In mature neurons AMPA receptors cluster at excitatory synapses primarily on dendritic spines, whereas GABA<sub>A</sub> receptors cluster at inhibitory synapses mainly on the soma and dendritic shafts. The molecular mechanisms underlying the precise sorting of these receptors remain unclear. By directly studying the constitutive exocytic vesicles of AMPA and GABA<sub>A</sub> receptors in vitro and in vivo, we demonstrate that they are initially sorted into different vesicles in the Golgi apparatus and inserted into distinct domains of the plasma membrane. These insertions are dependent on distinct Rab GTPases and SNARE complexes. The insertion of AMPA receptors requires SNAP25–syntaxin1A/VAMP2 complexes, whereas insertion of GABA<sub>A</sub> receptors relies on SNAP23–syntaxin1A/VAMP2 complexes. These SNARE complexes affect surface targeting of AMPA or GABA<sub>A</sub> receptors and synaptic transmission. Our studies reveal vesicular sorting mechanisms controlling the constitutive exocytosis of AMPA and GABA<sub>A</sub> receptors, which are critical for the regulation of excitatory and inhibitory responses in neurons.

Critical for many intracellular membrane trafficking events. The family is subdivided into v-SNAREs (synaptobrevin/VAMP, vesicle-associated membrane proteins) and t-SNAREs (syntaxins and SNAP25, synaptosomal-associated protein of 25 kDa) based on their localization on trafficking vesicles or target membranes, respectively. To mediate vesicle fusion with target membranes, SNARE proteins form a four-helix bundle (SNARE complex) consisting of two coiled-coil domains from SNAP25, one coiled-coil domain from syntaxin, and a coiled-coil domain from VAMPs (18). Formation of the helical bundle can be disrupted by neurotoxins, which specifically cleave different SNARE proteins (19). Each SNARE subfamily is composed of genes with high homology but different tissue specificity and subcellular localization. It remains to be determined whether individual SNAREs play specific roles in regulating the membrane trafficking of individual proteins. To address how AMPA and GABA<sub>A</sub> receptors are sorted in the exocytic pathway and what molecules are involved in regulating exocytosis of these receptors, we specifically studied constitutive exocytosis of AMPA and GABA<sub>A</sub> receptor subunits using total internal reflection fluorescence microscopy (TIRFM) in combination with immunocytochemistry, electrophysiology, and electron microscopy methods. Together, we revealed that AMPA and GABA<sub>A</sub> receptors are initially sorted into different vesicles in the Golgi apparatus and delivered to different domains at the plasma membrane and are regulated by specific Rab proteins and critical for many intracellular membrane trafficking events.
SNARE complexes. These results reveal fundamental mechanisms underlying the sorting of excitatory and inhibitory neurotransmitter receptors in neurons and uncover the specific trafficking machinery involved in the constitutive exocytosis of each receptor type.

Results

Dynamic Events of AMPA and GABA<sub>A</sub> Receptors on the Plasma Membrane of Hippocampal Pyramidal Neurons. To visualize individual exocytosis events of AMPA or GABA<sub>A</sub> receptors in living hippocampal neurons, we used TIRFM to specifically image trafficking events at or immediately beneath the plasma membrane in contact with the coverslip (100–200 nm) (20). To further ensure the imaging of exocytic events, superepictronic pHluorin (pHuorin or pH) was chosen to tag the extrasynaptic N terminus of AMPA and GABA<sub>A</sub> receptors. pHuorin is an EGFP variant that fluoresces brightly at pH 7.4 and is fully quenched in the lumen of secretory organelles having a pH <6 (21). Therefore, after exocytosis the fluorescent signal of pHuorin-tagged receptors dramatically increases under the exposure of imaging solution with pH 7.4 (14, 15, 22). pHuorin-tagged GluA2 (pH-GluA2) and γ2S (pH-γ2S) were used for the study, because these subunits are common subunits of AMPA and GABA<sub>A</sub> receptor complexes in hippocampus, respectively. Previous studies have confirmed that the pHuorin tag does not affect trafficking of these receptor subunits in neurons (15, 23).

pHuorin-tagged GluA2 or γ2S was expressed in dissociated hippocampal neurons and directly visualized under TIRFM. Before recording, the entire cell surface in the TIRF field was photobleached to eliminate signals from preexisting surface receptors and isolate new exocytic events (22). We observed robust dynamic events of pH-GluA2 and pH-γ2S throughout the plasma membranes. Most events of GluA2 and γ2S occurred on the extrasynaptic membrane in the cell body and dendritic shafts (Fig. 1 and Movies S1 and S2). We did not observe events of pH-GluA2 on dendritic spines. These dynamic events transiently occurred at high frequency: 95.8% events of pH-GluA2 lasted less than 7 s with the mean duration around 2.8 s, whereas 96.7% events of pH-γ2S lasted less than 7 s with mean duration around 2.1 s (Fig. 1 B and C). The mean event duration of pH-GluA2 was significantly longer than that of pH-γ2S (Fig. S1L). There are 22 ± 2 events per second per 100 μm<sup>2</sup> for pH-γ2S and 15 ± 1 events per second per 100 μm<sup>2</sup> for pH-GluA2. These frequencies remained stable under imaging with higher frame rate (Fig. S1B). To confirm that these events are on the plasma membrane we performed an acidification–neutralization test (24), which included 15–30 s of TIRF imaging in pH 7.4 extracellular solution, then a fast perfusion for 15–30 s with pH 5.5 extracellular solution, followed by a return to pH 7.4 extracellular solution. Most of dynamic events of pH-GluA2 and pH-γ2S quenched upon acidic perfusion and recovered immediately after renunciation (Fig. 1 D and Fig. S1C). In addition, when neurons were perfused with the pH 7.4 solution containing ammonium chloride (NH<sub>4</sub>Cl), which rapidly alkalized all of the acidic intracellular pools and revealed intracellular pH receptors (21), the frequency of the dynamic TIRF events remained constant. These results strongly suggest that the dynamic events under TIRFM present on the plasma membrane. Moreover, the frequency of these events was not regulated by neuronal activity, which was acutely suppressed or enhanced by brief application of TTX or KCI, respectively (Fig. S1D), suggesting these are constitutive trafficking events. Overall, these results indicate that the transient exocytotic events of GluA2 and γ2S under TIRFM are constitutive dynamics of receptors on the plasma membrane. We noticed that most of the events of GluA2 and γ2S have dim fluorescence intensity, suggesting that each event contains a low number of receptor subunits. To confirm this observation, we measured the number of fluorescent receptors per event (22). Based on the knowledge that the fluorescent intensity of the EGFP monomer is similar to the intensity of pHuorin in the environment of pH 7.4 (24), we compared the fluorescent intensity.
of EGFP monomer to the intensity of single events of pH-GluA2 and pH-γ2S under TIRFM. EGFP monomers were confirmed by their blinking dynamics and single-step photobleaching property (22) (Fig. S1E). The intensity of EGFP monomers, pH-GluA2-containing vesicles, and pH-γ2S-containing vesicles follows Gaussian distributions (Fig. S1 F–H). The peak intensities of fitted Gaussian curves for EGFP monomers, pH-GluA2, and pH-γ2S events indicate that each pH-GluA2 event contains on average two pH-GluA2 subunits (2.2 ± 0.1 subunits per event), whereas each pH-γ2S event contains around four pH-γ2S subunits (3.9 ± 0.2 subunits per event). We and others have previously characterized larger, much less frequent GluA2 and GluA1 insertion events that have slower kinetics and are distinct from these rapid insertion events (14, 15, 22). Because of the much lower frequency of these larger events (two to six insertions per minute) they did not significantly contribute to the quantitation and characterization of the smaller events.

Dynamic TIRF Events of GluA2 and γ2S Are Exocytic Events. Several lines of evidence suggest that the dynamic surface events of GluA2 and γ2S under TIRFM are exocytic events. First, the exocytic feature is supported by the stereotypic dynamics of these events under TIRFM. As demonstrated in Fig. 1E, when two subunits of the same receptor differentially tagged with pHluorin and a red fluorescent protein (pH-sensitive; for example, tdTomato) and delivered in the same exocytic vesicle (coinjection), they exhibit different dynamics under TIRFM. The pH-insensitive red fluorescent protein is excited immediately when the exocytic vesicle enters the TIRF field. However, pHluorin remains quenched until it is exposed to the extracellular space (pH 7.4) after the exocytosis. Therefore, the red fluorescence increases in advance of the green fluorescence (Fig. 1F).

To investigate whether the events of GluA2 and γ2S also exhibit this stereotypic dynamics of exocytosis under TIRF, we first tagged GluA2 and γ2S with pH-sensitive red fluorescent protein tdTomato and characterized the tagged receptor subunits (Fig. S2). In live hippocampal neurons tdt-GluA2 or tdt-γ2S colocalized with EGFP-GluA2 or EGFP-γ2S, respectively (Fig. S2 A and D). In addition, tdt-GluA2 (Fig. S2 B and C) and tdt-γ2S (Fig. S2 E and F) also colocalized well with endogenous GluA1 and 2/3, respectively, indicating that these tdTomato-tagged receptors trafficked similarly to endogenous receptors. Moreover, tdt-GluA2 and tdt-γ2S could be stained in live cells with anti-tdTomato antibody, indicating that these receptors were expressed on the surface (Figs. S3 and S4). Total and surface tdt-GluA2 colocalized with the excitatory postsynaptic marker PSD95 (postsynaptic density protein 95) (Fig. S3 A and B) and the presynaptic marker VGlut (vesicular glutamate transporter) (Fig. S3 C and D). Similarly, total and surface tdt-γ2S colocalized with the inhibitory postsynaptic marker gephyrin (Fig. S4 A and B) and the presynaptic marker VGAT (vesicular GABA transporter) (Fig. S4 C and D). These data suggest that tdTomato-tagged GluA2 and γ2S are properly trafficked and targeted in hippocampal neurons.

We then coexpressed pH receptor and tdt receptor and simultaneously visualized their exocytosis under dual-color TIRFM with 488-nm and 568-nm lasers to excite green and red fluorescent proteins, respectively. Dynamics of events containing both green and red fluorescence signals (coinjection events) were analyzed. As expected, in the coinjection events of pH-GluA2 and tdt-GluA2, the fluorescence of tdt-GluA2 increased earlier than that of pH-GluA2 (Fig. 1 G and H). Similar dynamics was also observed in the coinjection events of pH-γ2S and tdt-γ2S (Fig. 1 I and J). The particular dynamics of these coinjections under TIRF strongly suggest that they are exocytic events. In many events, we also observed that the fluorescence of tdTomato receptor decayed faster than the pHluorin fluorescence. This phenomenon is likely caused by the photoinstability of tdTomato compared to pHluorin (26).

The second evidence of exocytosis is based on the results of botulinum toxin (Botox) treatments. Receptor exocytosis occurs when an intracellular vesicle, which carries assembled receptor complexes, fuses to the plasma membrane and the receptor complexes are delivered to the plasma membrane (27). This process highly depends on SNARE proteins, which can be cleaved by different Bottoxs (19). Therefore, we tested the effects of Botox on the TIRF event frequencies of pH-GluA2 and pH-γ2S. Botox B, which cleaves VAMP2 (Fig. S5 C and D), reduced frequency of both GluA2 and γ2S events (Fig. 1K). Similarly, Botox C, which cleaves rat SNAP25, syntaxin1A, 1B, 2, and 3 (Fig. S5 E–J), inhibited events of GluA2 and γ2S (Fig. 1L). Notably, we detected a low degree of the incomplete blockade of exocytic events, which was commonly reported with botulinum toxin treatments. This is likely due to the inability of Bottoxs to prototelyze SNARE proteins in assembled complexes (Fig. S5) (28).

Finally, we also observed receptor dispersion to the surrounding regions after exocytosis, which is another stereotypic dynamic of exocytic events (14). As shown in Fig. S6, pH-GluA2 events showed increased fluorescence in the surrounding region while the fluorescence in the insertion spot decayed (Fig. S6 A and B). The appearance of fluorescence peak in the surrounding region was significantly delayed in comparison with the one in the insertion center, strongly indicating the receptor dispersion after the insertion (Fig. S6 C and D). A similar phenomenon was observed for pH-γ2S events (Fig. S6 E–H). In addition, many events showed the separation of receptor subunits during this dispersion process (Fig. S6 A and E), suggesting that each inserted receptor complex can diffuse independently.

In summary, the Botox sensitivity and stereotypic dynamics of these events under TIRFM strongly suggest that they are exocytic events of GluA2 and γ2S.

Exocytosis of AMPA and GABA<sub>A</sub> Receptors Is Mediated by Different SNAPS. Although Botox B and C inhibited exocytosis of both GluA2 and γ2S, Botox A, which cleaves SNAP25 (Fig. S5 A and B), showed different effects on exocytosis of pH-GluA2 and pH-γ2S. As shown in Fig. 2A, Botox A inhibited exocytosis of GluA2, but not γ2S. These Botox-treatment results indicate that exocytosis of both AMPA and GABA<sub>A</sub> receptors require VAMP2 and syntaxins, but the different receptors likely require different SNAPS: SNAP25 mediates AMPA receptor insertion, whereas a Botox A-insensitive SNAP mediates GABA<sub>A</sub> receptor insertion.

Three Botox A-insensitive SNAPS (SNAP23, SNAP29, and SNAP47) are expressed in rat hippocampal neurons (29). All three SNAPS are known to regulate membrane fusion events in neurons (13, 16, 17, 30–32). We examined the effect of shRNAs that specifically knock down each SNAP (Fig. S7 A–E) on the exocytosis frequencies of AMPA and GABA<sub>A</sub> receptors, compared with a control shRNA with a sequence not targeting any known vertebrate genes. Consistent with Botox A treatment, SNAP25 knockdown reduced the frequency of exocytosis of GluA2, but not γ2S, and this inhibition was fully rescued by shRNA-resistant SNAP25 but not SNAP25 (Fig. 2B). However, knockdown of SNAP23 blocked exocytosis of γ2S, but not GluA2, and this effect was rescued by shRNA-resistant SNAP23 but not SNAP23 (Fig. 2C). The effects of SNAP25 and SNAP23 shRNAs were observed on both somatic and dendritic exocytosis of pH-GluA2 and pH-γ2S (Fig. S7 J–M). Knockdown of SNAP29 or SNAP47 did not significantly affect exocytosis of either GluA2 or γ2S (Fig. S7 N and O). These results demonstrated that SNAP25 and SNAP23 specifically mediate the constitutive exocytosis of GluA2- and γ2S-containing receptors, respectively.

If SNAP25 and SNAP23 knockdown reduced constitutive exocytosis of AMPA and GABA<sub>A</sub> receptors, respectively, we would predict that this would decrease the surface expression of these receptors. To test this possibility, we examined surface levels of endogenous GluA2 and γ2 by immunostaining of surface receptors.

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while the SNAPs were specifically knocked down. Indeed, SNAP25 shRNA significantly reduced surface GluA2 levels in hippocampal neurons. This effect was rescued by shRNA-resistant SNAP25 but not SNAP23 (Fig. 2 D and E). Conversely, SNAP23 shRNA drastically reduced surface γ2 levels, which was rescued by shRNA-resistant SNAP23 but not SNAP25 (Fig. 2 F and G). However, knockdown of SNAP23 or SNAP25 did not affect surface levels of GluA2 or γ2, respectively (Fig. S8).

We then asked whether SNAP25 and SNAP23 regulate surface expression of endogenous GluA2 and γ2 subunits at synapses, respectively. In hippocampal neurons knockdown of SNAP25, rather than SNAP23, reduced synaptic surface GluA2 which colocalized with the excitatory presynaptic marker VGluT (Fig. 3 A and B). However, knockdown of SNAP23, but not SNAP25, significantly reduced synaptic surface levels of γ2, which colocalized with the inhibitory presynaptic marker VGAT (Fig. 3 C and D). Given our observations that most insertions of GluA2 and γ2S occur at extrasynaptic sites (Movies S1 and S2), these reductions in synaptic surface GluA2 and γ2 could result from the depletion of extrasynaptic surface receptors, which supply synaptic receptor pools by lateral diffusion to the postsynaptic membrane. These data suggest that SNAP25 and SNAP23 regulate not only the surface expression of GluA2 and γ2 subunits throughout the entire neuron, but also specifically regulate the synaptic surface expression of GluA2 and γ2, respectively.

The reduced surface expressions of GluA2 and γ2 at synapses suggest that SNAP25 and SNAP23 may affect AMPA and GABA<sub>γ</sub> receptor-mediated synaptic transmission. To test this hypothesis, whole-cell patch-clamp recording was used to examine AMPA receptor-mediated mEPSCs (miniature excitatory postsynaptic currents) and GABA<sub>γ</sub> receptor-mediated mIPSCs (miniature inhibitory postsynaptic currents) in hippocampal neurons with SNAP25 or SNAP23 knockdown by specific shRNAs. Strikingly, knockdown of SNAP25, but not SNAP23, preferentially reduced AMPA receptor-mediated mEPSC amplitude (Fig. 3 E and F), whereas knockdown of SNAP23, but not SNAP25, significantly reduced GABA<sub>γ</sub> receptor-mediated mIPSC amplitude (Fig. 3 G and H). These results are consistent with the specific effects of SNAP25 and SNAP23 on constitutive exocytosis and synaptic surface levels of GluA2 and γ2S, respectively. The SNAP25 or SNAP23-dependent exocytosis of AMPA or GABA<sub>γ</sub> receptors significantly affects excitatory and inhibitory synaptic transmission in neurons at the basal state.

Overall, our results reveal important postsynaptic roles of SNAP25 and SNAP23 on constitutive insertions, surface expression of GluA2 and γ2, and basal synaptic transmission mediated by AMPA and GABA<sub>γ</sub> receptors, respectively. The distinct functions of SNAP25 and SNAP23 support the model that AMPA and GABA<sub>γ</sub> receptors are inserted into the plasma membrane via different vesicles that are under regulation of specific SNAPs.

Exocytosis of AMPA and GABA<sub>γ</sub> Receptors Is Mediated by Syntaxin1 and VAMP2. We further investigated other SNARE components necessary for fusion of AMPA or GABA<sub>γ</sub> receptor-containing vesicles to the plasma membrane. In rat hippocampal neurons, five syntaxins are expressed on the plasma membrane: syntaxin1A, syntaxin1B, syntaxin2, syntaxin3, and syntaxin4 (33). All syntaxins, except syntaxin4, can be cleaved by Botoc A (Fig. S5 F–J) (34). SNAP25 and SNAP23 have higher affinities to syntaxin1A, 1B, and syntaxin4 than to syntaxin2 and 3 (35–37). We therefore...
tested whether syntaxin1A, 1B, or syntaxin4 could be the t-SNAREs mediating exocytosis of GluA2 or γ2S. We used specific shRNAs to knock down these three syntaxins (Fig. S7 F–H) and examined their effect on exocytosis of GluA2 or γ2S. Knockdown of syntaxin1A and 1B significantly reduced exocytic frequencies of both GluA2 and γ2S. This effect could be rescued by shRNA-resistant syntaxin1A and 1B, respectively (Fig. 4 A and B). Knockdown of syntaxin4 did not affect exocytosis of either GluA2 or γ2S (Fig. S7P). In conclusion, the constitutive insertion of GluA2- or γ2S-containing vesicles into the plasma membrane is commonly mediated by two t-SNAREs: syntaxin1A and 1B.

There are two VAMPs, VAMP1 and VAMP2, specifically expressed in the rat brain, including hippocampus (38–40). To test which VAMPs are important for pH-GluA2 and pH-γ2S exocytosis, we used shRNAs to specifically knock down VAMP1 and VAMP2 (Fig. S7I) and investigated their effects on the exocytosis of GluA2 and γ2S. Consistent with our previous observation that Botox B, which cleaves VAMP2, reduced the exocytic frequency of both pH-GluA2 and pH-γ2S (Fig. 1K), depletion of VAMP2 significantly reduced exocytic frequencies of both pH-GluA2 and pH-γ2S (Fig. 4C). However, knockdown of VAMP1, which is not cleaved by Botox B, had no effect on exocytosis of either pH-GluA2 or pH-γ2S (Fig. S7Q). Together, these results demonstrate that VAMP2, but not VAMP1, serves as a v-SNARE mediating the constitutive exocytosis of both GluA2- and γ2S-containing AMPA and GABA<sub>A</sub> receptors.

**Exocytosis of AMPA and GABA<sub>A</sub> Receptors Is Differentially Regulated by Specific Rab Proteins.** Surface receptors can be delivered to the plasma membrane along different trafficking pathways, such as the de novo exocytic pathway originating from Golgi apparatus and recycling pathways involving early and recycling endosomes. These exocytic pathways are regulated by the small GTPase Rab protein family (41). To investigate the source of the receptor-containing vesicles, we coexpressed pHluorin-tagged receptors with dominant negative Rab proteins that interfere with specific trafficking pathways. A dominant negative Rab8 [Rab8(T22N)], which blocks vesicle trafficking from the Golgi apparatus to the plasma membrane, reduced the exocytic frequency of both GluA2 and γ2S (Fig. 4D). Dominant negative Rab4, 5, and 11 [Rab4(S22N), Rab5(S34N), and Rab11(S25N)], which block different steps in the vesicle recycling pathway including sorting from early endosomes to the plasma membrane, endocytosis, and trafficking from recycling endosomes to the plasma membrane, only significantly inhibited exocytosis of GluA2, but not γ2S (Fig. 4 E–G). These results suggest that constitutive exocytic events of GluA2 include both de novo exocytic and recycling events, whereas constitutive exocytic events of γ2S are mostly de novo exocytic events.

**Exocytosis of AMPA and GABA<sub>A</sub> Receptors Targets Different Zones on the Plasma Membrane.** Exocytic events of pH-GluA2 and pH-γ2S not only occurred under different molecular mechanisms, but also show distinct spatial targeting on the plasma membrane.
Using an intensity-based program, exocytic events of pH-GluA2 and pH-γ2S were automatically isolated (Fig. S9A). Strikingly, we found that the pH-GluA2 exocytic events occur in the central region of the plasma membrane in contact with the coverslip, whereas the pH-γ2S exocytic events distribute in the peripheral region of the plasma membrane (Fig. 5A and Movies S3 and S4).

To confirm the spatial segregation of exocytosis of pH-GluA2 and pH-γ2S, we coexpressed GluA2 or γ2S tagged with tdTomato or pHluorin in the same cell and simultaneously visualized their exocytic events using dual-color TIRFM. Consistent with the previous observations, exocytic events of pH-γ2S and tdT-GluA2 have different distributions on the somatic plasma membrane in contact with the coverslip. Vesicles containing pH-γ2S are mainly targeted to the outer peripheral region of the soma, whereas tdt-GluA2-containing vesicles are preferentially targeted to the inner central region of the soma (Movie S5).

Quantification of these observations, by counting the number of exocytic events along the long axis of the somatic region, confirmed that exocytic vesicles of pH-γ2S and tdt-GluA2 are spatially segregated on the somatic plasma membrane (Fig. 5B–D).

To rule out any potential artifacts of the fluorescent tags, we swapped the fluorescent tags on the two receptor subunits and confirmed exocytosis of pH-GluA2 and tdt-GluA2 have different distributions on the somatic plasma membrane (Fig. 5E). Exocytic events of pH-GluA2 and tdt-GluA2 occur with a similar inner central-somatic distribution as tdt-GluA2 and tdt-GluA2, respectively (Fig. 5F). Exocytic events of pH-γ2S and tdt-γ2S have the same outer peripheral-somatic distribution (Fig. 5G). In addition, the distribution of pH-GluA2 exocytic events was not affected by the coexpression of other AMPA receptor subunits, such as GluA1 and GluA3 (Fig. S9B–E). Overall, our observations suggest that the exocytic events of excitatory AMPA receptors and inhibitory GABAA receptors are spatially segregated. The exocytosis of GluA2, a subunit present in most AMPA receptors, occurs at the outer region of the soma in contact with the coverslip, whereas the exocytosis of γ2S, a subunit present in most inhibitory GABAA receptors, occurs at the outer region of the soma in contact with the coverslip.

Interestingly, this differential surface targeting of exocytosis of GluA2 and γ2S is regulated by Rab proteins. Whereas dominant negative Rab8 did not change the distribution of GluA2 exocytosis, which mostly occurs at the inner region of the somatic membrane, the residual exocytic events of GluA2 after expression of dominant negative Rab4, Rab5, or Rab11 were distributed more evenly across the somatic membrane (Fig. S9H). The distribution of γ2S exocytosis was not affected by any of the Rab mutants (Fig. S5I). These data suggest that differential targeting of GluA2 and γ2S exocytosis on the plasma membrane potentially reflect specific exocytic pathways for each receptor. Although some AMPA-containing AMPA receptors can be delivered to the plasma membrane through the de novo exocytic pathway, the majority of AMPA receptors are delivered through recycling vesicles and inserted into the inner region of the soma in contact with the coverslip. However, most γ2S-containing GABAA receptors are delivered to the plasma membrane through de novo exocytic vesicles, which specifically insert at the outer regions of the soma.

**AMPA and GABAA Receptors Exit the Golgi Apparatus As Different Vesicles.** Because the constitutive exocytosis of both AMPA and GABAA receptors seems to occur through a de novo exocytic pathway originating from the Golgi apparatus, we further asked whether the two receptor types are trafficked by different vesicles after they exit the Golgi. To image post-Golgi trafficking of receptors, we coexpressed EGFP- or tdTomato-tagged GluA2 and γ2S and then incubated transfected neurons at 20 °C to inhibit vesicle budding from the Golgi apparatus (42). Under this condition, we observed the accumulation of GluA2 and γ2S in the Golgi apparatus (Fig. S10A). After the 20 °C incubation, live neurons were imaged at 32 °C when post-Golgi trafficking is restored (43). We first examined whether the same receptor with different fluorescent tags is cotrafficked in the post-Golgi route by coexpressing EGFP-γ2S and tdT-γ2S, or EGFP-GluA2 and tdT-GluA2. We were able to visualize trafficking vesicles containing both EGFP-γ2S and tdT-γ2S (Fig. S10B and Movie S6), or both EGFP-GluA2 and tdT-GluA2 (Fig. S10C and Movie S6), indicating cotrafficking of these differentially tagged receptor subunits. However, we also observed many vesicles containing EGFP- or tdTomato-tagged subunits alone. This is likely due to the low number of receptors in each vesicle (Fig. S1E–H) and the sensitivity of detection. In contrast, when we coexpressed EGFP-GluA2 and tdT-γ2S (Fig. S7) or EGFP-γ2S and tdT-GluA2 (Fig. S8), we very rarely observed the cotrafficking of GluA2 and γ2S. The percentage of cotrafficking events of different receptor pairs is significantly lower than that of same
receptor pairs (Fig. 6C), suggesting that vesicles exiting the Golgi carry preferentially GluA2 or γ2S alone. These results indicate that GluA2 and γ2S receptors are trafficked in separate vesicles after they exit the Golgi apparatus.

Endogenous AMPA and GABA<sub>A</sub> Receptors Are Sorted into Different Vesicles. Our results in cultured hippocampal neurons strongly suggest that AMPA and GABA<sub>A</sub> receptors are sorted into different intracellular vesicles before exocytosis. To further investigate whether endogenous receptors were also sorted into separate intracellular vesicle compartments in vivo we performed double-label immunogold EM studies in microsome-enriched fractions (P3) from adult rat brain. Rat brain homogenates were fractionated by differential centrifugation (44) and the fractions were characterized using markers of major intracellular organelles and vesicles (Fig. 7A). The P3 fraction contains membranes from the endoplasmic reticulum (ERP72), lysosomes (LAMP1), early endosomes (EEA1), recycling endosomes (synaxin13), and Golgi apparatus (TGN38). Other SNARE proteins, such as SNAP23, SNAP25, and VAMP2, were also present in the P3 fraction. Furthermore, GluA2 and γ2 are enriched in P3 fraction. The EM morphology of P3 fraction showed that the P3 pellet contained intact vesicular structures with different sizes (Fig. 7B and C).

Double-immunogold labeling was performed on thin sections of the P3 pellet after a light fixation (Fig. 7D). The morphology of small intracellular trafficking vesicles, which are 50–300 nm in diameter (44), was largely preserved under this condition. GluA2 and γ2 were labeled by specific primary antibodies and secondary antibodies conjugated to 6-nm and 12-nm gold particles, respectively. The majority of vesicles contained only a single type of receptor whereas 12% of the vesicles contained both GluA2 and γ2. We observed that 37% of
and $\gamma_2$ receptors preferentially insert in the central region of the soma, whereas vesicles containing $\gamma_2$ receptors preferentially insert in the periphery of the soma. This result was surprising and indicated that AMPA and GABA$\gamma_2$ receptors are not only differentially sorted into distinct vesicles but also targeted to distinct zones of the somatic plasma membrane during exocytosis.

Discussion

AMPA and GABA$\gamma_2$ receptors are selectively targeted to excitatory and inhibitory synapses (3), respectively. However, it is not clear when and how AMPA and GABA$\gamma_2$ receptors are sorted and trafficked into their target zones. To investigate this important question, we performed live TIRF imaging to directly visualize the constitutive exocytic vesicles of AMPA and GABA$\gamma_2$ receptors. In combination with immunocytchemistry, electrophysiology, and electron microscopy studies, we found that the exocytic sorting of these two receptor types follows the “vesicle sorting model” (Fig. 7F). AMPA and GABA$\gamma_2$ are initially sorted into different vesicle populations in the Golgi apparatus. The majority of GABA$\gamma_2$ receptors are directly delivered to the plasma membrane through the de novo exocytic pathway under the regulation of Rab8. The SNAP23-syntaxin1–VAMP2 complex mediates the fusion of GABA$\gamma_2$ receptor-containing vesicles to the plasma membrane. However, exocytosis of AMPA receptors includes not only the Rab8-mediated de novo pathway but also the recycling pathway regulated by Rab4, 5, and 11. The fusion between AMPA receptor-containing vesicle and the plasma membrane is mediated by the SNAP25–syntaxin1–VAMP2 complex. In addition, we observed that vesicles containing AMPA receptors preferentially insert in the central region of the soma, whereas vesicles containing GABA$\gamma_2$ receptors preferentially insert in the periphery of the soma. This result was surprising and indicated that AMPA and GABA$\gamma_2$ receptors are not only differentially sorted into distinct vesicles but also targeted to distinct zones of the somatic plasma membrane during exocytosis.

This sorting of the major excitatory and inhibitory receptors in the somatodendritic region is reminiscent of the polarized trafficking of apical versus basolateral proteins in epithelial cells (45, 46) and somatodendritic versus axonal proteins in neurons (47, 48), which involves vesicular sorting in TGN and endosomes. Previous studies and our current research suggest a general strategy that proteins that function at different subdomains of the cell are sorted early into separate vesicle populations. This early sorting maximally ensures the independent targeting and regulation of each protein.

**Fig. 6.** GluA2 and $\gamma_2$ are trafficked in different vesicles when they exit the Golgi apparatus. (A) Time series of a post-Golgi trafficking vesicle containing only EGFP-GluA2, but not tdt-$\gamma_2$, as indicated by arrows at corresponding locations. (Top) EGFP-GluA2. (Middle) tdt-$\gamma_2$. (Bottom) Overlay of top and middle panels. (Scale bar: 2.5 $\mu$m.) The kymographs show the trafficking of the vesicle along its trajectory for EGFP-GluA2, tdt-$\gamma_2$ and overlaid signal. (B) Time series of a post-Golgi trafficking vesicle containing only EGFP-$\gamma_2$, but not tdt-GluA2. (C) Quantification of cotrafficking events of EGFP- and tdt-tagged receptors after exit the Golgi apparatus. Asterisks indicate statistical significances.

**Fig. 7.** Endogenous AMPA and GABA$\gamma_2$ receptors are sorted into different vesicles. (A) Subcellular fractionation of adult rat brain. Each fraction was normalized based on protein concentration. H, whole brain homogenate; P1, cell debris and nuclei; P2, washed synaptosomal fraction; P3, microsomal pellet; S1, postnuclear supernatant; S2, postsynaptosomal fraction; S3, soluble protein fraction. (B and C) Morphology of vesicles in P3 fraction under EM. (Scale bars: B, 500 nm; C, 100 nm.) (D) Double-immunogold EM of GluA2 and $\gamma_2$ in P3 sections. GluA2 and $\gamma_2$ were labeled by 6-nm (arrows) and 12-nm (arrow heads) immunogold beads, respectively. (Scale bar: 100 nm.) (E) Quantification of vesicles containing GluA2 or $\gamma_2$ observed under double-immunogold EM. Green: GluA2-only vesicles (37%) of all vesicles. Magenta: $\gamma_2$-only vesicles (51%). Purple: GluA2 and $\gamma_2$-containing vesicles (12%). n = 73. (F) Vesicular sorting model for constitutive exocytosis of AMPA and GABA$\gamma_2$ receptors.
Moreover, it is surprising that AMPA and GABA_{A} receptors are delivered into distinct domains of the somatic membrane. Our data suggest the vesicles targeted at the central and peripheral regions of the soma originate from endocytic pathways and de novo exocytic pathways, respectively. This phenomenon indicates the presence of specialized zones on the plasma membrane for different exocytic pathways. Why would neurons deliver AMPA and GABA_{A} receptors to different locations and through different pathways on the cell soma? In hippocampal neurons inhibitory synapses are often localized on proximal dendrites and the soma, whereas excitatory synapses are distributed both at proximal and distal dendrites (3, 49). The direct exocytosis of GABA_{A} receptors to the peripheral somatic membrane would place the receptors near the location of inhibitory synapses. However, many AMPA receptors have to travel long distances to reach excitatory synapses on distal dendrites. The high level of constitutive exocytosis of AMPA receptors in the cell soma suggests that lateral diffusion of AMPA receptors from the somatic cell surface to proximal and possibly distal dendrites may play a significant role in maintaining surface and synaptic AMPA receptors. Consistent with this interpretation, a previous study had suggested that endogenous AMPA receptors are mostly exocytosed and recycled at extrasynaptic somatic sites (50). In addition, AMPA receptors may also be delivered through other trafficking pathways. For example, the transport of AMPA receptor-containing vesicles along microtubules certainly delivers AMPA receptors out to distal dendrites for local exocytosis into the extrasynaptic dendritic plasma membrane. It is possible that AMPA receptor containing recycling vesicles preferentially travel along microtubules assisting in the peripheral delivery of the receptors. Moreover, local translation of AMPA receptors subunits in dendrites will also likely play a role in the delivery of AMPA receptors to distal dendrites (1).

The constitutive exocytotic events characterized here are distinct from previously reported activity-dependent AMPA receptors exocytic events from our laboratory and others (14, 15, 22). Those events for GluA1 and GluA2 homomers and heteromers are brighter and occur much less frequently and have slower kinetics (14, 15). The brighter, long-lasting GluA2 events are moderately regulated by neuronal activity and require the binding of NSF and RNA editing of Q/R site in GluA2 (15). The GluA1 events are significantly regulated by neuronal activity, as well as the binding of NSF and RNA editing of Q/R site in GluA1 (11, 14, 22). The brighter and slower events of pHluorin-GluA1 contain around 50 receptor subunits (22). In contrast, we discovered constitutive exocytic events of GluA2 and y2, which transiently occur at higher frequency and contain fewer than 10 receptor subunits per vesicle. These observations together suggest that activity-dependent and constitutive exocytic events originate from different vesicle populations with distinct properties. However, these two types of exocytosis share a common feature, which is that they both target extrasynaptic sites on the somatic membrane and dendritic shafts. Following the initial extrasynaptic exocytosis, the specialized synaptic clustering of AMPA and GABA_{A} receptors is finally achieved by lateral diffusion of receptors from extrasynaptic pools to the synaptic membrane and stabilization of the receptors on specific postsynaptic membranes by scaffolding proteins (51).

The exocytic events of GluA1 have been observed in spines when neuronal activity is stimulated (11, 52) but we and others have rarely observed spine exocytosis even in active neuronal cultures (10, 14, 22). The roles of SNARE complexes on constitutive trafficking or basal surface level of GABA_{A} and AMPA receptors have been reported in many studies. However, the results are not fully consistent. In terms of GABA_{A} receptor, slices from SNAP25 null animals showed an up-regulation of postsynaptic surface GABA_{A} receptors (8), suggesting that SNAP25 is not necessary in GABA_{A} receptor exocytosis and is in agreement with our results. Conversely, the surface and total levels of GABA_{A} receptor α1 subunit did not change in SNAP23^{-/-} neurons (13). However, knockdown of SNAP23 by lentiviral-mediated shRNA expression only induced a modest reduction of surface AMPA receptors and no significant change of surface AMPA receptor levels was detected in SNAP23^{-/-} mice (13), supporting our conclusion that SNAP23 is not required for AMPA receptor exocytosis. However, AMPA receptor surface level was not affected by knockdown of SNAP25 expression with lentiviral-mediated shRNA (13). No postsynaptic defects were detected after application of glutamate agonists in SNAP25-deficient neurons (53). In addition, it has been shown that Botox B rapidly reduced the amplitude of basal AMPA receptor-mediated EPSCs (7) and VAMP2 is required for constitutive delivery of AMPA receptors to the plasma membrane (16), consistent with our observation. In contrast, it has been shown that Botox B application had no effect on basal excitatory synaptic transmission (5). Tetanus toxin, which also cleaves VAMP2 and other Botox B-sensitive VAMPs (19), did not change amplitude of basal AMPA mEPSCs (6). What could be responsible for these contradictory results? First, most previous studies have not directly measured exocytic events. The surface receptor levels or synaptic current amplitude reflect the effects of multiple trafficking steps, including receptor exocytosis, endocytosis, lateral diffusion, and stabilization. So, it is critical to investigate roles of a certain molecule while isolating a particular trafficking event, as we have done here using TIRFM to specifically isolate exocytosis. Second, perturbation of trafficking events by genetic ablation and lentiviral-mediated knockdown of particular genes could induce compensatory expression of other mechanistically related proteins. For this reason, we have used acute neurotoxin treatments and short-term shRNA-mediated knockdown to complement each other. Third, it has been shown that surface levels of postsynaptic receptors, especially AMPA receptors, are regulated by long-lasting homeostatic changes in global neuronal activity, so called “synaptic scaling” (54). SNARE complexes are critical for presynaptic neurotransmitter release (55), and knockdown of particular SNARE components by genetic or virus-based shRNA approaches could possibly modulate neuronal activity in the whole preparation and indirectly affect postsynaptic receptors. Therefore, disruption of SNARE proteins at the single-cell level by sparse transfection of shRNAs, as we have done here, is more reliable when studying SNARE function in postsynaptic receptor trafficking to demonstrate that effects are cell-autonomous and independent of network activity.

In summary, by directly studying the constitutive exocytosis of AMPA and GABA_{A} receptors, we found that the segregation of AMPA and GABA_{A} receptors occurs early during intracellular vesicle trafficking. AMPA or GABA_{A} receptor-containing vesicles are sorted in the Golgi and exit via distinct exocytic vesicles. AMPA receptors are highly targeted to recycling pathways, whereas GABA_{A} receptors are not. Moreover, these distinct exocytic events occur in different regions of the cell surface. AMPA and GABA_{A} receptor exocytic events share certain properties but are also distinct in several aspects and are differentially regulated by specific SNARE complexes and Rab proteins. These results demonstrate the neuron's capacity to elaborately sort different postsynaptic receptor complexes to regulate excitatory and inhibitory transmission.

Methods

Animal Use. All animal experiments were performed with approval by the Animal Care and Use Committee at Johns Hopkins University School of Medicine.

Fusion Constructs. pHluorin-, EGFP-, and tdTomato-GluA2 were constructed in pcDNA3.1 hygro- vector by inserting the fluorescent protein into GluA2 between the photoactivatable GTPase (51). The pHluorin-, EGFP-, and tdTomato-GluA2 were constructed in pcDNA3.1 hygro-vector by inserting the photoactivatable GTPase into GluA2 between the photoactivatable GTPase and palmitoylation of GluA1 (14). These brighter and slower events of pHluorin-GluA1 contain around 50 receptor subunits (22). In contrast, we discovered constitutive exocytic events of GluA2 and y2, which transiently occur at higher frequency and contain fewer than 10 receptor subunits per vesicle. These observations together suggest that activity-dependent and constitutive exocytic events originate from different vesicle populations with distinct properties. However, these two types of exocytosis share a common feature, which is that they both target extrasynaptic sites on the somatic membrane and dendritic shafts. Following the initial extrasynaptic exocytosis, the specialized synaptic clustering of AMPA and GABA_{A} receptors is finally achieved by lateral diffusion of receptors from extrasynaptic pools to the synaptic membrane and stabilization of the receptors on specific postsynaptic membranes by scaffolding proteins (51).

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