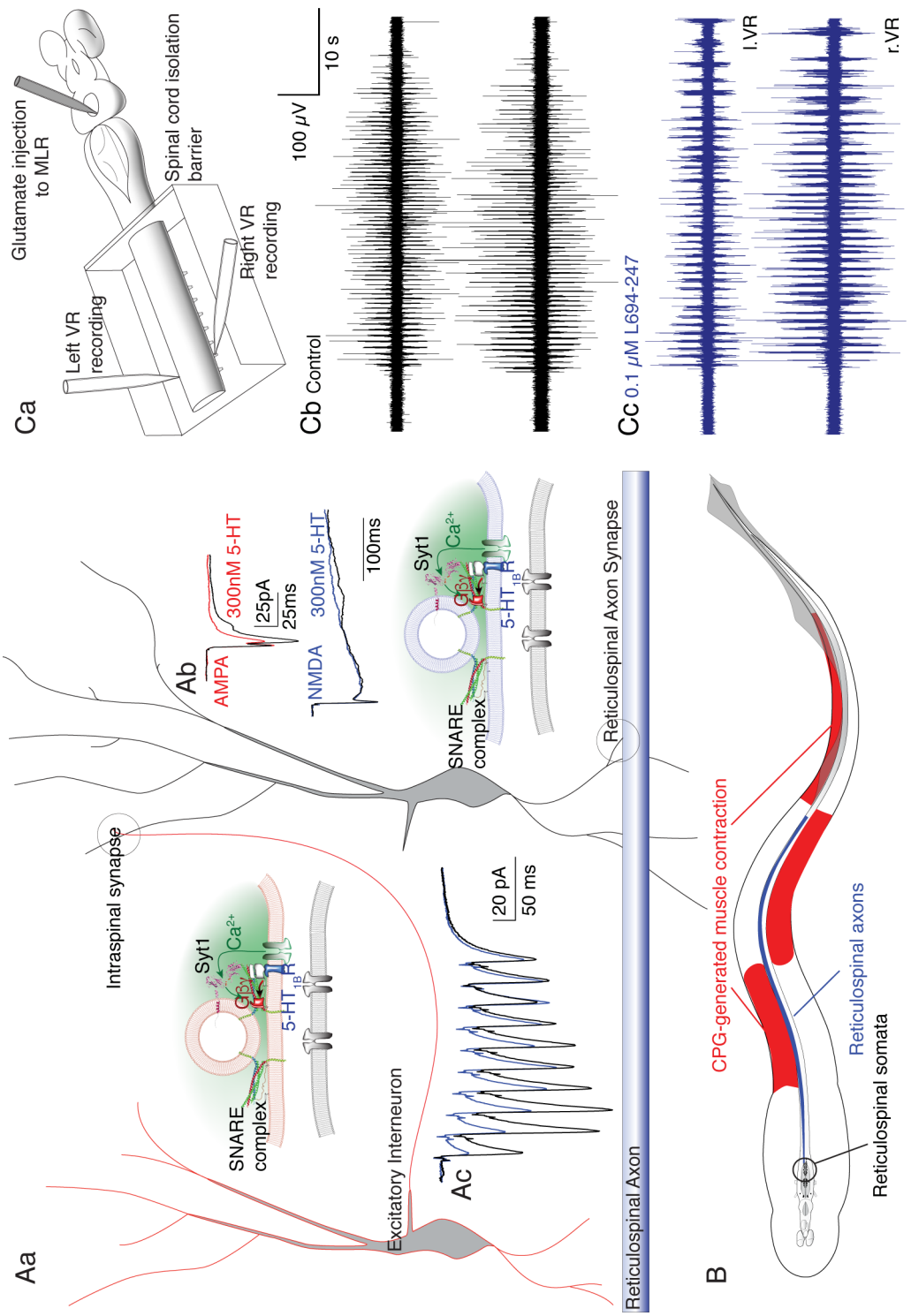

Fig. 5 Modulatory properties of presynaptic 5-HT receptors on synaptic transmission and motor behavior. **A** 5-HT inhibits excitatory synaptic transmission in the lamprey spinal cord, from both large descending command neurons—the reticulospinal neurons—and intraspinal excitatory interneurons. **Ab** AMPA receptor-mediated EPSCs recorded in the presence of D-AP5 to block NMDA receptor-mediated responses are inhibited by 5-HT (red). The same dose of 5-HT did not inhibit NMDA receptor-mediated EPSCs (blue) recorded in NBQX to block AMPA receptors. **Ac** Repetitive stimulation causes accumulation of presynaptic Ca^{2+} and loss of 5-HT-mediated inhibition. Paired recording between a giant axon and a postsynaptic neuron. Repetitive presynaptic action potentials cause a facilitating postsynaptic response (black). 5-HT (blue) inhibits at the start but not the end of the train. **B** The spinal motor circuitry activated by descending reticulospinal axons generates oscillations across the midline and a traveling wave along the rostro-caudal axis. The neural output that causes this movement is generated by spinal circuits including the excitatory synapses shown in (**A**). This can be activated by stimulation of the brainstem region that causes excitation of reticulospinal neurons. Shown in the schematic (**Ca**). The isolated brain-spinal cord preparation in which stimulation of the brainstem evokes fictive locomotion of alternating activity in left–right pairs of ventral roots. A split bath allows application of drugs selectively to the spinal compartment. **Cb** Stimulation of the MLR in the brainstem evokes episodes of spinal fictive locomotion. **Cc** application of the selective 5-$HT_{1B/1D}$ agonist L694-247, slows the alternating pattern without altering total output spiking. (Color figure online)

Conclusions

Presynaptic $G_{i/o}$ coupled GPCRs are ubiquitous, but signal through a variety of mechanisms. One of those mechanisms, whereby Gβγ targets the SNARE complex to compete with synaptotagmin1 binding, leads to complex synaptic modulation and a form of circuit plasticity that is extraordinarily synergistic with the activation of postsynaptic serotonin receptors. There is substantial debate on the nature of synaptic vesicle fusion and the protein-lipid machinery that drives this critical synaptic function. Fusion of vesicles may be complete and convey quantal properties to synaptic events [127, 128]. But fusion may also be incomplete and more nuanced [129]. This debate has extended to the present with evidence that kiss and run fusion [57, 130] can differentially modulate postsynaptic receptor activation [112], while the frequency of this form of transmission has been challenged [135].

Perhaps an answer to this conundrum lies in Gβγ's target—competing with Ca^{2+}-synaptotagmin interactions at SNARE complexes [76, 101], and the ability of presynaptic receptors to modulate fusion properties of synaptic vesicles, causing kiss-and-run fusion [58, 107]. It would appear that presynaptic GPCR activation provides a variety of routes to evoke synaptic plasticity. In particular Gβγ interaction with the SNARE complex opens the possibility for sophisticated modulation of synaptic vesicle fusion [58, 100, 131], that this modulation creates complex synaptic modulation far beyond simple changes in release probability [106, 107], and that these synaptic changes have implications for our understanding of circuit functions and behavior [98, 106]. In addition, the capacity to modulate neurotransmitter release independently of changes in presynaptic Ca^{2+} may preserve other effects of Ca^{2+} at the presynaptic terminal even during modulation. The requirement for such complex modulation of neurotransmitter release is apparent from effects on lamprey locomotion—effects that serotonin also causes in mammalian models of locomotion [132, 133]. Thus, both the target of 5-HT-liberated Gβγ at the SNARE complex in which inhibition is modulated by Ca^{2+}-dependent competition with synaptotagmin, and the effects of Gβγ on vesicle fusion are necessary to maintain the modulatory role of 5-HT in the spinal cord [106]. If, in contrast, 5-HT merely inhibited neurotransmitter release, no descending excitation would remain, no locomotion would occur, and other spinal modulatory effects of 5-HT would be moot in behaving vertebrates.

Gβγ SNARE complex interactions also allow for the possibility of differentially modulating evoked and spontaneous release of neurotransmitter. This mechanism of modulation does not significantly alter spontaneous release of glutamate in the hippocampal CA1-subiculum [32], nor in lamprey spinal cord [96]. However, it does in chromaffin cells [131]. Differences in SNARE complex proteins [134–136] or possible Ca^{2+} sensors [80, 137] utilized for these different fusion mechanisms might account for these various effects of GPCRs.

For the future, it is clear that the mechanisms by which Gβγ targeting of SNARE complexes leads to modification of the mode of synaptic vesicle fusion causing kiss-and-run fusion are important across a range of systems that require SNARE-dependent vesicle fusion. Thus, in central synapses this mode of fusion both allows selective inhibition of particular postsynaptic receptors [106, 107], but also a large dynamic range of presynaptic inhibition, while in endocrine large dense core fusion events, this mode may allow for selective release of neurotransmitters within or surrounding the dense core matrix [138, 139]. We have demonstrated the interaction between synaptotagmin1, Gβγ and the SNARE complex. Nevertheless, competition between Gβγ and syt1—the tagmin responsible for evoked release—at SNARE complexes is unlikely to account for the loss of Gβγ–SNARE interaction during trains (Fig. 5) because the high $[Ca^{2+}]$ that allows Ca^{2+}–syt1 binding, does not sufficiently sum. It seems more likely that presynaptic modulation requires multiple fusogenic Ca^{2+} sensors which differentially interact with different Gβγ modulators. Possible candidate C2AB domain containing proteins have been implicated in the modulation of fusion during stimulus trains. These include synaptotagmin 7 [82] and Doc2α and β [80, 140], and warrant further investigation. Finally, the Gβγ–SNARE interaction and its modulation of exocytosis downstream of presynaptic calcium entry is an important neuromodulatory mechanism in a large diversity of circuits, mediating multiple physiologic behaviors. Future studies should focus on identifying the $G_{i/o}$-coupled GPCRs that signal via Gβγ–SNARE.

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