POSTSYNAPTIC EXOCYTOSIS, ENDOCYTOSIS AND RECYCLING: MECHANISMS, REGULATION AND PHYSIOLOGICAL RELEVANCE IN SYNAPTIC FUNCTION AND PLASTICITY

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REVIEW
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γ2 $GABA_AR$ Trafficking and the Consequences of Human Genetic Variation

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GABA type A receptors ($GABA_ARs$) mediate the majority of fast inhibitory neurotransmission in the central nervous system (CNS). Most prevalent as heteropentamers composed of two α, two β, and a γ2 subunit, these ligand–gated ionotropic chloride channels are capable of extensive genetic diversity (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3). Part of this selective $GABA_AR$ assembly arises from the critical role for γ2 in maintaining synaptic receptor localization and function. Accordingly, mutations in this subunit account for over half of the known epilepsy-associated genetic anomalies identified in $GABA_ARs$. Fundamental structure–function studies and cellular pathology investigations have revealed dynamic $GABA_AR$ trafficking and synaptic scaffolding as critical regulators of GABAergic inhibition. Here, we introduce *in vitro* and *in vivo* findings regarding the specific role of the γ2 subunit in receptor trafficking. We then examine γ2 subunit human genetic variation and assess disease related phenotypes and the potential role of altered $GABA_AR$ trafficking. Finally, we discuss new-age imaging techniques and their potential to provide novel insight into critical regulatory mechanisms of $GABA_AR$ function.

Keywords: $GABA_A$ receptor, trafficking, genetic variation, human, epilepsy, imaging

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INTRODUCTION

The adult central nervous system (CNS) is critically dependent on fast inhibitory neurotransmission evoked by $GABA_A$ receptors ($GABA_ARs$). $GABA_ARs$ are ligand-gated ionotropic chloride (Cl^-) channels ubiquitously expressed throughout the CNS that play a fundamental role in restraining and sculpting neuronal activity. Disruptions in $GABA_AR$ dependent neurotransmission leads to insufficient inhibitory effects throughout the brain, contributing to the pathogenesis of epilepsy, neurodevelopmental disorders, depression, schizophrenia and stroke (Hines et al., 2012). Activation of $GABA_ARs$ by the neurotransmitter GABA induces ion channel opening, Cl^- influx, and subsequent membrane hyperpolarization. These heteropentameric structures are predominantly composed of two α (α1-6), two β (β1-3), and either a γ (γ1-3) or a δ subunit (Olsen and Sieghart, 2009) (**Figures 1A,B**). $GABA_ARs$ belong to the Cys-loop superfamily of pentameric ligand-gated ion channels (pLGICs) including strychnine-sensitive glycine receptors, nicotinic acetylcholine (nACh) receptors, and 5-hydroxytryptamine type-3 (5-HT3) receptors. Individual subunits have a common structure consisting of a large N-terminus extracellular domain (ECD) that participates in endogenous ligand binding, a transmembrane domain (TM) comprised of four α-helical regions (M1-4) and a

barely extruding extracellular C-terminus. The M2 region of the subunits forms the ion channel pore. The hydrophobic M regions are connected by a small intracellular loop between M1-M2 and a much larger intracellular domain (ICD; previously termed intracellular loop) between M3 and M4 (Sigel and Steinmann, 2012) that mediates interactions with intracellular proteins critical for receptor trafficking and synaptic clustering (**Figure 1C**). Recently, GABA$_A$R structures for the human β3 homopentamer bound to benzamidine (Miller and Aricescu, 2014), chimeric α5TM/β3ECD bound to the neurosteroid allopregnanolone (Miller et al., 2017), and human α1β2γ2 heteropentamer bound to GABA and the benzodiazepine site antagonist Flumazenil (Zhu et al., 2018) were resolved, advancing our growing understanding of GABA$_A$R molecular architecture. Importantly, nearly all pLGIC family structural data lacks the large ICD (Nemecz et al., 2016) (exception 5-HT3 receptor; Hassaine et al., 2014), leaving functionally relevant information about this region left undiscovered.

Presynaptic terminal release of GABA onto postsynaptically clustered GABA$_A$Rs initiates fast, transient receptor activation. In contrast, activation of extrasynaptic GABA$_A$Rs by ambient "spill over" GABA generates a persistent tonic current (**Figure 1D**). Most GABA$_A$Rs evoking fast synaptic inhibition in the mature cortex contain α1β2γ2 subunits, although α/β content can vary widely (Olsen and Sieghart, 2009), prompting a unifying role of γ2 in synaptic function. Importantly, the benzodiazepine drug class selectively binds between the interface of a γ2 subunit and either an α1/2/3/5 subunit to potentiate GABA$_A$R function and elicit behavioral effects including sedative/hypnotic, anti-convulsant, myorelaxant, and/or anti-anxiety effects (Vinkers and Olivier, 2012) (**Figures 1A,B**). Here we summarize (1) known molecular interactors and mechanisms regulating γ2 trafficking (2) the importance of this subunit physiologically and human γ2 genetic variants compromising structure and function *in vitro* and *in vivo* and (3) application of modern imaging techniques to discover novel insight into synaptic GABA$_A$R modulation.

γ2 SUBUNIT TRAFFICKING AND INTERACTORS

Biosynthetic Trafficking and Insertion

During biosynthesis, GABA$_A$R subunits are first assembled in the endoplasmic reticulum (ER) and then transported to the Golgi apparatus (Golgi) for further maturation (**Figure 2**). Forward trafficking of γ2-GABA$_A$Rs from the ER is negatively regulated by Cleft lip and palate transmembrane protein (CLPTM1) *in vitro* and *in vivo* (**Figure 2**) (Ge et al., 2018). Overexpressing CLPTM1 reduces surface and synaptic levels of γ2, resulting in reduced amplitude and frequency of inhibitory postsynaptic current (IPSC), where the opposite effect is seen by CLPTM1 knockdown (KD). Importantly, CLPTM1 also regulates tonic inhibition and interacts with the extrasynaptic subunits α4 and δ, suggesting this protein non-selectively binds many GABA$_A$R subtypes. Upon entry into the Golgi, the γ2 subunit undergoes palmitoylation via the Golgi-specific DHHC zinc finger enzyme (GODZ; also known as ZDHHC3) (Keller et al., 2004; Fang et al., 2006). This process is key for receptor clustering, innervation, and inhibitory strength *in vitro* and *in vivo* (Keller et al., 2004; Fang et al., 2006; Kilpatrick et al., 2016). GABA$_A$R forward trafficking to the cell surface depends on the microtubule-dependent molecular motor kinesins (KIFs) (**Figure 2**). The KIF21B protein co-precipitates with the GABA$_A$R γ2 subunit (Labonte et al., 2014). RNA KD of KIF21B reduces receptor surface levels and the intensity of extrasynaptic γ2 clusters, but does not affect synaptic GABA$_A$Rs levels. Additionally, the KIF5 family plays a critical role in trans-Golgi to surface GABA$_A$R trafficking (Twelvetrees et al., 2010). Conditional knockout (KO) of KIF5A in mice results in deficits of GABA$_A$R plasma membrane levels, epilepsy phenotypes, and high lethality rate within 21 days postnatal (Nakajima et al., 2012).

Notably, KIF5A (not KIF5B, KIF5C) selectively interacts with the GABA$_A$R-associated protein (GABARAP) *in vivo* (Nakajima et al., 2012). The well-characterized GABARAP (**Figure 2**) is part of the ubiquitin-like protein (UBL) family implicated in numerous cellular processes (van der Veen and Ploegh, 2012). GABARAP interacts with GABA$_A$R γ subunits and microtubules, is heavily localized at the Golgi apparatus and cell surface (Wang et al., 1999), and overexpression augments GABA$_A$R plasma membrane levels (Leil et al., 2004). However, GABARAP KO mice have unhindered distribution of γ2-GABA$_A$Rs and gephyrin, suggesting functional redundancy with other trafficking proteins (O'Sullivan et al., 2005). Some evidence suggests GABARAP preferentially associates with serine phosphorylated γ2-GABA$_A$Rs, while dephosphorylation by protein phosphatase 1 (PP1) decreases this interaction (Qian et al., 2011).

A number of GABARAP interacting proteins mediate GABA$_A$R trafficking or localization (**Figure 2**). For instance, increased association with the PDZ domain-containing protein GRIP is seemingly involved in NMDA receptor-dependent GABA$_A$R synaptic plasticity (Marsden et al., 2007). The phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2) and the *N*-ethylmaleimide-sensitive factor ATPase (NSF) interact with GABA$_A$Rs both indirectly via GABARAP and directly with β subunits (**Figure 2**) (Kanematsu et al., 2002; Terunuma et al., 2004; Goto et al., 2005; Mizokami et al., 2007). NSF is a key component of SNARE-mediated fusion and is involved in receptor cell surface transit (Chou et al., 2010). Notably, the γ2 subunit and PRIP share an overlapping binding site on GABARAP (Kanematsu et al., 2002). PRIP1/2 KO mice demonstrate diminished benzodiazepine sensitivity and Zn^{2+} modulation concurrent with lower plasma membrane GABA$_A$R expression, consistent with impaired γ2 subunit trafficking. KO of PRIP-1, the primary brain subtype, leads to mice displaying an epileptic phenotype that can be successfully suppressed by diazepam (DZP), but interictal discharges persist (Zhu et al., 2012). Interestingly, DZP potentiation of miniature inhibitory postsynaptic currents (mIPSC) remains unchanged, but baseline and DZP potentiated tonic GABA current amplitude in PRIP-1 KO neurons was reduced. PRIP-1

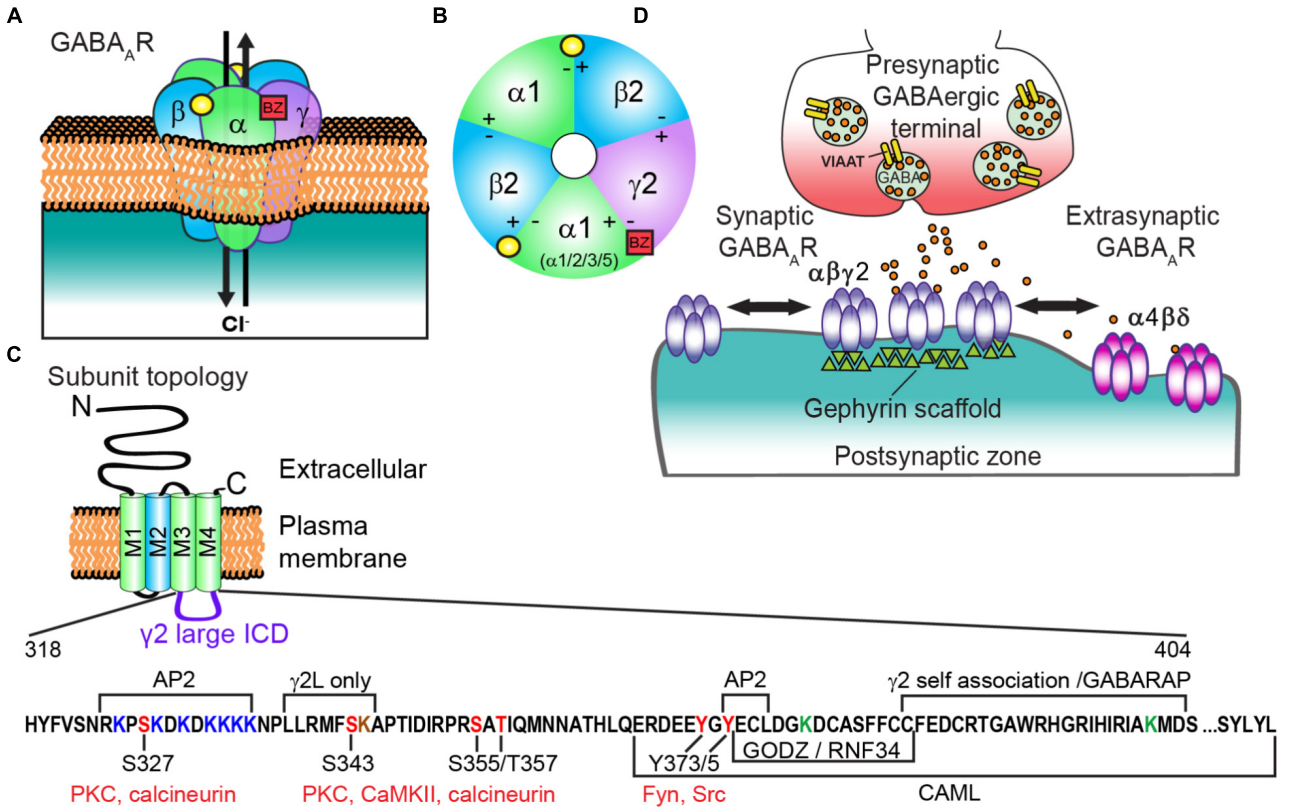

FIGURE 1 | Generic GABA$_A$R structure and subunit topology; and regulatory sites of the γ2 intracellular domain (ICD). **(A)** GABA$_A$R heteropentamer composed of αβγ subunits. Binding of the neurotransmitter GABA (yellow circle) at the αβ interface triggers ion channel opening and allows the rapid influx of Cl^- and membrane hyperpolarization in the mature nervous system. **(B)** Extracellular representation of the most prevalent cortical receptor subtype composed of α1β2γ2 subunits showing all five subunits contributing to the central ion pore and the general binding sites of GABA (yellow circle) and benzodiazepines (BZs) (red square). BZs bind at the interface of an α1/2/3/5 and γ subunit. **(C)** All subunits have a common topology including an extracellular N-terminal domain (ECD), short C-terminal tail, and four transmembrane regions (M1-4) which compose the transmembrane domain (TM). M2 (blue) contributes to formation of the receptor ion channel pore, while the ICD between M3 and M4 contains sites of phosphorylation and protein interactions that modulate channel function and/or trafficking. The γ2 L isoform intracellular domain (ICD = AA 318-404, residue numbering does not include signal peptide) is shown here with identified regulatory sites and regions of protein interaction. Seven lysine residues (red) contribute to γ2-containing GABA$_A$R ubiquitination and endo-lysosomal targeting in HEK cells, with mutation of three additional lysine residues needed to block receptor downregulation by E3 ligase RNF34 overexpression (ICD green Ks and K259 in smaller M1-M2 loop not shown in diagram). Note the γ2L specific K344 residue (brown) has not been tested in ubiquitination studies **(D)** GABA$_A$Rs composed of α(1-3)βγ subunits are largely synaptically localized via gephyrin interactions and contribute to phasic currents, whereas α(4 or 6)βδ receptors are extrasynaptic and generate tonic current.

KO and PRIP1/2 double KO mice show anxiety-related behaviors and abnormal locomotion related to GABA$_A$R dysfunction and reduced benzodiazepine sensitivity. Recently the Rho GTPase Activating Protein 32 (ARHGAP32) isoform 1 (PX-RICS) was shown to form an adaptor complex with GABARAP and the scaffold proteins 14-3-3ζ/θ to facilitate γ2-GABA$_A$Rs forward trafficking via dynein/dynactin and promote surface expression (Nakamura T. et al., 2016). KO of *PX-RICS* in mice generates an Autism Spectrum Disorder (ASD) phenotype with increased susceptibility to kainate-induced epileptic seizures, decreased GABA$_A$R plasma membrane levels, and lowered mIPSC amplitude. Transgenic overexpression of 14-3-3ζ in mice protects against neuronal death caused by prolonged seizures (Brennan et al., 2013). In contrast, 14-3-3ζ mutations or deletions have been identified in patients with pathology associated with GABA$_A$R deficits including schizophrenia, autism and generalized epilepsy (Tenney et al., 2011; Fromer et al., 2014; Toma et al., 2014).

Synaptic Accumulation and Functional Regulation

Following insertion at the plasma membrane, γ2-GABA$_A$Rs undergo Brownian diffusion until interaction with the inhibitory postsynaptic scaffolding protein gephyrin causes constraint and accumulation (**Figures 1D**, **2**). Specifically, GABA$_A$R α1/2/3/5 and β2/3 subunits (at lower affinity) mediate gephyrin-receptor binding (Tretter et al., 2008, 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013; Brady and Jacob, 2015). While no direct interaction between γ2 and gephyrin has been identified, the synaptic levels of these proteins are intimately tied, shown by KO studies of gephyrin (Kneussel et al., 1999) and γ2 (Schweizer et al., 2003). Interestingly, chimeric studies indicate

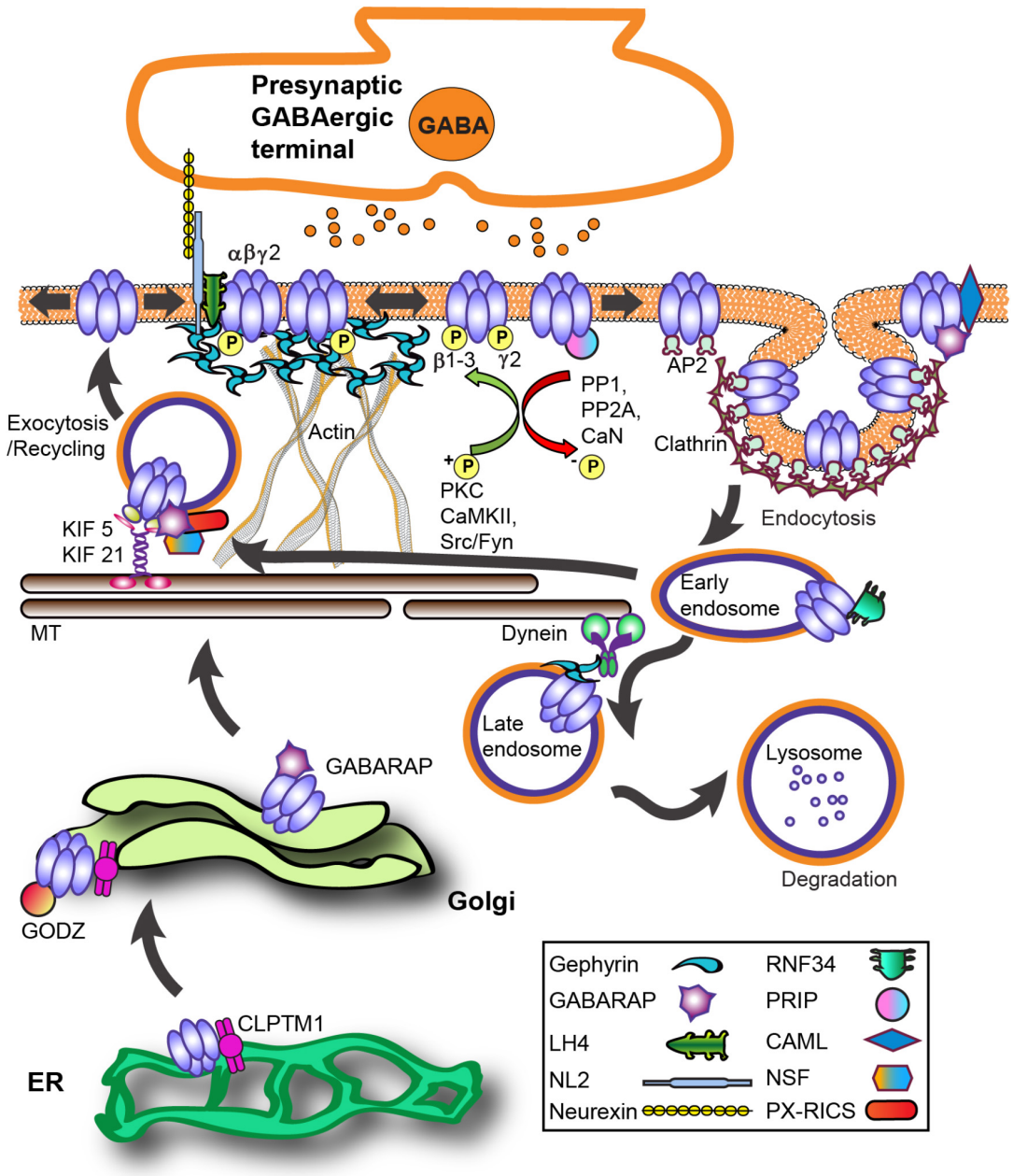

FIGURE 2 | GABA$_A$R trafficking and key interacting proteins at GABAergic synapses. The process of GABA$_A$R synthesis, assembly and forward trafficking is highly regulated. Forward trafficking of γ2-GABA$_A$Rs from the ER is negatively regulated by CLPTM1. Subunits are assembled into pentameric receptors in the endoplasmic reticulum (ER) where proper folding allows receptors to avoid proteosomal degradation and exit to the Golgi. In the Golgi, palmitoylation of γ subunits by the palmitoyltransferase GODZ is a key step in promoting forward trafficking to the synapse. GABARAP interacts with γ subunits and microtubules and overexpression augments receptor plasma membrane levels. PX-RICS forms an adaptor complex with GABARAP to facilitate γ2-GABA$_A$Rs forward trafficking. PRIP1/2 and NSF interact with GABA$_A$Rs both indirectly via GABARAP and directly with β subunits. The kinesin KIF5 is the main microtubule (MT)-dependent motor transporting inhibitory synapse components although recent work shows KIF21 contributes to extrasynaptic receptor delivery. LH4 forms a complex between γ2 and NL2. NL2 is central in GABA$_A$R synapse development via its trans-synaptic association with axonal neurexins and also binds gephyrin. GABA$_A$Rs primarily undergo clathrin-dependent endocytosis via β and γ subunit interactions with the clathrin-adaptor protein 2 (AP2) complex. Phosphorylation of AP2-interaction motifs within receptor subunits increases cell-surface receptor levels and enhances GABA$_A$R neurotransmission by reducing AP2 binding to receptors. After internalization, clathrin-coated vesicles fuse with early endosomes, allowing for subsequent receptor recycling or targeting for degradation in lysosomes. CAML interaction with the γ2 subunit promotes forward trafficking and recycling. Ubiquitination of GABA$_A$R contributes to lysosomal targeting, with the ubiquitin E3 ligase RNF34 directly interacting with the γ2 subunit. Protein abbreviations: CAML (calcium-modulating cyclophilin ligand), CLPTM1 (Cleft lip and palate transmembrane protein), GABARAP (GABAAR - associated protein), GODZ (Golgi-specific DHHC zinc finger enzyme), KIF 5/21 (microtubule-dependent molecular motor kinesins), LH4 (lipoma HMGIC fusion partner-like protein 4), NL2 (neuroligin 2), NSF (*N*-ethylmaleimide-sensitive factor ATPase), PRIP (phospholipase C-related catalytically inactive proteins), PX-RICS [Rho GTPase Activating Protein 32 (ARHGAP32) isoform 1], RNF34 (ring finger protein 34 E3 ligase).

the γ2 M4 is sufficient to cause GABA$_A$R accumulation opposite GABAergic terminals, while the large ICD of γ2 is necessary for gephyrin recruitment and rescue of synaptic function in γ2 KO cultured neurons (Alldred et al., 2005). It is likely that an indirect interaction occurs between γ2 and gephyrin across a bridge of other key synaptic proteins. Recently, six unrelated patients were identified with microdeletions in the gephyrin gene resulting in a range of neurodevelopmental deficits including ASD, schizophrenia or epilepsy (Lionel et al., 2013). The recently discovered GABA$_A$R regulatory Lhfpl (GARLH) family proteins lipoma HMGIC fusion partner-like 3 and 4 (LH3 and LH4) forms a native complex between γ2 and the transsynaptic protein neuroligin 2 (NL2) (**Figure 2**) (Yamasaki et al., 2017). NL2 is central in GABA$_A$R synapse development via its trans-synaptic association with axonal neurexins (Sudhof, 2008). Diminishing LH4 levels in culture and *in vivo* dramatically reduced γ2-GABA$_A$R and gephyrin synaptic clustering and inhibitory strength (Davenport et al., 2017; Yamasaki et al., 2017). Curiously, despite the dramatic reduction in synaptic inhibition, epilepsy susceptibility or overt behavioral phenotypes in these mice have yet to be reported in the constitutive LH4 KO mouse. Importantly, gephyrin is known to directly bind the intracellular domain of NL2 (Poulopoulos et al., 2009). Thus γ2 subunit-LH4-NL2-gephyrin interactions could provide a molecular framework to support γ2's role in GABA$_A$R synaptic recruitment and maintenance.

Synaptic plasticity, or the dynamic modulation of synaptic output, is heavily influenced by receptor phosphorylation via altering channel function or receptor trafficking. Phosphoregulation of γ2 S327 is an important mediator of GABA$_A$R retention at synapses. Detailed electrophysiology and *in vivo* studies have identified the PKCε isoform specifically phosphorylates the γ2 S327 residue (**Figures 1C**, **2**), ultimately fine-tuning responsiveness to ethanol and benzodiazepines (Qi et al., 2007). Additionally, protocols that induce calcium-entry via glutamate application, strong NMDA receptor activation, or robust neuronal activity enhance receptor lateral mobility, decrease synaptic cluster size, and reduce mIPSC amplitude via the phosphatase calcineurin (CaN) (Bannai et al., 2009) and dephosphorylation of the γ2 subunit S327 residue (**Figures 1C**, **2**) (Muir et al., 2010). More broadly, activation of all PKC isoforms by 1 h PMA (PKC activator; 30 nM) treatment decreases surface γ2-GABA$_A$R levels that can be reversed by specific inhibition of PKCε catalytic activity in HEK cells and PKCε specific activation reduces GABA$_A$R current amplitude (Chou et al., 2010). This effect was in part attributed to changes in GABA$_A$R trafficking occurring though PKCε association and phosphorylation of NSF. The scaffolding protein 14-3-3-θ acts as a bridge for the PKCγ isoform to interact with γ2 in cerebellar Purkinje neurons and N2a cells (Qian et al., 2012). 14-3-3-θ KD in mice by siRNA microinjection reduces γ2-GABA$_A$R overall serine phosphorylation, while KD of 14-3-3-θ or PKCγ reverses the PMA (200 nM, 30 min) induced upregulation of C cell surface expression in N2a cells. These apparently conflicting reports on PKC kinase family modulation highlights the complexity of this signaling pathway in γ2-GABA$_A$R regulation, with varied effects dependent on the pharmacological agents used, treatment times, model, and PKC isoforms.

An important consideration for γ2 subunit regulation is its presence in a short (γ2S) or long (γ2L) isoform; the γ2L isoform has 8 additional amino acids (LLRMFSFK) in the large ICD with the serine site (S343) capable of being phosphorylated by Protein kinase C (PKC) and Calcium/calmodulin-dependent protein kinase type II (CaMKII) (**Figure 1C**) (Whiting et al., 1990; Moss et al., 1992; McDonald and Moss, 1994). Expression levels of γ2S remain constant throughout development, while γ2L levels increase during neuronal maturation (Wang and Burt, 1991). Early *in vitro* expression studies found that the additional amino acids in the γ2L subunit may play a role in the response to diazepam and be critical for ethanol enhancement of GABA current (Wafford et al., 1991). Both mutation of S343 to a phosphomimetic aspartate or to non-phosphorylatable valine resulted in cell surface trafficking of γ2L when expressed alone, similar to γ2S (Boileau et al., 2010). This work also proposed an accessory protein role for γ2S as an external modulator of GABA$_A$R function to confer zinc blockade protection for receptors. When comparing synaptic clustering of γ2L vs. γ2S subunit large ICD (partial subunit chimeras) in spinal cord neurons, postsynaptic γ2L ICD chimera accumulation is higher, and can be enhanced by PKC activation by phorbol ester phorbol-12,13-dibutyrate (PDBu) and reversed by mutating the S343 residue of γ2L (Meier and Grantyn, 2004). The physiological role of CaMKII direct phosphorylation on γ2 has not yet been described, although CaMKII is required for a type of inhibitory long term potentiation (iLTP) in Purkinje neurons known as rebound potentiation (Kano et al., 1996) and increased association between the γ2 subunit and GABARAP (Kawaguchi and Hirano, 2007). CaMKII plays other critical roles in GABAergic plasticity including promoting receptor surface levels (Wang et al., 1995; Marsden et al., 2007, 2010; Saliba et al., 2012) and recruitment of the synaptic scaffold protein gephyrin, while reducing GABA$_A$R lateral diffusion (Petrini et al., 2014).

Internalization

Non-synaptic GABA$_A$Rs on the cell surface are capable of undergoing internalization (Bogdanov et al., 2006), a fundamental cellular process that regulates receptor signaling and function (**Figure 2**). GABA$_A$R internalization is primarily clathrin-mediated in concert with GTPase dynamin activity and the adaptor protein AP2 complex (Kittler et al., 2000), although clathrin-independent GABA$_A$R endocytosis has been described (Cinar and Barnes, 2001; Rowland et al., 2006). AP2 interacts with the ICD of GABA$_A$R β subunits and the extrasynaptic δ subunit in a phospho-dependent manner (McDonald et al., 1998; Brandon et al., 2002, 2003; Herring et al., 2005; Kittler et al., 2005; Smith et al., 2008; Gonzalez et al., 2012; Smith et al., 2012). The γ2 subunit also contains two AP2 interaction domains on its ICD, a 12 basic amino acid region and a classical YGYECL motif (Smith et al., 2008) (**Figure 1C**). Phosphorylation at Y365/367 residues within the YGYECL motif by the non-receptor tyrosine-protein kinases

Fyn and Src family kinases (Moss et al., 1995; Brandon et al., 2001; Jurd et al., 2010) reduces AP2 binding, as does mutation of Y365/7 to phenylalanine (Kittler et al., 2008; Tretter et al., 2009). Homozygous tyrosine to phenylalanine (Y365/7F) knock-in mice are developmentally lethal, suggesting phosphoregulation of these residues is critical for GABA$_A$R function or trafficking *in vivo*. Heterozygous Y365/7F knock-in mutant mice show inhibition of AP2 binding to the γ2 subunit, surface and synaptic accumulation of receptors and ultimately spatial memory deficits (Tretter et al., 2009). Further investigation revealed that brain-derived neurotrophic factor (BDNF) enhances Y365/7 phosphorylation and stabilizes γ2-containing GABA$_A$R, consistent with heterozygous Y365/7F mice showing an anti-depressant phenotype in the forced swim task and tail-suspension test and increased neurogenesis effects that are resistant to further enhancement by BDNF (Vithlani et al., 2013).

GABA$_A$R endocytosis can be increased by stimuli of opposite polarities, either excitotoxic protocols such as *in vitro* seizure (Goodkin et al., 2005, 2008; Naylor et al., 2005; Lorenz-Guertin et al., 2017) and oxygen-glucose deprivation (OGD) (Arancibia-Carcamo et al., 2009), or by prolonged inhibition with agonist exposure (Chaumont et al., 2013; Gutierrez et al., 2014). Internalization is in part regulated by phosphatase activity under these conditions. For example, inhibition of CaN or the serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A) reverses a status epilepticus induced decrease in surface γ2-GABA$_A$Rs and mIPSC amplitude (Joshi et al., 2015). Importantly, genetic GABA$_A$R mutants also affect intracellular trafficking. For instance, the γ2 R82Q (numbering without signal peptide R43Q) mutation linked to childhood absence epilepsy and febrile seizures (FS) showed increased basal receptor endocytosis rates relative to wild-type (Chaumont et al., 2013). In summary, endogenous signaling pathways, pharmacological treatments, and pathological stimuli or genetic variation can modulate GABA$_A$R endocytosis networks [kinase and phosphatase regulation reviewed in Lorenz-Guertin and Jacob (2017)].

Recycling/Lysosomal Degradation

Internalized GABA$_A$Rs can either be recycled back to the cell surface or targeted for degradation at lysosomes (**Figure 2**) (Kittler et al., 2004; Arancibia-Carcamo et al., 2009). Interaction of the integral membrane protein calcium-modulating cyclophilin ligand (CAML) with the γ2 subunit cytoplasmic and fourth transmembrane domain regions promotes forward trafficking and recycling (Yuan et al., 2008). Neurons lacking CAML demonstrate diminished recycling of endocytosed GABA$_A$Rs and decreased inhibitory strength. Broad PKC activity is implicated as a negative regulator of GABA$_A$R recycling activity following internalization (Connolly et al., 1999). 5-HT2 serotonergic negative modulation of GABA$_A$R currents is also thought to occur through a PKC-RACK1 (receptor for activated C kinase) mechanism (Feng et al., 2001).

Synaptic receptors destined for degradation undergo ubiquitination of 7 lysine residues within the ICD of the γ2 subunit (**Figure 1C**) (Arancibia-Carcamo et al., 2009). Lysine to arginine (K7R) mutation at these ubiquitination sites diminishes late endosome targeting of receptors in heterologous cells, and reverses loss of surface receptor clusters following OGD treatment (Arancibia-Carcamo et al., 2009). The ring finger protein 34 (RNF34) E3 ligase directly binds the γ2 ICD, co-immunoprecipitates with γ2 *in vivo* and can be identified at inhibitory synapses (**Figure 2**) (Jin et al., 2014). Interestingly, the short 14 amino acid motif in the γ2 ICD sufficient for RNF34 binding is identical to the GODZ binding region (**Figure 1C**), and is also highly conserved among the γ subunits. γ2-GABA$_A$R degradation is accelerated upon overexpression of RNF34 resulting in smaller GABA$_A$R synaptic clusters and diminished inhibitory current strength. Proteosomal and lysosomal inhibitor experiments suggest RNF34 ubiquitination of γ2 contributes to degradation by both of these pathways in HEK cells. Notably, co-expression of RNF34 with the γ2 ubiquitin resistant K7R mutant did not inhibit degradation of this subunit. On the contrary, additional lysine mutations (K8R, K9R, K10R) were able to prevent downregulation of γ2 by RNF34, suggesting these residues may be important for ubiquitination-degradation.

Only a handful of stimuli clearly induce lysosomal degradation of GABA$_A$Rs, likely due to the receptor's crucial role in maintaining neuronal inhibition and the tight regulation of receptor surface levels that must therefore occur. Our lab previously found 24 h benzodiazepine treatment in cultured hippocampal neurons enhances lysosomal-mediated degradation of α2-containing receptors (Jacob et al., 2012). More recently, we identified that a GABA$_A$R antagonist bicuculline acute seizure model also induces lysosomal targeting of surface GABA$_A$Rs in cultured cortical neurons (Lorenz-Guertin et al., 2017). It is likely that stimulus specific subunit ubiquitination patterns ultimately dictate receptor fate. This remains a highly understudied area of research in GABA$_A$R trafficking.

Proteomics

The network of proteins governing inhibitory synapse clustering, trafficking, and plasticity are unresolved, as evidenced by three recent *in vivo* inhibitory synapse proteomic screenings utilizing either knock-in mice expressing GFP-tagged α2 subunit (Nakamura Y. et al., 2016), adeno-associated viral (AAV) expression of fusion proteins including gephyrin (Uezu et al., 2016), or mice expressing a Thy1-His6-Flag-YFP-γ2 subunit transgene (Ge et al., 2018). Initial analysis from these experiments has revealed novel inhibitory protein constituents including the metabotropic glutamate receptor subunit mGluR5, the Dbl family GEF Ephexin, metabotropic GABA B receptor (GABA$_B$R) auxiliary subunit KCTD12, and inhibitory synaptic regulator protein 1 (InSyn1) (Nakamura Y. et al., 2016; Uezu et al., 2016). Most recently, tandem affinity purification proteomics revealed the critical GABA$_A$R forward trafficking component CLPTM1, and two novel interactors including integral membrane protein 2C (ITM2C) and Golgi glycoprotein 1 (GLG1) (Ge et al., 2018). Considering new candidate interactor proteins are identified with slight

derivations in methodology (140 in Uezu et al., 2016; 149 in Nakamura Y. et al., 2016; 39 additional in Ge et al., 2018), future investigations will need to both confirm the validity and importance of these observed proteins in GABA$_A$R function and modulation.

Genetic Knockdown and Knockout of γ2 in Rodents

Due to the fundamental importance of γ2 GABA$_A$R inhibition in the CNS, embryonic KO animals die within days of birth (Gunther et al., 1995). Developmentally delayed KO of γ2 using a CaMKIICre transgene expression system results in mice who are phenotypically normal 3 weeks post-natal, but by week 4 exhibit a rapid decline in health including epileptic episodes and eventually death (Schweizer et al., 2003). A large drop in gephyrin immunoreactivity also occurs coincident with loss of γ2 expression without changing GABAergic presynaptic innervation as measured by vesicular inhibitory amino acid transporter (VIAAT) levels.

Partial KD of brain wide γ2 levels results in impaired behavior including an enhanced anxious-depressive phenotype (Crestani et al., 1999; Chandra et al., 2005; Earnheart et al., 2007; Shen et al., 2010). In addition, heterozygous $\gamma2^{+/-}$ mice show defective spine maturation and synaptogenesis (Ren et al., 2015). Ablating forebrain γ2 expression in embryonic glutamatergic neurons using homozygous EMX1Cre-induced inactivation also recapitulated the depressive-anxiety phenotype and reduced hippocampal neurogenesis similar to total heterozygous γ2 KO mice (Earnheart et al., 2007). In contrast, KD of γ2 in neurons at post-natal day 13/14 did not affect hippocampal neurogenesis, but anxiety- and depressive-like behavior still formed (Shen et al., 2012). Numerous studies have examined brain-region or cell-type specific γ2 KD or KO describing circuit specific roles that will not be discussed here (Buhr et al., 1997; Wingrove et al., 1997; Wulff et al., 2007, 2009; Lee et al., 2010; Leppa et al., 2011, 2016; Zecharia et al., 2012; Stojakovic et al., 2018).

Homozygous deletion of γ2L in mice results in near complete replacement with γ2S subunit (Homanics et al., 1999). When examining γ2 isoform specific ablation, *in vitro* findings (refer to earlier discussion in Synaptic Accumulation and Functional Regulation) would suggest GABA$_A$R incorporating γ2L vs. γ2S would incur distinct changes in functional and pharmacological properties of GABA$_A$R. Yet, this isoform switch did not result in changed responsiveness to ethanol in behavioral or electrophysiology experiments, although a mild increase in anxiety was observed (Homanics et al., 1999). Interestingly, the $\gamma2L^{-/-}$ mice did show a modest increase in behavioral sensitivity and GABA$_A$R affinity for benzodiazepine agonists (Quinlan et al., 2000). Isoform switching of γ2 *in vivo* has been described to occur in response to certain cues such as chronic intermittent ethanol administration in rats (Petrie et al., 2001; Cagetti et al., 2003) and in schizophrenic brains of humans (Huntsman et al., 1998). The relevance of γ2 isoform switching and predominance to pathophysiology *in vivo* remains poorly understood.

HUMAN GENETIC VARIATION OF γ2 AND PATHOLOGICAL IMPLICATIONS

Pathology Arises From γ2 Genetic Anomalies in Humans

Amongst all the subunit genes, mutations in *GABRG2* encoding the γ2 subunit are most commonly linked to epileptogenesis (Macdonald et al., 2012). Indeed, heterozygous γ2 R82Q mutant mice were one of the first *in vivo* models for childhood absence epilepsy, recapitulating a familial mutation phenotype including onset, behavior, and treatment responsiveness (Tan et al., 2007). *GABRG2* genetic anomalies including missense, nonsense, frameshift, splice-site, insertion and deletion mutations are associated with epilepsy phenotypes ranging from mild FS to moderate generalized tonic-clonic seizures or more severe disorders such as Dravet syndrome (DS) or epileptic encephalopathies (further information found in Kang and Macdonald, 2016). In order to bridge the gap between known γ2 trafficking mechanisms, identified protein interaction sites and human pathology, we examined γ2 subunit genetic variation using the Genome Aggregation Database (gnomAD) (Lek et al., 2016), a dataset of exome sequence data from 123,136 individuals and whole genome sequencing from 15,496 unrelated individuals without any severe pediatric disease and their first-degree relatives. We focused specifically on synonymous (codon substitutions result in no amino acid sequence change) and non-synonymous (alter amino acid sequence) mutations. Although synonymous codon changes were previously labeled as "silent" mutations and thought to have limited consequences, recent data indicates these may also impact function and contribute to disease through effects on *cis*-regulatory elements, mRNA structure, and protein expression. Non-synonymous mutations that result in a stop codon are referred to as nonsense mutations whereas missense mutations result in the exchange of one amino acid for another. Non-synonymous mutations may affect structural and functional properties and be associated with a disease condition; however, others may be functionally neutral and not related to a disease phenotype. Protein domains which show significant diversity in mutations identify regions of genetic flexibility, while regions with low allele frequency events (standard threshold of 0.1%) identify potentially pathogenic mutations that are not evolutionarily favored (Dudley et al., 2012). In the γ2S isoform, we identified and plotted the distribution of 104 synonymous and 122 non-synonymous missense variants (**Figure 3A**) (Jay and Brouwer, 2016). Five additional non-synonymous variants were found in the γ2L specific sequence (LLRMFSFK: L377R, R379W, R379Q, F381L, S382C), while no synonymous variants were identified (**Figure 3A**). Of note, there is a third putative γ2 isoform which appears conserved in humans and primates including the great apes and old world monkeys but absent in rodents that was not evaluated here for human genetic variation (ENST00000414552, Y211 is substituted by W, followed by 40 additional amino acids in the N-terminal extracellular domain). Overall, the latter half of the ECD, TM and linker regions showed low levels of missense variation when compared to synonymous variation (**Figure 3A**).

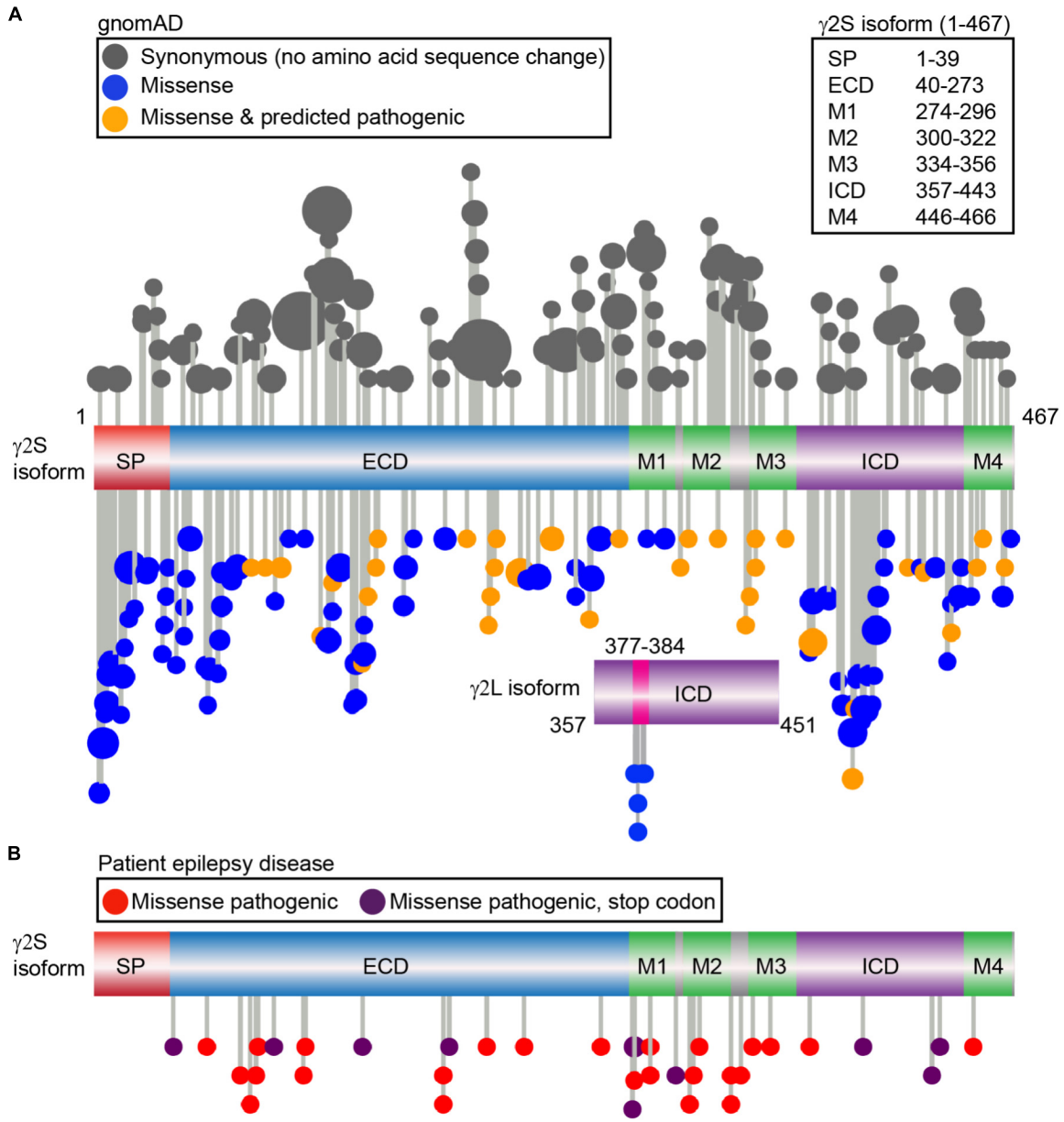

FIGURE 3 | Genetic variation of the γ2 GABA$_A$R subunit in gnomAD vs. genetic epilepsies. **(A)** The gnomAD dataset (individuals without any severe pediatric disease or their first-degree relatives) was used to identify a total of 104 control synonymous variants (gray) and 122 missense non-synonymous variants and plotted; each variant is represented by a lollipop marker that scales with allele frequency. Missense variants were categorized as neutral (blue) or deleterious (orange) through bioinformatics analysis using PROVEAN and SIFT predictions. Linear representation of the γ2 GABA$_A$R subunit with domains: signal peptide (SP; red); extracellular N-terminal region (ECD, blue), transmembrane domain including the four transmembrane helical regions (M1-M4, green); small loops between transmembrane regions (gray); and large intracellular domain between M3-M4 (ICD, purple). The residue numbers correspond to the γ2S sequence (UniProt P18507). The independent ICD below shows the additional residues present in the γ2L isoform (UniProt P18507-2), and 5 distinct missense variants identified, predicted as neutral by PROVEAN: L377R, R379W, R379Q, F381L, S382C. **(B)** Patient epilepsy disease related missense (red) and nonsense variants leading to early stop codons (dark purple) were compiled as described in Material and Methods and plotted on the linear protein structure. All genetic variant AA residue numbering includes signal peptide.

We next turned to the patient epilepsy disease case variants to determine if these are over-represented in similar regions. Disease case variants were gathered from National Center for Biotechnical Information (NCBI), ClinVar, and Human Gene Mutation Databases (HGMD), yielding a total of 49 pathogenic or likely pathogenic mutations including 25 missense, 11 nonsense, 9 frameshift, and 4 intron splice variants. The distribution of the 36 epilepsy-related missense and nonsense mutations was mapped across the γ2 subunit protein domains (**Figure 3B**). The 11 γ2 nonsense variants resulted in early stop

codons (X) throughout the following domains: (1) ECD = Q40X, L91X, R136X, Y180X, G273X; (2) M1 = Y274X (2 unique stop codon mutant variants), W295X; (3) ICD = Q390X, R425X, W429X. The 25 γ2 subunit missense mutations showed wider distribution throughout the ECD, M1-4, M2-M3 linker and ICD regions. Comparison of the disease-associated and gnomAD missense variants identified significantly greater percentages of epilepsy related variants in the M2 and M2-M3 linker regions (**Table 1**). In contrast, signal peptide missense mutations were not found and ICD missense mutations were less prevalent in epilepsy patients (**Table 1**).

In the field of medical genomics, identification of potentially pathological mutations is a significant challenge, prompting the development of multiple bioinformatics methods to assess non-synonymous variants. We used the sequence homology-based genetic analysis bioinformatics programs PROVEAN (Protein Variation Effect Analyzer) and SIFT (Sorting Intolerant from Tolerant) to assess non-synonymous variants in the gnomAD population and predict the effects on γ2 subunit biological function. Interestingly, 35 of the 122 non-synonymous gnomAD variants were also predicted to be putatively damaging/deleterious by both of the two bioinformatics tools (scoring agreement at 81.9%, **Figure 3A**, orange colored variants). Neutral scored non-synonymous variants included S386P and T388A (aka S355 and T357 phosphorylation sites, **Figure 1C**). None of the γ2L isoform missense variants were predicted by PROVEAN as damaging, although S382C (aka S343, the PKC/CaMKII phosphorylation site, see earlier Synaptic Accumulation and Functional Regulation, **Figure 1C**) was predicted as possibly damaging by SIFT. Among the gnomAD population six variants were identified that overlapped the epilepsy patient missense group (L57F, N79S, M199V, R177Q, A334T, R363Q): three were predicted as deleterious (N79S, M199V, A334T) and 3 as neutral (L57F, R177Q, R363Q). PROVEAN and SIFT bioinformatics analysis of the 25 epilepsy patient missense variants showed four as neutral (L57F, A106T, L307V and R363Q), two had conflicting predictions (L74V, R304K), and all others were scored as damaging. As the gnomAD population is relatively free from significant clinical disorders, this implies masking by epistatic genetic interactions, consistent with phenotypic variability seen in epilepsy patients and animal epilepsy models. In addition, although *in silico* prediction tools show overall robust performance, particularly when software are used in combination (Leong et al., 2015; Masica and Karchin, 2016), this suggests pathological variants can be missed. Improving clinically admissible predictions from these *in silico* tools is a current high priority focus in medical bioinformatics (Masica and Karchin, 2016; Ernst et al., 2018). To expand our insight into the cellular pathology underlying the thirty-six patient cases, we next cross-examined database information (NCBI, ClinVar, HGMD) and the current literature for disease phenotypic and cellular study based analysis.

Patient Epilepsy Phenotypes

The most common patient phenotypes associated with nonsense and missense mutations ranged in severity and included FS, generalized tonic-clonic seizures (GTCS), GTCS with FS, genetic epilepsy with FS (GEFS), genetic epilepsy (GE), DS, and epileptic encephalopathy with severe global developmental delays (EEDD). FS are a relatively mild pathology which occur in the presence of fevers and display tonic-clonic seizure activity in individuals between 6 months and 5 years of age (Boillot et al., 2015). FS which have prolonged episode duration and occur past 6 years of age are termed FS+ and are generally associated with increased risk for developing epilepsy later in life. Moderate forms of epilepsy include GTCS and GE both with and without FS, where FS can co-occur with persistent seizure episodes past childhood and can present intense seizure activity more commonly known as a "grand mal" seizure as in the case of GTCS (Johnston et al., 2014; Wang et al., 2016; Fisher et al., 2017). The most severe phenotypes reported are DS and EEDD. In particular, DS is subset of epileptic encephalopathy and is characterized by a wide range of seizure type activity as well as psychomotor development delays, ataxia and hyperkinesis emerging between the ages of 1–4 (Ishii et al., 2014; Fisher et al., 2017). In contrast, EEDD have broader phenotypic manifestations and deficits as a result of global neurodevelopmental impairments with treatment-resistant seizures (Shen et al., 2017). Less common reported patient phenotypes included myoclonic epilepsy, absence seizures, complex partial seizures, tonic infantile spasms, tonic seizures, Rolandic epilepsy, and ASD with learning difficulties. *In vitro* studies have been invaluable in gaining in depth understanding of etiology, cellular pathology, and functional effects of these epilepsy patient variants.

γ2 Subunit Disease Case Analysis

In vitro studies on 17 of the γ2 pathogenic variants have revealed reduced surface expression in 15 cases, in part resulting from ER retention and trafficking defects (**Table 2**). The severe disease DS epilepsy phenotype is associated with three nonsense mutations (Q40X, R136X, Q390X) and one missense (P302L) mutation (**Table 2**). The early occurrence of Q40X and R136X within the ECD resulted in premature termination codons (PTCs) and mRNA degradation via nonsense mediated mRNA decay (NMD) with decreased γ2 protein levels. The introduction of upstream PTCs limited the availability of trafficable γ2, diminished overall receptor surface expression and synaptic localization and resulted in significant GABAergic deficits (Ishii et al., 2014). Conversely, the Q390X (previously known as Q351X) mutation occurs in the ICD and escapes NMD but is instead subject to ubiquitin-proteasome degradation (Kang et al., 2013). *In vitro* experiments found Q390X to have comparable mRNA levels to other late sequence nonsense mutations but dissimilar protein expression due to different degradation rates. Q390X displayed a substantially longer half-life as compared to wild-type γ2 and other nonsense mutant subunits in addition to an increased ability to oligomerize with and sequester wild-type α and β subunits. This alternative disruption in receptor trafficking provides evidence that expressed non-functional truncated subunits may be modifiers of epilepsy phenotype severity. Interestingly, P302L was the only missense mutation reported in

a patient with DS (Hernandez et al., 2017). Of note, this mutation resides in M2 and contributes to the formation of the ion channel pore which likely explains its severe phenotype. This is supported by P302L mutant electrophysiological studies and structural modeling which suggests a shift in pore activity resulting in slow activation, low conductance states, and fast desensitization of GABA$_A$R (Hernandez et al., 2017). In contrast, all six cases of EEDD were found in patients with missense mutations (A106T, I107T, P282S, R323W, R323Q, F343L) dispersed throughout structural domains (ECD, M1 and M2) and exhibited additional epileptic phenotypes such as GTCS, GEFS, and tonic seizures (Shen et al., 2017). In fact, the I107T mutation is located in the ECD which typically tolerates missense mutations as evidenced by relatively mild phenotypes; however, this mutation was found to exhibit the most severe cellular pathologies as compared to other disease variants emphasizing the need to further investigate these mutations and their ramifications on cellular processes.

The moderate epileptic phenotype GEFS without co-occurring conditions was observed in three cases with two missense (P83S and K328M) and one nonsense (W429X) variants reported with structural locations in the ECD, M2-M3 linker, and ICD, respectively (**Table 2**). P83S was found to reduce GABA-evoked whole cell currents mainly through a plasma membrane and trafficking-dependent manner (Lachance-Touchette et al., 2011; Huang et al., 2014; Bennett et al., 2017). In contrast, K328M (previously known as K289M) is found in the short extracellular loop between the M2-M3 regions and was found to increase receptor deactivation, implicating this region in receptor kinetic properties (Macdonald et al., 2012). Conversely, W429X displayed less drastic protein degradation and subunit oligomerization pathologies compared to the previously discussed DS variant Q390X (Wang et al., 2016). The later downstream incidence of W429X combined with slightly higher surface expression compared to Q390X may explain the milder epilepsy phenotype (Sun et al., 2008; Macdonald et al., 2012; Kang et al., 2013; Wang et al., 2016).

Throughout the reviewed mutations, only two variants (L57F and N79S) deviated from a pathology associated with reduced γ2 containing GABA$_A$R plasma membrane levels and were located in the ECD. L57F was present in an individual with GE and found to have normal surface and trafficking characteristics compared to wild-type γ2 receptors; however, altered current density properties and function were observed possibly due to minor structural perturbations in the α1-helix of the ECD (Hernandez et al., 2016). Comparatively, the N79S mutation was the sole occurrence of GTCS without co-occurring phenotypes and presented slight but significant impairments in plasma membrane levels and peak current amplitude (Huang et al., 2014) suggesting it is more of a susceptibility variant as opposed to an epilepsy mutation (Shi et al., 2010; Migita et al., 2013; Huang et al., 2014). Moreover, the resilience of the ECD is further supported by R82Q (previously known as R43Q), a well characterized missense mutation associated with mild phenotypic manifestations like FS and absence seizures with trafficking deficient pathologies (Macdonald et al., 2012). Overall, the 13 frameshift and intron splice variant mutations analyzed were associated with mild phenotypes, though further studies are needed to elucidate their pathological mechanisms (**Table 3**). However, frameshift mutations within the ICD (E402Dfs*3 generating a stop codon at Y404X critical Src/Fyn phospho site discussed earlier; and S443delC resulting in an altered and elongated carboxy terminus with +50 novel AA) were associated with more moderate-severe phenotypes like GTCS and GEFS+ underscoring the importance for intracellular regulation via the ICD (Macdonald et al., 2012).

In summary, both deficits in GABA$_A$R surface trafficking and the functional role of specific γ2 subunit regions are critical factors modulating phenotypic outcome, with some missense mutations resulting in phenotypes as severe as nonsense mutations. Furthermore, expressed non-functional truncated subunits may be correlated with more severe manifestations and be modifiers of disease phenotypes. Disease case variants in the pore lining M2 region showed particularly severe phenotypes, consistent with the reduced genetic variation in this region in

TABLE 1 | Genetic variation across *GABRG2* domains.

Region	Residues	GnomAD missense	(*n* = 122)	Disease-associated missense	(*n* = 25)	*p*-value
		#	%	#	%	
Signal peptide	1–39	21	17.21	0	0.00	***0.025**
ECD	40–273	53	43.44	12	48.00	0.8255
M1	274–296	3	2.46	3	12.00	0.0616
M1-M2 loop	297–299	0	0.00	0	0.00	1
M2	300–325	2	1.64	3	12.00	***0.0348**
M2-M3 loop	326–333	2	1.64	3	12.00	***0.0348**
M3	334–356	3	2.46	2	8.00	0.2006
ICD	357–443	33	27.05	1	4.00	***0.0096**
M4	444–466	5	4.10	1	4.00	1
C-Term	467	0	0.00	0	0.00	1

*Coordinates based on GABRG2 (GenBank NM_000816.3 transcript variant 2 γ2S, Uniprot P18507). ECD, extracellular amino-terminal domain; M1–M4, transmembrane regions 1–4; ICD, intracellular domain; C-Term, carboxy-terminus. Fisher's exact t-test p-values are reported; *denotes statistical significance.*

TABLE 2 | *GABRG2* missense and nonsense patient mutations with associated cellular pathologies and reported clinical phenotypes.

		Cellular Pathology				Phenotype						Publication(s)
Region	**Variant**	**Trafficking**	**ER retention**	**Surface expression**	**Cell current**	**Febrile seizures**	**Generalized tonic-clonic seizures**	**Genetic epilepsy**	**Dravet syndrome**	**Epileptic encephalopathy**	**Other**	
ECD	Q40X	↓↓	↑	↓↓	↓↓				✓			Hirose et al., 2005; Huang et al., 2012; Macdonald et al., 2012; Ishii et al., 2014
ECD	L57F	–	✓	–	↓↓			✓				Hernandez et al., 2016
ECD	N79S	↓	–	↓	↓		✓					Shi et al., 2010; Migita et al., 2013; Huang et al., 2014
ECD	R82Q	↓	↑	↓	↓	✓					✓	Wallace et al., 2001; Bianchi et al., 2002; Bowser et al., 2002; Macdonald et al., 2003; Kang and Macdonald, 2004; Sancar and Czajkowski, 2004; Kang et al., 2006; Eugene et al., 2007; Frugier et al., 2007; Macdonald et al., 2012; Chaumont et al., 2013; Bennett et al., 2017
ECD	P83S	↓↓	↑	↓↓	↓↓	✓		✓				Lachance-Touchette et al., 2011; Huang et al., 2014; Bennett et al., 2017
ECD	A106T	↓↓	↑	↓↓	↓↓		✓			✓	✓	Shen et al., 2017
ECD	I107T	↓↓	↑	↓↓	↓↓					✓	✓	Shen et al., 2017
ECD	R136X	↓↓	↑	↓↓	↓↓	✓		✓	✓		✓	Kang et al., 2013; Johnston et al., 2014
ECD	G257R	↓↓	↑	↓↓	–						✓	Reinthaler et al., 2015
M1	P282S	↓↓	↑	↓↓	↓↓					✓		Shen et al., 2017
M2	P302L	–	↔	↓	↓				✓			Hernandez et al., 2017
M2	R323W	↓↓	↑	↓↓	↓↓		✓			✓	✓	Shen et al., 2017
M2	R323Q	↓↓	↑	↓↓	↓↓	✓	✓	✓		✓	✓	Carvill et al., 2013; Reinthaler et al., 2015; Shen et al., 2017
M2-M3 loop	K328M	↓↓	↑	↓↓	↓↓	✓		✓				Baulac et al., 2001; Bianchi et al., 2002; Macdonald et al., 2003, 2012; Ramakrishnan and Hess, 2004; Hirose et al., 2005; Kang et al., 2006; Eugene et al., 2007; Frugier et al., 2007; Bouthour et al., 2012; Bennett et al., 2017
M3	F343L	↓↓	↑	↓↓	↓↓					✓	✓	Shen et al., 2017
ICD	Q390X	↓↓	↑	↓↓	↓↓	✓		✓	✓			Singh et al., 1999; Harkin et al., 2002; Kang et al., 2006, 2009; Macdonald et al., 2012; Kang et al., 2013
ICD	W429X	↓↓	↑	↓↓	↓↓	✓		✓				Sun et al., 2008; Macdonald et al., 2012; Kang et al., 2013; Wang et al., 2016

Variants are organized by nucleotide sequence position, and variants ending in 'X' are nonsense mutations with all others being missense mutations. Other epileptic or developmental phenotypes include absence seizures, complex partial seizures, severe global developmental delay, tonic infantile spasms, autism disorder with learning difficulties, Rolandic epilepsy, or epilepsy with myoclonic-astatic seizures; ECD, extracellular amino-terminal domain; M1–M3, transmembrane regions 1–3; ICD, intracellular domain; ↓↓, reduced; ↑, increased; ✓, observed; ✓, unknown; -, not affected/changed; ↓, slightly reduced; ↔, possibly.

TABLE 3 | Patient frameshift mutations and intron splice variants associated or likely associated with various epilepsy phenotypes.

Region	Canonical sequence codon	Mutant sequence	Variant name	Mutation type	Phenotype(s)	Function effect(s)
ECD	ACT-CCA-AAA 58 59 60	ACA-CAA-AAG	P59Qfs*12	Frame shift	Febrile Seizures, Tonic-Clonic Seizures	Predicted to undergo NMD (Boillot et al., 2015).
ECD	TTT-GCG-CAA 117 118 119	TTT-TGC-GCA	A118Cfs*6	Frame shift	Febrile Seizures	Predicted to undergo NMD (Della Mina et al., 2015).
ECD	AAA-GCT-GAT 57 58 59	AAG-CTG-ATG	A158Lfs*13	Frame shift	Unknown	Predicted to cause loss of normal protein function either through protein truncation or NMD. #
ECD	CGA-GTG-CTC 177 178 179	CAG-TGC-TCT	R177Qfs*6	Frame shift	Childhood Absence Epilepsy, Febrile Seizures	Predicted to cause loss of normal protein function either through protein truncation or NMD. #
Intron 4	CTT-AGG-TTG Int4 Int4 184	CTG-AGG-TTG	549-3T > G	Intron Splice Variant	Unknown	Abnormal gene splicing; *in silico* assessment predicts altered protein function (Reinthaler et al., 2015).
Intron 6	TCC-GTG-AAG 256 Int6 Int6	TCC-GGG-AAG	IVS6 + 2T– > G	Intron Splice Variant	Childhood Absence Epilepsy, Febrile Seizures	Truncation; ER retention; undergo NMD; decreased surface γ2 subunit levels and GABA-evoked whole cell currents; and increased ER stress marker BIP (Kananura et al., 2002; Tian and Macdonald, 2012).
ECD	GGA-GAT-TAT 257 258 259	AGA-GAT-TAT	770-1G > A	Intron Splice Variant	Suspected to cause epilepsy	Predicted to cause abnormal gene splicing and undergo NMD or the production of an abnormal protein. #
M3	GTT-TGT-TTC 341 342 343	GTT-TTT-TCA	C342Ffs*50	Frame shift	Childhood Absence Epilepsy, Febrile Seizures	Not anticipated to result in NMD but expected to result in a truncated protein. #
FproveIntron 8	CAG-GCC-CCT Int8 377 378	CGG-GCC-CCT	1129-2A > G	Intron Splice Variant	Childhood Absence Epilepsy, Febrile Seizures	Not anticipated to undergo NMD, but likely alters RNA splicing and disrupts protein function. #
ICD	ATT-CAA-GAG 397 398 399	ATT-CGA-GAG	Q398Rfs*4	Frame shift	Unknown	Predicted to cause protein truncation. #
ICD	GAA-GAG-TAC 402 403 404	GAT-TCA-TGA	E402Dfs*3	Frame shift	Febrile Seizures, Temporal Lobe Encephalopathy, Generalized Tonic-Clonic Seizures, Focal seizures	Predicted to cause protein truncation (Boillot et al., 2015). #
ICD	TCC-TAT-GCT 443 444 445	TCT-ATG-TCT	S443delC	Frame shift	Genetic Epilepsy with Febrile Seizures Plus	Produced elongated peptide with 50 novel amino acids compared to γ2S; trafficking impairments, ER retention, decreased surface expression and whole cell currents (Tian et al., 2013).
M4	GTC-TCC-TAC 462 463 464	TCT-CCT-ACC	V462Sfs*33	Frame shift	Febrile Seizures	Predicted to escape NMD and produce elongated peptide with 32 novel amino acids as compared to γ2S (Boillot et al., 2015). #

Patient variants are ordered by nucleotide sequence position of GABRG2.
Nucleotides deleted (red) and inserted (green) for each variant are noted.
ECD, extracellular amino-terminal domain; M3-M4, transmembrane regions 3-4; ICD, intracellular domain; NMD, nonsense-mediated mRNA decay; introduction of downstream premature stop codon following specified number of codons (*); predicted function from GeneDX (#).

gnomAD non-synonymous variants. Clearly, *in vitro* studies of recombinant receptor trafficking, electrophysiology and assembly have provided important insight into the underlying cellular pathology and functional effects of these epilepsy patient variants. Greater understanding of the consequences of γ2 genetic variation, both for revealing disease mechanisms and for GABA$_A$R synaptic plasticity will be gained through application of innovative imaging methods in the neuronal context.

LOOKING FORWARD: IMAGING ADVANCES

Advancing imaging techniques are providing critical insight into GABA$_A$R trafficking extending beyond basic endo/exocytic trafficking of receptors. Live-cell imaging using pH-sensitive GFP (pHluorin) tagged GABA$_A$Rs subunits and fluorescence recovery after photobleaching (FRAP) experiments first identified GABA$_A$R synaptic retention, limiting diffusion at synaptic release sites, and the crucial role of gephyrin in this process (Jacob et al., 2005). Receptor subunits with pHluorin tags have further described GABA$_A$R surface levels and lysosomal degradation (Jacob et al., 2012; Lorenz-Guertin et al., 2017) and novel exocytic machinery and insertion sites of receptors (Gu et al., 2016). The pHluorin-FRAP technique is often performed in addition to the newer workhorse of diffusion studies, quantum dot (QD) single-particle tracking. QD studies have revealed precise quantitative properties of synaptic and extrasynaptic GABA$_A$R diffusion during baseline conditions (Renner et al., 2012), excitatory stimulation (including iLTP) (Bannai et al., 2009, 2015; Muir et al., 2010; Niwa et al., 2012; Muir and Kittler, 2014; Petrini et al., 2014), GABA$_A$R agonist and/or drug treatment (Gouzer et al., 2014; Levi et al., 2015; de Luca et al., 2017), GABA$_B$ receptor activation (Gerrow and Triller, 2014), purinergic (P2x2 receptor) activation (Shrivastava et al., 2011), and changes in gephyrin or radixin phosphorylation (Hausrat et al., 2015; Battaglia et al., 2018). Receptor functional regulation by changes in surface diffusion, perhaps completely independent of changes in surface levels, represents a paradigm shift in our basic understanding of synaptic plasticity. Indeed current studies of human genetic variants in recombinant systems are unlikely to detect these fundamentally important properties due to lack of a neuronal context, the appropriate GABA$_A$R subunit complement, interacting proteins, and general overexpression problems. For example, QD neuronal studies of the γ2 K328M disease variant revealed an additional phenotype of enhanced temperature sensitive receptor diffusion, likely contributing to the FS pathology in patients (Bouthour et al., 2012).

To address multiple trafficking questions within a single assay, our group recently engineered a GABA$_A$R γ2 subunit dual fluorescent sensor encoding a pHluorin tag and a fluorogen-activating peptide (FAP) (γ2^{pH}FAP) (Lorenz-Guertin et al., 2017). FAPs are antibody single chain variable fragments characterized to selectively bind inorganic dyes with high specificity and affinity (Szent-Gyorgyi et al., 2008). The dyes are non-fluorescent until bound by a FAP and individual dyes have unique characteristics including cell permeability, pH-sensitivity, fluorescent properties, and *in vivo* administration capability (Fisher et al., 2010; Grover et al., 2012; Saunders et al., 2012; Zhang et al., 2015; He et al., 2016). We have used the FAP-dye system in neurons to selectively examine cell surface GABA$_A$Rs undergoing internalization, early endosomal accumulation and targeting to late endosomes/lysosomes via confocal live-imaging (Lorenz-Guertin et al., 2017). Pulse-labeling γ2^{pH}FAP with cell impermeable dye allows for detection of surface receptor turnover rates independent of a change in total GABA$_A$R surface levels, as we demonstrated using a mild seizure protocol. As more GABA$_A$Rs subunits are engineered to express the FAP tag, and additional unique dyes are synthesized to address specific experimental questions, the utility of this imaging approach continues to grow.

Other innovative imaging approaches advancing our ability to detect changes in GABA$_A$R synaptic plasticity include optogenetic toolkits for controlling GABA$_A$R activity (Lin et al., 2014, 2015), spatially regulated GABA activation using two-photon photolysis (Oh et al., 2016), proximity ligation assays to measure endogenous protein interaction (Smith et al., 2014; Tseng et al., 2015; Ghosh et al., 2016), and super-resolution imaging and other fluorescent tools to examine inhibitory gephyrin scaffolding (Gross et al., 2013, 2016; Sigal et al., 2015; Maric et al., 2017; Pennacchietti et al., 2017). Fluorescence resonance energy transfer (FRET) techniques have been limitedly applied to studying GABA$_A$R trafficking or receptor subunit composition (Ding et al., 2010; Shrivastava et al., 2011), collectively suggesting imaging techniques will be a rich resource of novel GABA$_A$R knowledge.

CONCLUSION

In summary, we live in an unprecedented time for understanding human disease pathology and neurodevelopment through integration of "big data" on human genetic variation and protein interaction networks/interactomes, in combination with high resolution live-imaging approaches. Future efforts to resolve GABA$_A$R pathologies will benefit from connecting genetic variants to their cellular mechanisms of pathology within the complexity of neuronal signaling. Importantly, increased understanding of surface and intracellular pool regulated trafficking of GABA$_A$R will provide mechanisms to treat overall reduced receptor levels in various disease states. Future treatment of genetic epilepsy syndromes are likely to involve CRISPR-Cas9 gene editing (Ma et al., 2017), RNA focused REPAIR editing approaches, or application of improved drugs that act as chaperones to promote receptor trafficking. The new imaging based methods described here are particularly likely to show high utility in both identifying cellular pathology of human GABA$_A$R genetic variants and for drug screening efforts in a neuronal context.

MATERIALS AND METHODS

Data Mining of *GABRG2* Genetic Variation

The prevalence of γ2 subunit non-synonymous and synonymous variations in gnomAD[1], currently a dataset of exome sequence data from 123,136 individuals and whole genome sequencing from 15,496 unrelated individuals, was assessed and restricted to those meeting the "PASS" quality threshold (Lek et al., 2016). Individuals known to be affected by severe pediatric disease are not contained in this data set, or their first-degree relatives. Next "pathogenic" and "likely pathogenic" patient case variants not present in the gnomAD dataset were investigated in National Center for Biotechnical Information variation viewer (NCBIvv)[1], ClinVar, and Human Gene Mutation Databases (HGMD) utilizing the following search parameters: GRCh37.p13 annotation release 105 assembly and NM_000816.3 (transcript variant 2, γ2S). The search in NCBIvv identified 17 variants (accessed January 2018). The ClinVar search (accessed February 2018) confirmed 16/17 candidate variants with the outlier (R323W) having been newly identified in the literature (Shen et al., 2017)[2]. In addition to those confirmed, the ClinVar investigation produced 10 additional mutations. Some variants identified in ClinVar had associated predicted functions (submitted by GeneDX genetics company)[3]. Finally, HGMD (hgmd2018.1; accessed March 2018) interrogation uncovered 22 disease-causing mutations that were absent from NCBIvv and ClinVar inquiries[4]. Using these candidate case variants and their associated database information, the current literature was evaluated for disease phenotypic and cellular study based implications yielding a total of 49 pathogenic or likely pathogenic mutations including 25 missense, 11 nonsense, 9 frameshift, and 4 intron splice variants. We used lollipops-v.1.3.1 software (Jay and Brouwer, 2016) to plot the distribution of synonymous, non-synonymous and disease case mutations in *GABRG2* along a linear γ2S assembly (P18507, ENST00000361925) and a linear segment representation of the additional eight encoded amino acids within the ICD in the γ2L isoform (P18507-2, ENST00000356592). The missense and nonsense disease case variants studied at the cellular trafficking level were included in **Table 2**. The frameshift and intron splice variants were annotated in **Table 3**.

[1] http://gnomad.broadinstitute.org/transcript/ENST00000361925

[2] https://www.ncbi.nlm.nih.gov/clinvar

[3] https://www.genedx.com/

[4] http://www.hgmd.cf.ac.uk/ac/gene.php?gene=GABRG2

Bioinformatics Tools

PROVEAN (Protein Variation Effect Analyzer[5] (Choi et al., 2012) and SIFT (Sorting Intolerant from Tolerant) algorithms (Hu and Ng, 2013) are bioinformatics tools which predict whether an amino acid substitution or indel (insertion or deletion) has an impact on a protein's biological function using homology based genetic analysis. Currently PROVEAN provides scoring via both PROVEAN and SIFT algorithms. PROVEAN utilizes pairwise sequence alignment scores to generate pre-computed predictions at every amino acid position in all human and mouse protein sequences. Mutations are predicted to be deleterious or tolerant based on the prediction cutoff value of −2.5: scores smaller than −2.5 are considered deleterious. Similarly, SIFT predicts whether the amino acid substitution alter the protein function based on sequence homology and the physical properties of amino acids. The intolerant range of SIFT is $\leq$0.05 for predicted damaging/deleterious mutations and a score of $>$0.05 predicts the tolerant range.

WEB RESOURCES

- gnomAD, http://gnomad.broadinstitute.org/
- ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
- Human Gene Mutation Database, http://www.hgmd.org/
- Lollipops v.1.3.1, https://github.com/pbnjay/lollipops/releases
- UniProt, http://www.uniprot.org/

AUTHOR CONTRIBUTIONS

JL-G and TJ wrote and edited the sections "γ2 Subunit Trafficking and Interactors" and "Looking Forward: Imaging Advances." MB and TJ analyzed, wrote, and prepared the section "Human Genetic Variation of γ2 and Pathological Implications" and associated tables. TJ prepared all the figures. MB prepared all the tables.

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[5] http://provean.jcvi.org

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MINI REVIEW
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Mechanism of BDNF Modulation in GABAergic Synaptic Transmission in Healthy and Disease Brains

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In the mature healthy mammalian neuronal networks, γ-aminobutyric acid (GABA) mediates synaptic inhibition by acting on $GABA_A$ and $GABA_B$ receptors ($GABA_AR$, $GABA_BR$). In immature networks and during numerous pathological conditions the strength of GABAergic synaptic inhibition is much less pronounced. In these neurons the activation of $GABA_AR$ produces paradoxical depolarizing action that favors neuronal network excitation. The depolarizing action of $GABA_AR$ is a consequence of deregulated chloride ion homeostasis. In addition to depolarizing action of $GABA_AR$, the $GABA_BR$ mediated inhibition is also less efficient. One of the key molecules regulating the GABAergic synaptic transmission is the brain derived neurotrophic factor (BDNF). BDNF and its precursor proBDNF, can be released in an activity-dependent manner. Mature BDNF operates via its cognate receptors tropomyosin related kinase B (TrkB) whereas proBDNF binds the p75 neurotrophin receptor ($p75^{NTR}$). In this review article, we discuss recent finding illuminating how mBDNF-TrkB and proBDNF-$p75^{NTR}$ signaling pathways regulate GABA related neurotransmission under physiological conditions and during epilepsy.

Keywords: BDNF, TrkB, $p75^{NTR}$, GABA receptors, KCC2

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INTRODUCTION

A striking trait of early GABAergic transmission is that activation of γ-aminobutyric acid ($GABA_A$) receptors ($GABA_ARs$) causes membrane depolarization and Ca^{2+} influx in immature neurons (Ben-Ari et al., 1989, 2007; Ganguly et al., 2001). During this critical period, depolarizing $GABA_AR$ activity plays a major role in neuronal network construction (Ben-Ari et al., 2007; Wang and Kriegstein, 2008; Sernagor et al., 2010). Given this fundamental role it comes as no surprise that flawed GABAergic transmission is implicated in an array of brain disorders such as epilepsy (Ben-Ari and Holmes, 2005), autism spectrum disorder (ASD), Rett syndrome (Kuzirian and Paradis, 2011), schizophrenia (Lewis et al., 2005; Charych et al., 2009; Mueller et al., 2015) and major depressive disorder (Sanacora et al., 1999; Brambilla et al., 2003). GABAergic development relies heavily on brain derived neurotrophic factor (BDNF; Hong et al., 2008; Gottmann et al., 2009; Sakata et al., 2009; Kuzirian and Paradis, 2011), one of the most crucial regulator of synapse development and function in the developing and adult central nervous system (CNS; Lu et al., 2005; Cohen-Cory et al., 2010). BDNF can be secreted either as a precursor (proBDNF) or a mature form (mBDNF; Nagappan et al., 2009; Yang et al., 2009).

ProBDNF and mBDNF modulate the efficacy of synaptic responses via the tropomyosin-related kinase receptor B (TrkB) and the p75 neurotrophin receptor ($p75^{NTR}$), respectively (Lu et al., 2005). BDNF shapes the development of neuronal circuits, as well as the construction of inhibitory connections throughout life (Kovalchuk et al., 2004; Gubellini et al., 2005; Gottmann et al., 2009) and alterations in BDNF processing have been observed in diseases of the CNS, including schizophrenia, ASD and epilepsy (Binder et al., 2001; Carlino et al., 2011; Garcia et al., 2012). In this review article, we discuss the recent achievements in analysis of the development of GABAergic network with an emphasis on GABA and BDNF interplay. We particularly focus on ionotropic $GABA_A$ or metabotropic $GABA_B$ receptors activation in triggering the postsynaptic release of BDNF, which in turn regulates the maturation of GABAergic synapses. We then discuss how BDNF tunes up or down inhibitory transmission by acting on synthesis and trafficking of $GABA_A$Rs and KCC2 chloride ion transporters at the cell membrane. Finally, we focus on epilepsy, a pathology that highlights the links between GABA and BDNF.

BDNF AND INHIBITORY STRENGTH OF $GABA_A$ RECEPTORS

$GABA_A$Rs are ionotropic receptors that allow the bidirectional flux of chloride ions across the neuronal membrane. The direction of Cl^- flux depends on $[Cl^-]_i$ and the membrane potential, whereas the intensity of the flux depends on the number of activated $GABA_A$Rs. In mature healthy neurons the $[Cl\text{-}]_i$ is close to 4 mM, and the reversal potential of the ion flux through $GABA_A$Rs ($EGABA_A$) is ∼78–82 mV, close to the resting membrane potential (Tyzio et al., 2003; Khazipov et al., 2004). Hence, at rest, the activation of $GABA_A$Rs produces no or, at the most, a weak (1–2 mV) hyperpolarization or depolarization. The activation of $GABA_A$Rs during neuronal depolarization induced by the excitatory synapses allows massive Cl^- entry that provides strong hyperpolarizing force and effectively compensates or diminishes the strength of the excitatory signal. The increased $[Cl^-]_i$ is rapidly extruded by electroneutral neuron-specific potassium-chloride cotransporter KCC2 (Rivera et al., 1999). In immature neurons as well as in mature neurons during different pathologies (epilepsy (Cohen et al., 2002), acute trauma (Boulenguez et al., 2010), Rett syndrome (Banerjee et al., 2016), Down syndrome (Deidda et al., 2015), Huntington disease (Dargaei et al., 2018), ASD (Tyzio et al., 2014)) the activation of $GABA_A$Rs produces neuron depolarization reflecting increased resting level of $[Cl^-]_i$. This Cl^--dependent depolarization facilitated the activation of the neuronal network and contributes to the formation of pathological patterns of network activities (Ben-Ari et al., 2007; Moore et al., 2017). Thus, the inhibitory strength of $GABA_A$R mediated inhibition is determined by two complementary parameters: the amount of ion flux through opened $GABA_A$Rs and the $[Cl^-]_i$. The mBDNF and proBDNF do regulate these two parameters.

ProBDNF, mBDNF AND $GABA_A$R INTERPLAY

Expression patterns of BDNF and proBDNF are developmentally regulated. ProBDNF expression levels increase during the first postnatal weeks while mature BDNF peaks at a later period (Yang et al., 2014; Menshanov et al., 2015; Winnubst et al., 2015). ProBDNF can be cleaved under physiological conditions depending mainly on neuronal activity generated in the developing neuronal networks (Lessmann and Brigadski, 2009; Nagappan et al., 2009; Langlois et al., 2013). For instance, theta burst stimulation triggers the co-release of proBDNF and the serine protease, tissue Plasminogen Activator (t-PA) which converts plasminogen to plasmin yielding to mature BDNF, whereas low-frequency stimulation increases the amounts of proBDNF in the extracellular space (Nagappan et al., 2009). Overexpression of proBDNF in proBDNF-HA/$^+$ mice showed a decrease in dendritic arborization and spine density of hippocampal neurons as well as altered synaptic transmission (Yang et al., 2014). In developing rat hippocampal neurons, proBDNF/$p75^{NTR}$ signaling has been reported to induces a long-lasting depression of $GABA_A$R-mediated synaptic activity (Langlois et al., 2013), whereas endogenous BDNF/TrkB signaling is required for the induction of GABAergic long-term-potentiation (Gubellini et al., 2005).

In the cerebral cortex, BDNF/TrkB signaling controls the development of interneurons (Yuan et al., 2016) and the expression of the presynaptic GABA synthetic enzyme GAD65 (Sánchez-Huertas and Rico, 2011). In the cerebellum, BDNF promotes the formation of inhibitory synapses (Chen et al., 2016). Postsynaptically, BDNF and proBDNF are critical to control the $GABA_A$Rs trafficking between synaptic sites and endosomal compartments. The cell membrane expression of $GABA_A$Rs depends on their phosphorylation level (Nakamura et al., 2015). Thus, dephosphorylation of the $GABA_A$R ß3 subunits triggers the association with the assembly polypeptide 2 (AP2) complex which leads to a clathrin-mediated internalization (Kittler et al., 2000; Nakamura et al., 2015). In fact, BDNF/TrkB signaling inhibits the internalization of $GABA_A$Rs through activation of the phosphoinositide-3 kinase (PI-3 kinase) and PKC pathways (**Figure 1**). This ability of BDNF to modulate $GABA_A$Rs endocytosis and activity is likely to occur due to an inhibition of their interaction with the protein phosphatase 2A complex (PP2A), a downstream target of PI-3 kinase (Jovanovic et al., 2004; Vasudevan et al., 2011). Inversely, application of proBDNF to cultured rat hippocampal neurons cause a reduction in GABAergic synaptic transmission by promoting dephosphorylation and internalization of $GABA_A$R ß3 subunits through the RhoA–Rock–PTEN (phosphatase and tensin homolog) signaling cascade (Riffault et al., 2014). The underlying molecular mechanism of PTEN-mediated dephosphorylation and downregulation of $GABA_A$Rs remains to be determined but may involve the inhibition of PI3-kinase activity and the subsequent upregulation of PP2A activity. Accordingly, PTEN activated by $p75^{NTR}$ is a major negative regulator of the PI3-kinase signaling cascade (Song et al., 2010). Thus, the cell surface expression levels of $GABA_A$Rs

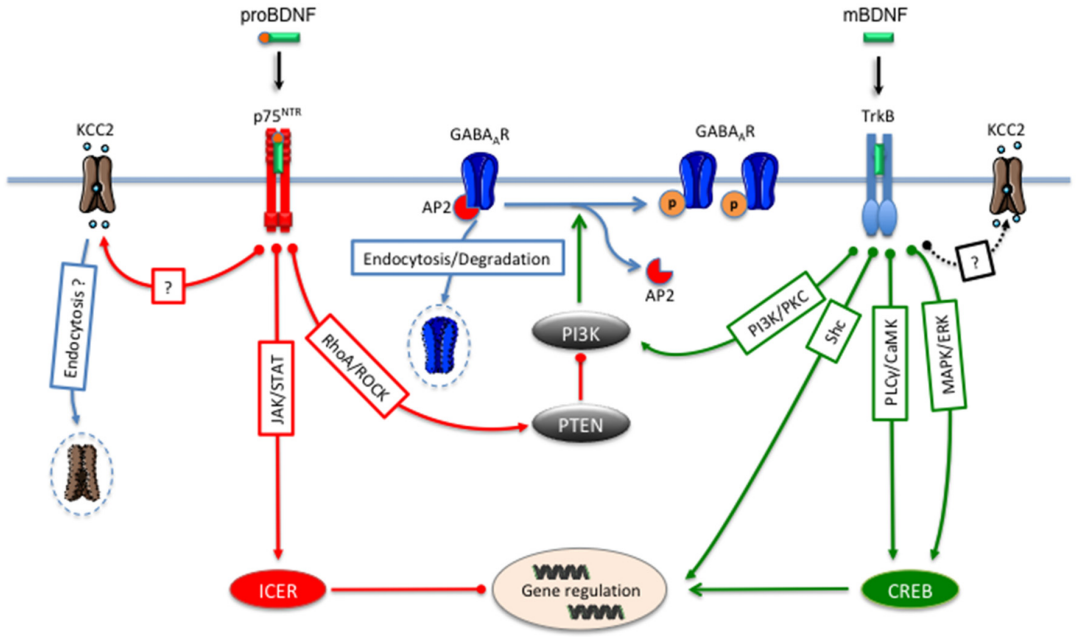

FIGURE 1 | mBDNF/TrkB and proBDNF/p75^{NTR} signaling pathways regulate γ-aminobutyric acid (GABA) neurotransmission. Activation of TrkB receptors by mBDNF leads to an inhibition of GABA$_A$R endocytosis and a consequent increase in the cell surface expression of these receptors through the PI 3-kinase and the PKC signaling pathway. At the transcriptional level, BDNF/TrkB signaling regulates GABA$_A$R and KCC2 gene expression through the Shc, PLC/CaMK or MAP/ERK pathways. Activation of p75^{NTR} by proBDNF decreases GABA$_A$Rs cell surface expression through the RhoA/ROCK/PTEN pathway that leads to the dephosphorylation of GABA$_A$R and endocytosis and degradation of internalized receptors. At the transcriptional level, proBNDF/P75^{NTR} leads to the repression of GABA$_A$R synthesis through the JAK2/STAT3/ICER pathway. The proBDNF/75^{NTR} signaling also decreases KCC2 expression.

can be settled by the competition between mBDNF/TrkB and proBDNF/p75^{NTR} intracellular cascades on the PTEN/PI3-kinase-mediated activation of PP2A. After endocytosis, the proBDNF/p75^{NTR}/Rho-ROCK pathway moved internalized GABA$_A$Rs to late endosomes and finally to lysosomes for degradation (Riffault et al., 2014).

The BDNF may also be involved in GABA$_A$Rs clustering at synaptic sites through the regulation of the main scaffolding protein gephyrin. Indeed, in immature rat hippocampal neuronal cultures BDNF enhanced the expression and clustering of gephyrin, which in turn leads to an increase in the density of GABA$_A$Rs-gephyrin containing complexes at postsynaptic sites (González, 2014). Conversely, in cultured mouse amygdala neurons, rapid application of BDNF decreased the cell surface expression of GABA$_A$Rs-gephyrin complexes whereas long-term treatment with BDNF elicits opposite effects (Mou et al., 2013). BDNF can exert different roles depending on the developmental stages (young vs. adult neurons) but also in function of the brain structures or according to the delivery mode (rapid vs. long-term treatment). These opposing responses of BDNF on GABA$_A$RS clustering may reflect the differences in the kinetics of TrkB activation (Ji et al., 2010) and may contribute to the homeostatic regulation of GABAergic synaptic strength (Tyagarajan and Fritschy, 2010; Vlachos et al., 2013; Brady et al., 2018).

After its release into the synaptic cleft, the activity of GABA is terminated by the reuptake of the neurotransmitter, a process mediated by the GABA transporters (GATs). The surface expression of GABA transporter-1 (GAT-1), the major GABA transporter expressed by both neurons and astrocytes (Guastella et al., 1990), is upregulated in neuronal cells by BDNF-mediated tyrosine kinase-dependent phosphorylation (Law et al., 2000; Whitworth and Quick, 2001). However, the neurotrophin was found to inhibit GAT-1-mediated GABA transport at the isolated nerve endings (Vaz et al., 2008), suggesting that this effect is very localized, to delay GABA uptake by the nerve terminal, thereby enhancing synaptic actions of GABA. In contrast with the effects at the synapse, BDNF may accelerate the uptake of GABA at extrasynaptic sites, allowing replenishment of neuronal pools of GABA. Furthermore, BDNF enhances GABA transport in rat cortical astrocytes by modulating the trafficking of GAT-1 from the plasma membrane (Vaz et al., 2011).

BDNF also regulates genes transcription of GABA$_A$R subunits (Bell-Horner et al., 2006) GAD65 (Sánchez-Huertas and Rico, 2011) and GATs (Vaz et al., 2011), through the recruitment of the ERK-MAP kinase cascade, which activates the cAMP-response element (CRE)-binding protein (CREB; **Figure 1**; Yoshii and Constantine-Paton, 2010). In an opposite way, the downstream signaling pathway triggered by proBDNF/p75^{NTR} activates the JAK-STAT pathway leading to the induction of the cAMP early repressor ICER, which mediates the downregulation of GABA$_A$Rs ß3 gene synthesis (**Figure 1**). Interestingly, the activation of this pathway precedes the decrease of GABA$_A$Rs ß3 cell surface expression (Riffault et al., 2014).

Other reports have also suggested that in rat visual cortex and cerebellar Purkinje cells, the BDNF/TrkB signaling modulates $GABA_A$Rs mediated currents through the PLCγ-Ca^{2+} and CaMK pathways (Cheng and Yeh, 2003; Mizoguchi et al., 2003). In immature cultured rat hippocampal and hypothalamic neurons, the BDNF/TrkB dependent increase in $GABA_A$Rs plasma membrane expression occurs when activation of $GABA_A$Rs lead to a depolarization of the membrane potential, which in turn triggers the release of BDNF (Obrietan et al., 2002; Porcher et al., 2011). In more mature cultured rat hippocampal neurons and murine cerebellar granule cells, BDNF decreases the plasma membrane expression of $GABA_A$Rs (Brünig et al., 2001; Cheng and Yeh, 2003). In parallel, BDNF/trkB signaling reduces the excitability of parvalbumin-positive interneurons in the mouse dentate gyrus (Holm et al., 2009). Surprisingly, these neurons do not express the proBDNF receptor $p75^{NTR}$ (Dougherty and Milner, 1999; Holm et al., 2009). The change in the regulation of $GABA_A$Rs cell surface expression by BDNF coincides with a shift in GABA polarity (depolarization to hyperpolarization), attributed to the activity of KCC2 (Rivera et al., 1999) which is also regulated by both forms of BDNF. A recent study showed that increased proBDNF/$p75^{NTR}$ signaling disrupts the developmental GABAergic sequence by maintaining a depolarizing GABA response in a KCC2-dependent manner in mature cortical neurons (Riffault et al., 2018). In developing neurons, BDNF increases KCC2 expression on the level of mRNA transcription (Aguado et al., 2003; Rivera et al., 2004; Ludwig et al., 2011). In line with these observations, it was shown that the expression of KCC2 is significantly decreased in $trkB^{-/-}$ mice hippocampi (Carmona et al., 2006) whereas, in adult neurons BDNF decreases both mRNA and protein KCC2 (Rivera et al., 2002, 2004; Wake et al., 2007; Shulga et al., 2008; Boulenguez et al., 2010). In accordance with these results, neurons in the dorsal horn of the spinal cord treated with BDNF showed a depolarizing shift of the GABA reversal potential (Coull et al., 2003, 2005). The actions of BDNF/TrkB signaling on GABAergic synapses are developmentally regulated, with BDNF leading to an increase of KCC2 expression in immature neurons through activation of Shc pathway, and a decrease in adult neurons through activation of both Shc and PLCγ cascades (Rivera et al., 2002, 2004; **Figure 1**).

Altogether, these findings suggest that the relative availability of the two forms of BDNF, pro and mature, could affect the excitatory/inhibitory balance during the development by regulating the polarity and the synaptic strength of GABAergic transmission.

$GABA_B$R AND BDNF INTERPLAY

Similarly to BDNF, a crucial factor regulating the development of inhibitory transmission is GABA itself (Ben-Ari et al., 2007; Gaiarsa et al., 2011). In the neocortex, extracellular GABA signaling regulates the development of GABAergic inhibition through $GABA_A$ and $GABA_B$ receptors. During the developmental period, ambient GABA may also participate in neuronal network construction and synaptogenesis. In the visual cortex of mice, Chattopadhyaya et al. (2007) demonstrated that the tonic activation of $GABA_A$ and $GABA_B$ receptors regulates the axonal branching of basket-cell interneurons. They reported that reducing GABA levels in a single basket cell results in a decrease of perisomatic GABAergic inputs on the pyramidal cells. This deficit of synaptic transmission is partially restored by GABA uptake blocker or $GABA_A$ and $GABA_B$ receptor agonists. In agreement with this study, knockout of the $GABA_{B1}$ subunit leads to altered maturation of GABAergic synaptic transmission in murine hippocampal neurons and synaptic activation of $GABA_B$Rs promotes the development of GABAergic synapses (Fiorentino et al., 2009). The mechanisms are not fully understood but may likely involve the BDNF/TrkB signaling. Indeed, the trophic action of $GABA_B$Rs was prevented by BDNF scavenger (TRkB-IgG) and not observed in BDNF KO mice (Fiorentino et al., 2009). Moreover, the stimulation of $GABA_B$Rs induce a calcium-dependent release of BDNF via the PLC-PKC signaling cascade and L-type voltage-gated calcium channels (Fiorentino et al., 2009; Kuczewski et al., 2011). Finally, in the developing rat hippocampus, it was shown that activation of $GABA_B$Rs also increased the phosphorylation levels of the α-CamKII, which play a critical role in BDNF release (Fischer et al., 2005; Kolarow et al., 2007; Xu et al., 2008). Therefore, postsynaptic calcium increase and phosphorylation of α-CamKII may underlie the $GABA_B$-R-mediated release of BDNF. Interestingly, the regulated secretion of BDNF following $GABA_B$ receptor activation increases the number of $GABA_A$ ß2/3 subunits receptors at the postsynaptic membrane (Kuczewski et al., 2011). Thus, the interplay between $GABA_B$Rs activation and the subsequent BDNF secretion in developing hippocampal neurons contribute to the functional maturation of GABAergic synaptic transmission.

BDNF AND GABA INTERPLAY IN EPILEPSY

Epilepsy is a brain disorder characterized by the appearance of spontaneous recurrent seizures due to network hyperexcitability (Fischer et al., 2005). Neurotrophic signaling pathways are over-activated after status epilepticus (SE) and seem to contribute to epileptogenesis by promoting neuronal cell deaths and rewiring of excitatory networks (Koyama et al., 2004; Unsain et al., 2008; Goldberg and Coulter, 2013). Similarly, changes in GABAergic neurotransmission and altered neuronal Cl^- homeostasis are considered to play a crucial role in epileptogenesis. Initial studies regarding the contribution of BDNF to epilepsy led to conflicting conclusions, with intrahippocampal BDNF perfusion or intraventricular injection of the BDNF scavenger TrkB-IgG, both being protective in a model of dorsal hippocampal kindling (Reibel et al., 2000; Binder et al., 2001). However, further studies reported that epileptogenesis was suppressed in mice with conditional deletion of TrkB in the brain (He et al., 2004) as well as in mice carrying a TrkB gene mutation that uncouples TrkB from the PLCγ (He et al., 2010). Interestingly, elevated levels of BDNF and TrkB following seizure activity or bath application of BDNF on hippocampal neurons trigger a down-regulation of KCC2 surface expression and a subsequent increase in neuronal excitability which most likely contributes to the establishment

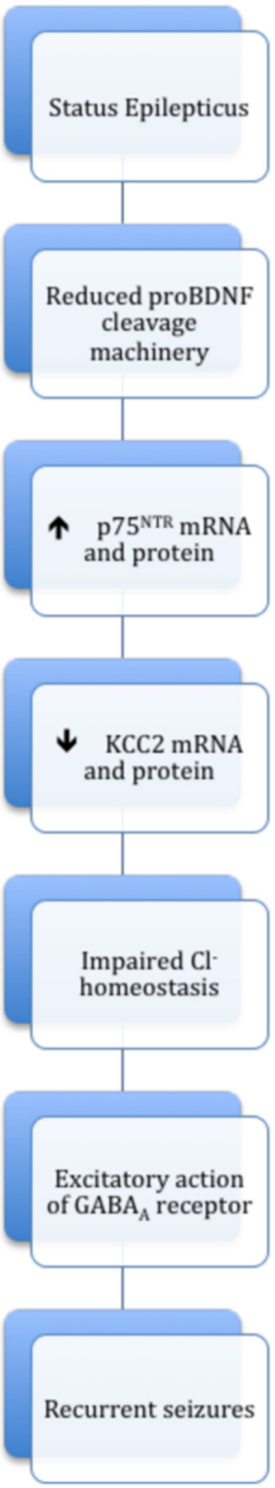

FIGURE 2 | Scheme summarizing the causal relationship between proBDNF/p75^{NTR} and depolarizing action of GABA during epileptogenesis. Elevated amounts of proBDNF following status epilepticus (SE) are associated with reduced proBDNF cleavage machinery and increased expression of p75^{NTR}. The proBDNF/p75NTR response donwregulates KCC2, which promotes a chloride homeostasis dysregulation leading to an excitatory action of GABA and facilitate recurrent seizures.

of recurrent seizures (Rivera et al., 2002; Wake et al., 2007). In addition to the pro-epileptogenic effect of mBDNF, it has been shown that proBDNF and p75^{NTR} are markedly increased after Pilocarpine-induced seizures. The elevated amounts of proBDNF following SE are associated with reduced proBDNF cleavage machinery that results from acute decreases in tPA/plasminogen proteolytic cascade and increases in API-1, an inhibitor of proBDNF cleavage (Reibel et al., 2000; Binder et al., 2001). Furthermore, two recent studies showed that proBDNF/p75^{NTR} response following SE selectively downregulates KCC2, which in turn promotes a chloride homeostasis dysregulation leading to an excitatory action of $GABA_A$ receptors and facilitate epileptiform discharges (Kourdougli et al., 2017; Riffault et al., 2018; **Figure 2**). Interestingly, blockade of p75^{NTR} during the earliest phase of epileptogenesis restores KCC2 levels and reduces seizures frequency (Kourdougli et al., 2017; Riffault et al., 2018). These results suggest that proBDNF/p75^{NTR} play a critical role in the mechanisms of epileptogenesis (see **Figure 2**). It should be pointed, however, that apart from these pro-epileptogenic actions, BDNF could exert anti-epileptic effects (Paradiso et al., 2009; Bovolenta et al., 2010). Several observations support the view that at least part of the pro-epileptogenic actions of pro- or mature-BDNF relies on an alteration of GABAergic inhibition. Thus, although BDNF exerts beneficial effects on developing GABAergic synapses, exogenous applications of this neurotrophin decrease the efficacy of GABAergic inhibition on mature neurons (Berninger et al., 1995; Mizoguchi et al., 2003). In cultured hippocampal neurons, proBDNF promotes $GABA_A$ receptor endocytosis and degradation (Riffault et al., 2014) and BDNF has been reported to reduce the probability of GABA release (Mizoguchi et al., 2003). At the transcriptional level, BDNF/TrkB signaling causes the repression of $GABA_A$Rs α1 subunit gene through the activation of JAK-STAT pathway following SE (Lund et al., 2008). An important feature of epileptogenesis is a downregulation of KCC2 expression both in human epileptogenic tissues (Aronica et al., 2007; Huberfeld et al., 2007; Munakata et al., 2007; Shimizu-Okabe et al., 2011; Kahle et al., 2014) and in animal models of epilepsy (Jin et al., 2005; Kourdougli et al., 2017; Riffault et al., 2018). In patients with temporal lobe epilepsy, the decrease in KCC2 expression results in depolarizing GABAergic events in a minority of subicular pyramidal cells that contribute to inter-ictal like activity (Cohen et al., 2002; Huberfeld et al., 2007). These findings are consistent with reports of KCC2 downregulation and changes in the polarity of GABAergic response in animal models of epilepsy (Huberfeld et al., 2007; Barmashenko et al., 2011; Shimizu-Okabe et al., 2011; Kourdougli et al., 2017; Riffault et al., 2018). Because both forms of BDNF regulate the expression of KCC2 (Rivera et al., 1999; Ludwig et al., 2011), the decrease observed in epileptic tissues could be due to an imbalance between mBDNF/TrkB and proBDNF/p75^{NTR} signaling during the first postnatal weeks causing an impaired or delayed functional maturation of GABAergic inhibition. Alternatively, an excess of BDNF production and secretion associated with reductions in proBDNF cleavage in epileptic tissues (Ernfors et al., 1991; Thomas et al., 2016) could account for the decrease in KCC2 expression (**Figure 2**).

Altogether, these findings show a complex picture in which BDNF signaling can influence the pathogenicity of epilepsy both ways. Further studies will be necessary to precise the role of the extracellular proBDNF/mBDNF ratio in GABAergic transmission during neuronal development and in different types of epilepsies.

Unveiling the mode of action of BDNF in the development and functioning of the GABAergic network is a promising quest for developing new cures of a number of neurological diseases. BDNF influences the development and functioning of the GABAergic network which in turn controls BDNF levels. As a result of this interaction, impairment of one of the two systems will most disturb the other, and since each of them is fundamental to normal CNS functioning, this will potentially lead to a host of neurological conditions. As of today, there is hope that investigation of the molecular pathways mediating the trophic action of BDNF may provide new insights into the normal development of the GABAergic network, providing new therapeutic strategies to improve the symptoms in a broad spectrum of GABA-related pathologies.

AUTHOR CONTRIBUTIONS

The review was conceptualized, written and edited by each of the authors. CP was the supervisor.

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REVIEW
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Recent Findings on AMPA Receptor Recycling

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α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs) are tetrameric protein complexes that mediate most of the fast-excitatory transmission in response to the neurotransmitter glutamate in neurons. The abundance of AMPA-Rs at the surface of excitatory synapses establishes the strength of the response to glutamate. It is thus evident that neurons need to tightly regulate this feature, particularly in the context of all synaptic plasticity events, which are considered the biological correlates of higher cognitive functions such as learning and memory. AMPA-R levels at the synapse are regulated by insertion of newly synthesized receptors, lateral diffusion on the plasma membrane and endosomal cycling. The latter is likely the most important especially for synaptic plasticity. This process starts with the endocytosis of the receptor from the cell surface and is followed by either degradation, if the receptor is directed to the lysosomal compartment, or reinsertion at the cell surface through a specialized endosomal compartment called recycling endosomes. Although the basic steps of this process have been discovered, the details and participation of additional regulatory proteins are still being discovered. In this review article, we describe the most recent findings shedding light on this crucial mechanism of synaptic regulation.

Keywords: AMPA-Rs recycling, LTP, LTD, recycling endosomes, homeostatic plasticity

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INTRODUCTION

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs) are tetrameric ionotropic receptors made up of preassembled dimers of four different, although highly homologous, subunits: GluA1-4. The most common dimers in the adult central nervous system of mammals are GluA1/2 and GluA2/3 (Huganir and Nicoll, 2013).

AMPA-Rs respond to the binding of the neurotransmitter glutamate by opening their central channel thus leading to the entry of sodium (and calcium if lacking the GluA2 subunit) and the exit of potassium ions allowing for depolarization of the postsynaptic neuron (Scannevin and Huganir, 2000; Henley and Wilkinson, 2016).

These receptors are crucial for basal excitatory transmission and are among the most important in synaptic plasticity phenomena, namely, Hebbian (long term potentiation, LTP or long term depression, LTD) or homeostatic plasticity, since their abundance at the postsynapse regulates the strength of the response to presynaptic release of glutamate (Henley and Wilkinson, 2016).

In particular, the increase of AMPA-Rs is typical, and necessary, for all of the forms of LTP and in the response to prolonged activity blockade. On the other hand, LTD and long-lasting activity enhancement lead to AMPA-R reduction at the postsynapse (Huganir and Nicoll, 2013).

Apart from the insertion of receptors newly synthesized (either distally or locally; Ju et al., 2004), two main pathways are exploited by neurons to regulate AMPA-R presence: lateral surface membrane diffusion from extrasynaptic sites (Tardin et al., 2003; Groc et al., 2004) and cycling between synaptic surface and intracellular endosomal compartments (Shi et al., 1999).

In this review article, we will focus our attention on the latter of the two processes since it is probably the one that has been most extensively described in the literature (for other reviews, see Hirling, 2009; Hanley, 2010; van der Sluijs and Hoogenraad, 2011; Henley and Wilkinson, 2013; Widagdo et al., 2017).

AMPA-Rs, similarly to many other surface proteins, are not only localized at the postsynaptic density but are also found in intracellular compartments such as in early endosomes, where they localize upon endocytosis, in late endosomes, where they are directed for degradation, and in recycling endosomes, specialized organelles that are able to translocate to the cell surface for delivery of transmembrane proteins (Scannevin and Huganir, 2000; Henley and Wilkinson, 2016). This system, namely, the endosomal system, is crucial for the regulation of surface proteins levels in almost every cell of the human body (Maxfield and McGraw, 2004).

This pathway leads to the existence of a continuous cycling of AMPA-Rs between these compartments and provides neurons with a pool of inactive intracellular receptors that are ready to be replenished or rapidly delivered upon stimuli such as synaptic plasticity (Hirling, 2009).

Mammalian cells present two pathways of recycling, one named "long loop" that involves the transport of endocytosed molecules to the pericentriolar endosomal system and one named "short loop" in which proteins are locally redirected back to the plasma membrane (Li and DiFiglia, 2012).

In the short loop, which in neurons can occur in close proximity to dendritic spines, endocytosed proteins are localized in a functional compartment named "sorting endosomes" in which their fate is decided. The pH of this compartment is acidic enough (pH $\sim$ 6) to dissociate the majority of ligands from their receptors (Maxfield and McGraw, 2004). The sorting endosome is composed of large vacuoles, which could mature and fuse with the late endosomal compartment, and of tubular structures, which are thought to become part of the recycling endosomal compartment (Li and DiFiglia, 2012).

Although exclusive markers for recycling endosomes are lacking, different proteins have been shown to participate in the function of recycling endosomes and are usually used to identify this compartment.

The most important of these is likely the small GTPase Ras-related protein Rab11 (Ren et al., 1998). The function of Rab11 is mediated by different effectors that include Rab11 family interacting proteins (Rab11-FIPs; Hales et al., 2001) and the motor proteins MyosinVa/b, which are thought to be the transporter of recycling endosomes (Lapierre et al., 2001; Hales et al., 2002) and are also specifically involved in AMPA-Rs recycling (Correia et al., 2008). Rab8 and Rab35 GTPases have been found to participate in the exocytosis of recycling endosomes (Kouranti et al., 2006; Brown et al., 2007; Jullié et al., 2014).

Neuron-specific features of exocytosis of recycling endosomes have been discovered. In addition to common rapid exocytosis and release of membrane proteins to the plasma membrane, neurons present to a greater extent a second modality of exocytosis named persistent or display (Jullié et al., 2014). In this modality, the recycling endosomes are fused in a "kiss and run" fashion with opening and closure of a fusion pore. This mechanism leads to the retention of receptors in the membrane of recycling endosome and thus in restricted areas of the plasma membrane. This phenomenon is the most prevalent when observing recycling endosomes containing the Transferrin Receptor, AMPA-Rs and β2 adrenergic receptor (Jullié et al., 2014). Evidence also suggests that recycling endosomes are subdivided into different pools containing different receptors (i.e., AMPA-Rs and β2 adrenergic receptors; Jullié et al., 2014). Many adaptor proteins participate in regulating all the steps of AMPA-R cycling (for review see Hirling, 2009; Anggono and Huganir, 2012; Bassani et al., 2013).

Our review article will focus on the most recent findings obtained both on the definition of the mechanism of AMPA-R recycling and on newly discovered adaptor proteins in basal constitutive recycling and recycling in synaptic plasticity (**Figure 1**).

BASAL CONSTITUTIVE RECYCLING

Basal recycling is the cycling of AMPA-Rs between the plasma membrane and the endosomal compartment that occurs under basal conditions, independently from synaptic plasticity. AMPA-Rs are believed to undergo endocytosis primarily through clathrin and dynamin (Carroll et al., 1999; Man et al., 2000; Anggono and Huganir, 2012), although a clathrin-dynamin-independent endocytosis mechanism relying on actin dynamics has been observed (Glebov et al., 2015). Endocytosis is thought to occur in the Endocytic Zone (EZ), a region localized just outside the postsynaptic density (Lu et al., 2007).

Once internalized, the receptors might be relocated to recycling endosomes for delivery back to the plasma membrane or to the lysosomal compartment for degradation.

While this is a general process of AMPA-R basal recycling, it is important to note that there are AMPA-R subunit specific mechanisms which will be discussed later in "Basal Constitutive Recycling" section.

The next paragraph will focus on the study of newly discovered adaptor proteins that regulate AMPA-R recycling.

Recent work has elucidated part of the basal motor proteins involved in the delivery of AMPA-R-containing recycling endosomes to the plasma membrane (Esteves da Silva et al., 2015). Esteves da Silva et al. (2015) have taken advantage of the recently developed chemically inducible dimerization system FRB-FKBP (Kapitein et al., 2010) to induce binding between motor proteins and Rab11-positive recycling endosomes that contain AMPA-Rs. This study showed that the microtubule motor KIF1C and the actin motor Myosin V are both involved in this process in accordance with previous findings (Setou et al.,

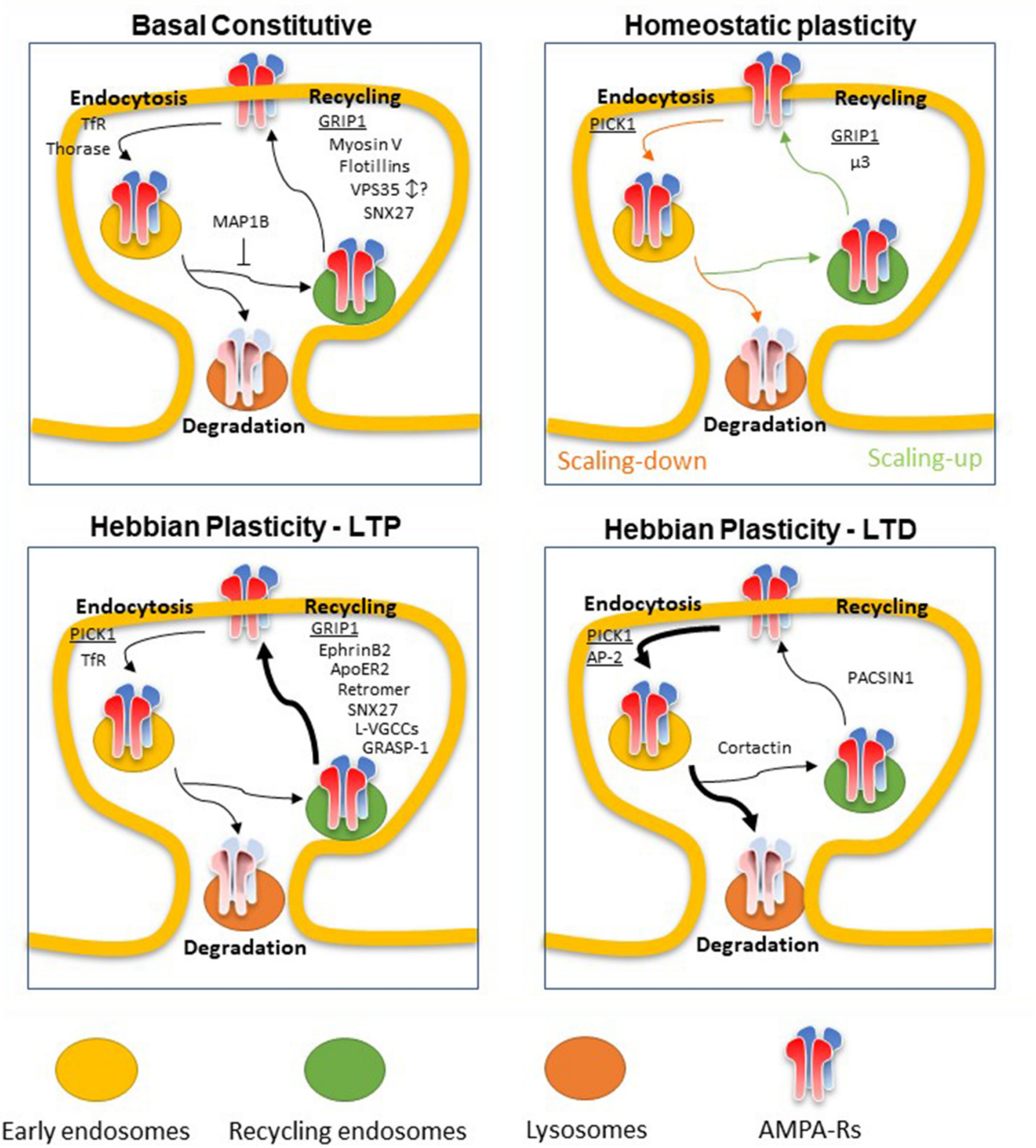

FIGURE 1 | Scheme of newly discovered adaptors of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs) recycling in Basal constitutive condition or upon Homeostatic or Hebbian (long term potentiation, LTP or long term depression, LTD) plasticity. Major proteins involved in AMPA-R recycling are underlined. Basal constitutive: AMPA-Rs are constitutively endocytosed and sorted between lysosomes for degradation or recycling endosomes for recycling back to the plasma membrane. Glutamate interacting protein 1 (GRIP1) is one of the main adaptors driving AMPA-R exocytosis by binding to the C-terminal tail of GluA2. Myosin V is the main actin motor involved in delivery of AMPA-Rs-containing recycling endosomes to the plasma membrane. Flotillins Reggie-1 and -2 promote the recycling of AMPA-Rs whereas TfR is mainly involved in its endocytosis in basal condition. Thorase was found to regulate AMPA-R endocytosis by disrupting the interaction between GRIP1 and AMPA-R subunit GluA2. The retromer complex, and more specifically vacuolar sorting proteins 35 (VPS35), and its adaptor SNX27 have been found to participate in AMPA-R recycling in basal condition. SNX27 was found to positively regulate the process whereas discording results have been found for VPS35. MAP1B was found to retain AMPA-Rs away from dendritic spines preventing the entry into the recycling endosomal system by interacting with GRIP1. Homeostatic plasticity: depending on whether the plasticity is a synaptic scaling-up (after chronic activity blockade, green arrows) or scaling-down (after chronic activity enhancement, orange arrows), AMPA-Rs are mainly internalized, with the help of PICK1 binding to GluA2 C-terminal tail and degraded or recycled back to the surface membrane thanks to GRIP1 action, respectively. μ3 subunit of the adaptor complex AP-3A promotes the recycling of AMPA-Rs to the plasma membrane in scaling-up phenomena. Hebbian plasticity-LTP: LTP induces an increase in synaptic abundance of AMPA-Rs mainly promoting the exocytosis of an internal pool of receptors. GRIP1 is one of the main adaptor proteins exerting this action whereas PICK1 has been found to induce GluA2-containing AMPA-R endocytosis also upon LTP stimuli, possibly to allow the temporary substitution with CP-AMPA-Rs. The retromer complex and its adaptor SNX27 have a positive

(*Continued*)

FIGURE 1 | Continued
effect on AMPA-R exocytosis during LTP. GRIP associated protein 1 (GRASP1), ApoER2 and ephrinB2 also promote AMPA-R surface delivery, through the interaction with GRIP1. L-VGCCs have been shown to have a crucial role, via the increase of intracellular calcium, in causing a complete fusion of recycling endosomes to the plasma membrane upon LTP stimuli. Hebbian plasticity-LTD: Long term depression is induced and maintained by an increase in internalization rates of AMPA-Rs followed by lysosomal degradation. PICK1 is crucial in AMPA-R removal from the surface membrane through its interaction with GluA2 and with the adaptor protein complex AP-2. Cortactin association with AMPA-Rs was found to counteract this process promoting the sorting of the receptors toward recycling endosomes. PACSIN1 was involved in promoting recycling endosomes exocytosis thus acting against the induction and maintenance of LTD.

2002; Wang et al., 2008). The motor myosin VI was instead found to participate in the removal of Rab11-positive recycling endosomes away from the synapse. In agreement with the well-known role of AMPA-Rs in regulating the strength of excitatory synapses, forcing the removal of Rab11-positive recycling endosomes from dendritic spines led to a general reduction of synapse strength both functionally and structurally.

In recent years, most of the studies have focused on the investigation of adaptors or regulatory proteins acting on AMPA-R recycling.

Among recycling regulatory proteins whose actions affect AMPA-Rs trafficking nonspecifically, the Rab11A binding proteins Reggie-1 and -2 were recently investigated in cultured hippocampal neurons and in a knockout mouse model (Bodrikov et al., 2017). These proteins, also known as flotillins, reside in lipid rafts and were previously found to bind Rab11A and SNX4, exerting crucial roles in the recycling of Transferrin Receptor, E-Cadherin, α5 and β1 integrins and T-Cell receptor in various cell lines (Stuermer et al., 2004; Stuermer, 2010; Solis et al., 2013; Hülsbusch et al., 2015) and in the sorting of N-Cadherin to the growth cone in neurons (Bodrikov et al., 2011). The exact mechanism of action of flotillins on Rab11A and/or SNX4 activities remains to be determined.

The authors discovered that the absence of Reggie proteins was linked with impaired recycling of AMPA-R subunit GluA1 together with *N*-methyl D-Aspartate (NMDA) receptor subunit GluN1 and N-cadherin under basal condition in neurons. A reduction of PSD-95 was also observed, leading to the hypothesis that Reggie proteins are general players in Rab11A-mediated recycling of synaptic proteins. This was also corroborated by the observation that the defects could be reversed by the overexpression of a constitutively active Rab11A (Bodrikov et al., 2017).

A recent study surprisingly discovered a regulatory effect of the TfR protein in the cycling of AMPA-Rs in neurons (Liu et al., 2016).

TfR, which binds to diferric transferrin and is crucial for iron homeostasis in the body, is highly expressed in neurons (Moos, 1996) and undergoes a high rate of cycling between the cell surface and the endosomal compartment in basal condition (West et al., 1997). For this reason, TfR has often been used as a control protein for studies of endocytosis and recycling of synaptic proteins, including AMPA-Rs. In this recent work, the absence of TfR appeared to cause a decrease in the association between AMPA-R subunit GluA2 with the endocytosis adaptor AP-2, thus slowing AMPA-R endocytosis. On the other hand, the recycling of AMPA-Rs occurred at faster rates with the net effect of higher levels of both GluA1 and GluA2 on the surface membrane. However, the authors propose that TfR acts indirectly on AMPA-Rs through regulation of the interaction of the latter with AP-2 (Liu et al., 2016). This possible competition mechanism remains to be directly demonstrated, and the possibility of more general unspecific defects of the recycling machinery upon TfR knockout needs to be ruled out.

The retromer complex is another group of proteins that have been shown to regulate the recycling machinery (Bonifacino and Hurley, 2008) and recent work has elucidated its effect on AMPA-R trafficking.

This complex, made up of the assembly of vacuolar sorting proteins 35 (VPS35), VPS26, VPS29 and different adaptor proteins, acts by sorting transmembrane protein away from lysosomal degradation for recycling from the endosomal compartment to the trans-Golgi network or to the plasma membrane (Bonifacino and Hurley, 2008). AMPA-Rs have been identified as one of the cargo of the retromer complex as demonstrated by the decrease in AMPA-evoked currents upon knockdown of VPS35 (Choy et al., 2014).

Different studies have linked the retromer complex to neurological and neurodegenerative conditions (Small and Petsko, 2015). Recently, the p.D620N mutation in VPS35 has been found to be associated with Parkinson's disease (Munsie et al., 2015). This mutant protein showed a loss of function effect lacking the activity of wild-type VPS35 in decreasing AMPA-mediated transmission both in mouse cortical neurons and dopaminergic neurons derived from human patients. VPS35 was shown to interact with GluA1, to a greater extent compared with GluA2, suggesting subunit specificity of the complex. This selective action on GluA1 also supports a more prominent role of the retromer complex in activity-dependent recycling of AMPA-Rs.

Apparently discording results have been observed in a mouse model in which VPS35 was in heterozygosity. Decreased levels of VPS35 were associated with impaired AMPA-Rs trafficking to the cell surface, with a more relevant effect on GluA1 and a consequent defect in dendritic spines density and maturation in the CA1 region of the hippocampus. Interestingly, these effects on dendritic spines were reverted by GluA2 overexpression (Tian et al., 2015).

The similarity of effects on AMPA-Rs upon opposite modulation of VPS35 levels suggests that the exact stoichiometry of VPS35 is crucial for an efficient action on AMPA-Rs recycling. Additional effort is needed in order to clarify this aspect.

Sorting nexin 27 has been identified as one of the adaptor proteins that regulate cargo binding of the retromer complex (Temkin et al., 2011). As other members of the sorting nexin family, it presents a phox-homology domain (PX) that binds phosphatidylinositol phosphate (PIP; Teasdale and Collins, 2012) and can thus interact with either endosomes or the plasma membrane. SNX27 also presents a peculiar PDZ domain through

which it can interact with GluA2 and GluA1 C-termini (Hussain et al., 2014). In addition, mutations or deficits in SNX27 have been associated with different neurological conditions in which impaired AMPA-mediated transmission plays a critical role (Wang et al., 2013; Damseh et al., 2015; Zhang et al., 2018). SNX27 positive regulation of AMPA-Rs exocytosis in cultured hippocampal neurons was assessed by surface staining upon either overexpression or knockdown of SNX27 and a role in this process was established for both the PX and PDZ domains (Hussain et al., 2014).

Glutamate interacting protein 1 (GRIP1) is believed to be one of the major adaptor proteins regulating the fate of AMPA-Rs during all steps of intracellular trafficking thanks to the binding of the C-termini of the receptor through its PDZ domains (Dong et al., 1997; Anggono and Huganir, 2012). GRIP1 was shown to also interact directly with the kinesin motors (Setou et al., 2002) or in a complex with liprin-α (Wyszynski et al., 2002), promoting AMPA-R transport to dendrites and dendritic spines. In addition, its interaction with NEEP21 has been involved in AMPA-R sorting and more specifically recycling (Alberi et al., 2005; Steiner et al., 2005). Although the major role of GRIP1 in promoting AMPA-Rs exocytosis is generally accepted, other contrasting effects have been observed when interfering with GRIP1 function with apparent regulation of endocytosis and intracellular retention (Daw et al., 2000; Lu and Ziff, 2005). The picture is complicated by the fact that the GRIP1 binding site on the GluA2 C-terminus is shared with PICK1, a protein well known for negatively regulating AMPA-R surface levels by enhancing internalization and retention. The selectivity of the binding is regulated by the phosphorylation status of Serine 880 and of Tyrosine 876 of GluA2 C-terminal tail (Matsuda et al., 1999; Chung et al., 2000; Fu et al., 2003; Hayashi and Huganir, 2004).

Recent work has elucidated another level of complexity of this sophisticated machinery. GRIP1 has been found to present the ability to bind simultaneously both AMPA-Rs and N-Cadherin and associating them with the KIF5 motor protein, thus promoting their contemporaneous dendritic delivery, which would provide synapses instantly with a more complete machinery for establishment and maturation (Heisler et al., 2014). Other work has elucidated the already described (Seog, 2004) unusual interaction between GRIP1 and the MAP1B light chain in regulating AMPA-R trafficking under basal conditions (Palenzuela et al., 2017). MAP1B decorates microtubules along the dendrites (Halpain and Dehmelt, 2006) and is directly involved in dendritic spines morphogenesis (Tortosa et al., 2011). MAP1B light chain overexpression was shown to impair AMPA-mediated currents due to a specific reduction in the surface levels of the GluA2 subunit of AMPA-Rs. This effect, which was not associated with any defects in LTP, was likely caused by a reduction in dendritic targeting of GRIP1 that is proposed to be trapped by MAP1B outside the dendritic spine. This mechanism describes an unusual role for GRIP1in reducing synaptic delivery of AMPA-Rs. However, further investigation is needed, especially because a MAP1B mutant lacking the microtubule binding domain was shown to retain the ability to impair AMPA-mediated currents, arguing against the binding of MAP1B to microtubules as the trapping mechanism for GRIP1-GluA2 complexes (Palenzuela et al., 2017).

AMPA-R association with GRIP1 has recently been found to also be regulated by the protein Thorase, an AAA+ ATPase encoded by the ATAD1 gene (Zhang et al., 2011). This mechanism is of particular interest considering that different mutations in the ATAD1 gene have been found in patients affected by lethal encephalopathy (Ahrens-Nicklas et al., 2017; Piard et al., 2018). Thorase was proposed to become part of the complex between GRIP1 and GluA2 to disrupt their interaction after ATP hydrolysis and promote AMPA-R endocytosis (Zhang et al., 2011).

Accordingly, thorase knockdown or knockout, and encephalopathy-associated mutations, were found to impair AMPA-R internalization, causing increased levels of the receptor on the surface membrane, thus leading to the exaggerated excitatory transmission typical of epilepsy (Zhang et al., 2011).

One of the most elusive aspects of AMPA-R trafficking is the specificity of the various mechanisms and adaptors identified for different AMPA-R subunits.

As stated above, the most abundant AMPA-R in the adult mammalian central nervous system is made up of GluA1/GluA2 and GluA2/GluA3 dimers (Wenthold et al., 1996; Lu et al., 2009; Huganir and Nicoll, 2013) with a minor presence of GluA1/GluA1 homomers and GluA1/GluA3 heteromers at the postsynapse in basal condition. The presence of GluA2 in most of the receptors leads to technical difficulties in identifying specific endogenous mechanisms. In addition, there has been lower interest in elucidating properties of the GluA3 subunit. Previous studies have investigated the differential properties of GluA1 and GluA2 in trafficking and function (Passafaro et al., 2001; Shi et al., 2001).

However, most of the studies published do not address the complete subunit composition of the tetrameric receptors being analyzed.

As a whole, the literature mainly suggests that GluA2/GluA3-containing AMPA-R undergo rapid constitutive cycling between the synapse surface and the endosomal compartment, whereas GluA1-containing receptors are slowly recycled in basal conditions and are more effectively transported upon stimulation such as LTP (Passafaro et al., 2001; Shi et al., 2001).

Different studies have suggested the existence of another dimer: the GluA1/GluA1 homomer (Plant et al., 2006; Jaafari et al., 2012). The absence of GluA2 in this receptor allows it to become permeable to calcium ions and classified GluA1 homomers as Calcium-permeable AMPA-Rs (CP-AMPA-Rs) in contrast to GluA2-containing Calcium-impermeable AMPA-Rs (CI-AMPA-Rs). Current models predict that CP-AMPA-Rs are rarely present at the synapse under basal conditions in the adult brain, whereas they are rapidly delivered in the first moments of potentiating synaptic plasticity phenomena and then quickly reinternalized and substituted by CI-AMPA-Rs (Hanley, 2014). This implies that CP-AMPA-Rs are already assembled and rapidly released from an intracellular compartment, possibly the recycling endosomes. Variations in CP-AMPA-R levels have been seen in brain development and found to be associated with different

pathological conditions including neuronal ischemia and cocaine addiction (Jaafari et al., 2012; Yuan and Bellone, 2013; Hanley, 2014). However, the precise mechanisms regulating CP-AMPA-Rs trafficking still need further clarification since most of the identified adaptor proteins are GluA2-specific interactors or binds indistinctly both GluA2 and GluA1.

A recent study addressed this topic in medium spiny neurons of the nucleus accumbens in mice (Werner et al., 2017). In these neurons, the accumulation of CP-AMPARs was demonstrated as a response to prolonged withdrawal after cocaine administration (Conrad et al., 2008). Werner et al. (2017) discovered that CP-AMPARs undergo faster endocytosis and recycling compared with CI-AMPARs in this paradigm.

However, further studies are needed to elucidate the specific properties of different AMPA-R tetramers to gain more precise knowledge of the behavior of these proteins.

SYNAPTIC PLASTICITY

Homeostatic Plasticity

Homeostatic plasticity, also referred to as synaptic scaling, is a phenomenon of synaptic potentiation or depression that occurs upon a long-lasting decrease or increase of synaptic responses that can be reproduced *in vitro* by chronic administration of Tetradotoxin (TTX) or Bicuculline, respectively (Turrigiano, 2008).

Synaptic potentiation or depression occur through the up- or downregulation, respectively, in the abundance of AMPA-Rs at the plasma membrane, and this synaptic scaling is strongly regulated by endocytosis or recycling of AMPA-Rs (Wierenga et al., 2005; Gainey et al., 2009).

These plasticity phenomena are mainly believed to be adaptation responses to chronic, non-physiological stimuli, to restore a normal circuit signaling.

The opposing roles of GRIP1 and PICK1 strongly participate in regulating AMPA-R-levels under basal condition (see "Basal Constitutive Recycling" section above) and in Hebbian plasticity phenomena through their interaction with the GluA2 C-terminal tail (see "Basal Constitutive Recycling" section). More recently, these proteins have been involved in homeostatic plasticity with similar antithetical effects. PICK1 was shown to be crucial in homeostatic downscaling as these phenomena appeared occluded in cultured neurons from PICK1 knockout animals (Anggono et al., 2011). On the other hand, GRIP1 binding to GluA2 was enhanced in synaptic upscaling (Gainey et al., 2015; Tan et al., 2015).

A recent study on CP- and CI-AMPA-Rs cited above (Werner et al., 2017) also investigated the differential contribution of these receptors in synaptic scaling phenomena. Both long-lasting activity blockade and enhancement appeared to affect CI-AMPA-Rs to a greater extent compared with CP-AMPA-Rs, inducing increased recycling and exocytosis in the scaling-up plasticity and increased endocytosis in scaling-down phenomena.

Another interesting study pointed out the importance of the μ subunit of AP-3 complex in regulating AMPA-R recycling in mice after sensory deprivation, an *in vivo* correlate of synaptic scaling-up (Steinmetz et al., 2016). AP-3 belongs to a family of adaptor proteins, the adaptor protein complexes (APC), that are well-known regulator of endosomal trafficking by acting as vesicle coats (Bonifacino, 2014; Guardia et al., 2018). There are five known adaptor protein complexes (AP-1, -2, -3, -4, -5), all of them composed of four different subunits (Bonifacino, 2014; Guardia et al., 2018). AP-2, AP-3 and AP-4 have been shown to affect AMPA-R transport in the endolysosomal system (Burbea et al., 2002; Lee et al., 2002; Margeta et al., 2009; Matsuda et al., 2013). AP-3A was previously found to be involved in directing AMPA-Rs towards degradation in the lysosomal compartment upon LTD stimulation through the interaction with the transmembrane AMPA-R regulatory protein (TARP) stargazin (Matsuda et al., 2013). Steinmetz et al. (2016) through a transcriptomic analysis in pyramidal neurons of Layer 4 of the visual cortex after sensory deprivation, found that the transcription of the μ subunit of AP-3A was increased. In contrast to our knowledge of the adaptor protein complex family where the subunits are believed to be obligated tetramers, the μ3 subunit appears to act independently from the complex to recruit AMPA-Rs to the recycling endosomes.

Hebbian Plasticity

Hebbian plasticity is considered the biological correlates of learning and memory. It refers to the ability of a pattern of stimuli with precise frequency and intensity to elicit the potentiation (LTP) or depotentiation (LTD) of synapses, reinforcing or weakening specific circuit connections. Both LTP and LTD rely on AMPA-R trafficking to modify the synaptic strength with increased surface delivery or endocytosis and degradation, respectively.

Hebbian Plasticity—LTP

Different forms of LTP exist physiologically with the main one being dependent on NMDA-Rs. NMDA-R activation causes the increase of intracellular calcium, which activates a series of signaling cascades leading to the insertion of more AMPA-Rs at the postsynapse (Nicoll et al., 1988; Huganir and Nicoll, 2013). These insertion events take place through different mechanisms including increased exocytosis of recycling endosomes (Park et al., 2004; Huganir and Nicoll, 2013).

Recently, a new LTP mechanism relying on the activation of a metabotropic activity of kainate receptors to induce increased surface delivery of AMPA-Rs was identified (Petrovic et al., 2017). Kainate receptors are glutamate receptors that act in concomitance with AMPA-Rs in producing the depolarization of the postsynaptic neuron (Carta et al., 2014). The amplitude of their responses is generally lower compared with that of AMPA-Rs. Surprisingly, the authors identified a new mechanism of LTP induction based on the kainate receptor-mediated action of a G protein, not yet identified, that induces a signaling cascade of activation of protein Kinase C and Phospholipase C with the concluding effect of liberating recycling endosomes-containing AMPA-Rs and thus potentiating the responses to glutamate.

A very interesting study further investigated the molecular mechanism underlying these phenomena in chemical-LTP

stimulated cultured neurons (Hiester et al., 2017). This work highlighted the need for a secondary calcium release through L-type voltage gated calcium channels (L-VGCCs) for the complete fusion of AMPA-R-containing recycling endosomes to the plasma membrane preventing its resealing without content release. Activation of NMDA-Rs without the subsequent activation of L-VGCCs, as with the application of specific inhibitors nimodipine, verapamil and diltiazem, appears to provide only the initial fusion of the vesicles with the membrane thus impairing the instauration of potentiation phenomena. Interestingly, the authors also showed, through high-resolution imaging experiments, that each synapse can contain multiple TfR-positive recycling endosomes.

The retromer complex, already introduced in the "Basal Constitutive Recycling" section, has been shown to also play a role in LTP-induced AMPA-R delivery. Very interestingly, depletion of the VPS35 subunit of the retromer complex *in vivo* in adult mice through lentiviral delivery of Sh-RNA caused the block of either NMDA-Rs- or L-Type Ca^{2+} channel-dependent LTP phenomena without affecting LTD (Temkin et al., 2017). These results are in agreement with the role of the retromer in AMPA-R delivery to the plasma membrane. However, in contrast to what described in the previous section, Temkin et al. (2017) did not observe any defect in basal AMPA-R-mediated transmission or in homeostatic plasticity-like treatment with retinoic acid. These findings argue against a general role for the retromer complex in all AMPA-R exocytosis phenomena as previously reported (Choy et al., 2014) and suggest on the other hand an LTP-specific involvement. Differences in model used possibly explain these inconsistencies; however, further research is needed to clarify these aspects.

Furthermore, the retromer adaptor SNX27 has recently been involved in LTP-driven AMPA-R delivery (Hussain et al., 2014; Loo et al., 2014). The work from Hussain et al. (2014) showed that the knockdown of SNX27 in cultured rat cortical neurons was sufficient to abolish the increase in surface-exposed GluA1 upon glycine treatment-induced chemical-LTP (Hussain et al., 2014). Loo and coworkers investigated a possible mechanism by which SNX27 exert its action on AMPA-R delivery. They observed that, shortly after glycine treatment, the membrane bound GTPase K-Ras, which is bound by Ca^{2+}-activated Calmodulin (Villalonga et al., 2001; Wu et al., 2011), increases its interaction with SNX27. Concomitantly, the same stimulus enhanced SNX27 interaction with the GluA1 AMPA-R subunit and its surface delivery. The authors suggest a direct link between all these proteins in connecting the increase in Ca^{2+} concentration that follows LTP stimuli to increased GluA1 surface; however, direct evidences for the existence of this multimeric complex is needed.

TfR activity on AMPA-R recycling during LTP phenomena was evaluated in the study cited in the "Basal Constitutive Recycling" section (Liu et al., 2016). The authors took advantage of super-ecliptic pHluorins to study internalization and recycling of GluA1 and GluA2 subunits of AMPA-Rs in TfR knockout mouse-derived neurons upon brief NMDA application, a treatment that is known to trigger AMPA-R internalization. Both subunits showed impairment in endocytosis and faster recycling, which is in accordance with the observed increase in the surface level of the receptor.

However, quite surprisingly, LTP was reduced in intensity in CA1 synapses of TfR knockout mice. This might be an effect of LTP occlusion with the levels of AMPA-Rs being too high to be further enhanced, although this would have generated an increased input-output relation that was, instead, decreased. Further investigations are needed to clarify this aspect.

A specific function in LTP for GRIP1-mediated AMPA-Rs insertion, already introduced in the previous chapters, involving the interaction between ephrinB2 and ApoER2 has recently been described (Pfennig et al., 2017). ApoER2 act as a receptor for the secreted extracellular matrix protein Reelin to exert its action in promoting neuron maturation and positioning during migration through Dab1 (D'Arcangelo et al., 1999; Trotter et al., 2013). EphrinB2 was shown to participate in this Reelin-dependent mechanism (Sentürk et al., 2011). This pathway was also involved in hippocampal synaptic plasticity through the recruitment of GRIP1 by ephrinB2 (Essmann et al., 2008). In this work, Pfennig et al. (2017) showed that the multimeric complex between Serine-9 phosphorylated ephrinB2, ApoER2, GRIP1 and GluA2 was recruited under the condition of enhanced neuronal activity following KCl treatment of cultured neurons. Interestingly, in mice, reduction of levels of either ApoER2 or GRIP1 and expression of a mutant ephrinB2 deficient for Serine 9 phosphorylation all caused slight impairments in LTP induction and maintenance, whereas the simultaneous presence of all these modulations greatly enhanced the LTP defects (Pfennig et al., 2017).

Another very interesting article was published on the role of GRIP associated protein 1 (GRASP1) in AMPA-R recycling and LTP (Chiu et al., 2017). GRASP1 is a neuronal-specific Ras-GEF that interacts with GRIP1 and AMPA-Rs (Ye et al., 2000), which has been proposed to promote the transition from Rab4-positive early endosomes to Rab11-positive recycling endosomes (Hoogenraad and van der Sluijs, 2010; Hoogenraad et al., 2010). In this work, Chiu et al. (2017) characterized a GRASP1 knockout mouse identifying the crucial role of the GRASP1, GRIP1 and AMPA-Rs association in allowing correct trafficking of the receptor. Animals deprived of GRASP1 indeed showed impairment in the surface levels of AMPA-R subunits GluA1 and GluA2 and defects in LTP induction and spatial memory behavioral tests. In addition, glycine treatment, an LTP-like stimulus, was observed to potentiate the association between the three proteins in cultured neurons.

Two different GRASP1 mutations, found in patients affected by intellectual disability, were also investigated. The mutations, although having opposite effects on the levels of interaction between GRASP1 and GRIP1, produced the same downstream reduction in surface levels of AMPA-Rs, suggesting a tightly regulated mechanism (Chiu et al., 2017).

Another group found GRASP1 in the context of AMPA-R recycling and LTP (Lu et al., 2017). The authors identified GRASP1 as a key protein whose transcription is regulated

by the translational regulator cytoplasmic polyadenylation element binding protein 2 (CPEB2). As expected, the absence of CPEB2 in a knockout mouse model led to reduction in the levels of GRASP1 and secondarily to a decrease in the surface level of AMPA-Rs, impaired LTP and defective performances in spatial and contextual fear memory tests.

Hebbian Plasticity—LTD

LTD induction is usually followed by a rapid internalization of AMPA-Rs with a consequent rerouting of the receptors towards the lysosomal compartment for degradation, thus preventing their entrance in the recycling endosomes system (Lüscher and Malenka, 2012; Huganir and Nicoll, 2013).

One of the most important player in these events is PICK1 (Hanley, 2008). This protein, as introduced in the "Basal Constitutive Recycling" section, binds to the C-terminus of GluA2 AMPA-R subunit and promotes its internalization (Xia et al., 1999; Perez et al., 2001; Terashima et al., 2004); this interaction was shown to be necessary for the induction of NMDA-R-dependent LTD (Kim et al., 2001; Terashima et al., 2008). On the other hand, PICK1 has also been shown to promote the intracellular retention of GluA2 in recycling endosomes, thus allowing maintenance of depotentiation phenomena (Lin and Huganir, 2007; Madsen et al., 2012). On the other hand, PICK1 was also associated with increased surface delivery of CP-AMPA-Rs which could explain why its overexpression induces synaptic potentiation phenomena that occludes further LTP induction whereas its knockdown blocks LTP (Terashima et al., 2004, 2008; Clem et al., 2010). A detailed mechanism of the role of PICK1 in these processes is still lacking; the most likely hypothesis is that PICK1-mediated removal of GluA2 occurs both in LTP (only in the first phases) and LTD (permanently) with the receptor being substituted by CP-AMPA-Rs only in the first case with a mechanism that still needs to be elucidated.

Recently, Fiuza et al. (2017) further elucidated the mechanism through which PICK1 mediates GluA2 removal from the surface membrane. The authors showed that PICK1 is recruited to clathrin-coated pits by interacting with the adaptor protein complex AP-2, an association enhanced by LTD-like NMDA-R activation. In addition, PICK1 was also able to promote dynamin polymerization by interacting with its GTPase domain. This work interestingly suggests that PICK1 not only links AMPA-Rs to the endocytic machinery to enhance its internalization but that it is directly able to promote the activity of two crucial players in endocytosis events, AP-2 and dynamin (Fiuza et al., 2017).

Another protein, protein kinase C and casein kinase II substrate in neurons (PACSIN1), was recently found to regulate AMPA-R recycling that occurs after LTD-like phenomena (Widagdo et al., 2016). PACSIN1 was already known to regulate endocytosis of GluA2 after NMDA-R stimulation through interaction with PICK1 in a phosphorylation-dependent manner (Anggono et al., 2013). The authors here identified a novel specific role of PACSIN1 in the recycling step of AMPA-Rs that follows internalization after NMDA-Rs stimulation. This activity was also mediated by the interaction of PACSIN1, through specific serine residues, with PICK1, which once again shows a bidirectional role in being able to regulate both internalization and exocytosis.

The protein Cortactin, which was previously associated with actin dynamics, was recently identified to bind the AMPA-R subunit GluA2, regulating its fate in the endo-lysosomal system (Parkinson et al., 2018). Cortactin was found to prevent specifically GluA2/GluA3-containing AMPA-R from being directed towards the lysosomal compartment and subsequently degraded in basal conditions, possibly by retaining them in the early endosomes and thus favoring their redirection to recycling endosomes. As expected, this association appeared to decrease in the context of a chemical LTD protocol, where GluA2-containing receptors are rapidly degraded. On the other hand, cortactin knockdown impaired the induction of LTD, likely due to an occlusion mechanism caused by reduced levels of surface AMPA-Rs.

CONCLUSION

Although AMPA-R recycling has been known for a long time now (Huganir and Nicoll, 2013), many details are still missing for a full picture to be obtained. The crucial role that the existence of this continuously cycling pool of receptors plays in the phenomena of synaptic plasticity, which are considered the biological correlates of the higher cognitive function of learning and memory, makes our in-depth understanding of this pathway extremely important. The importance of these processes is also inferable by the high number of recently published manuscripts that describe newly discovered adaptor proteins or new details of the exocytosis mechanism.

Thus, more efforts are needed to fully describe the mechanisms that produce the basal constitutive cycling of the receptor to then better characterize the molecular pathways that exploit this pool for all synaptic plasticity phenomena.

We would like to underline the importance of a comprehensive analysis of the AMPA-R subunits behavior when studying adaptor proteins, to further elucidate the specific mechanisms regulating different AMPA-R tetramers.

AUTHOR CONTRIBUTIONS

EM and MP conceived, wrote and revised the manuscript.

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REVIEW
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The Regulation of AMPA Receptor Endocytosis by Dynamic Protein-Protein Interactions

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The precise regulation of AMPA receptor (AMPAR) trafficking in neurons is crucial for excitatory neurotransmission, synaptic plasticity and the consequent formation and modification of neural circuits during brain development and learning. Clathrin-mediated endocytosis (CME) is an essential trafficking event for the activity-dependent removal of AMPARs from the neuronal plasma membrane, resulting in a reduction in synaptic strength known as long-term depression (LTD). The regulated AMPAR endocytosis that underlies LTD is caused by specific modes of synaptic activity, most notably stimulation of NMDA receptors (NMDARs) and metabotropic glutamate receptors (mGluRs). Numerous proteins associate with AMPAR subunits, directly or indirectly, to control their trafficking, and therefore the regulation of these protein-protein interactions in response to NMDAR or mGluR signaling is a critical feature of synaptic plasticity. This article reviews the protein-protein interactions that are dynamically regulated during synaptic plasticity to modulate AMPAR endocytosis, focussing on AMPAR binding proteins and proteins that bind the core endocytic machinery. In addition, the mechanisms for the regulation of protein-protein interactions are considered, as well as the functional consequences of these dynamic interactions on AMPAR endocytosis.

Keywords: synaptic plasticity, LTD (long term depression), clathrin, AP2 clathrin adaptor complex, PICK1, protein interacting with C-kinase 1

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INTRODUCTION

Since AMPA receptors (AMPARs) mediate the majority of fast synaptic excitation in the central nervous system, their regulation at the synapse is of fundamental importance to brain function. The formation of neuronal circuits during brain development and their subsequent modification during learning, forgetting and other aspects of memory processes require plasticity at excitatory synapses in the brain, manifested by changes in synaptic strength (Chater and Goda, 2014; Henley and Wilkinson, 2016). Long-term potentiation (LTP; an increase in synaptic strength) and long-term depression (LTD; a decrease in synaptic strength) are synapse-specific (Hebbian) forms of plasticity that have been the subject of intense research for many years and are now considered to be the major mechanisms that underlie such changes (Huganir and Nicoll, 2013). In addition, homeostatic plasticity, also known as synaptic scaling, involves a cell-wide adjustment of synaptic strength to maintain a stable output of a particular neuron during changes in neuronal circuit activity (Fernandes and Carvalho, 2016).

A major component of these forms of synaptic plasticity is the trafficking of AMPARs to or from synapses to increase or decrease the number of AMPARs localized at synapses,

and hence modulate the strength of synaptic transmission. The subject of this review article is AMPAR endocytosis, the consequence of which is the removal of receptors from the neuronal surface and hence from the synapse, leading to a decrease in synaptic strength (LTD). This process is essential for specific types of learning and memory systems (Griffiths et al., 2008; Connor and Wang, 2016; Migues et al., 2016). The precise regulation of AMPAR trafficking and hence of synaptic transmission is critical for the balance between maintaining memories/learned behaviors and modifying memories or storing new ones. In addition, a number of neurological disorders involves aberrant recruitment of AMPAR endocytosis mechanisms. This can cause pathological levels of synaptic depression or the internalization of specific AMPAR subtypes from the synapse as part of a process that results in the synaptic expression of Ca^{2+}-permeable AMPARs, which contribute to neuronal death (Hsieh et al., 2006; Liu et al., 2006; Dixon et al., 2009).

AMPARs are complexes comprising the core pore-forming subunits GluA1–4, as well as an increasing number of auxiliary subunits that play critical roles in regulating various aspects of AMPAR function (Henley and Wilkinson, 2016; Greger et al., 2017; Jacobi and von Engelhardt, 2018). Core and auxiliary subunits are integral membrane proteins and are subject to the basic cell biological trafficking processes of endocytosis, endosomal sorting, recycling and exocytosis that apply to the majority of transmembrane proteins in most mammalian cell types. In this review article, I will discuss the current state of knowledge about specific mechanisms of AMPAR endocytosis, focussing on dynamic protein-protein interactions modulated by signaling pathways downstream of synaptic stimuli that induce long-term changes in synaptic transmission. While much is known about how dynamic protein-protein interactions are orchestrated and regulated in the generalized endocytic process (McMahon and Boucrot, 2011; Daumke et al., 2014) surprisingly few protein interactions have been identified that are regulated by plasticity stimuli to control AMPAR endocytosis, despite the intensity of research into synaptic plasticity mechanisms in the past two decades.

AMPARs are thought to be rarely static, but instead are continually cycling between the synapse and the endosomal system (Luscher et al., 1999; Ehlers, 2000; Lee et al., 2004). In a process thought to be largely driven by the GluA2 subunit and its associated proteins, AMPARs diffuse laterally from the synapse and are endocytosed at plasma membrane sites adjacent to the post-synaptic density (PSD), proposed to be specialized endocytic zones (EZs; Lu et al., 2007; Opazo and Choquet, 2011). Following sorting in the early endosome, AMPARs are either targeted for degradation in lysosomes or recycled to the plasma membrane, with reinsertion taking place away from the PSD and lateral diffusion in the plane of the membrane resulting in the reincorporation of AMPARs at the synapse (Opazo and Choquet, 2011; van der Sluijs and Hoogenraad, 2011). This review article will not discuss the details of AMPAR endosomal sorting, which is also a critical determinant of synaptic strength and is itself subject to regulation as an important aspect of synaptic plasticity. Moreover, it is important to note that experimental quantification of AMPAR "internalization," for example in surface biotinylation or antibody-feeding assays, does not measure endocytosis *per se*, but is confounded by the amount of receptors that are retained in endosomal compartments or recycled to the plasma membrane. For example, dissociating a protein-protein interaction that blocks the NMDA-induced loss of surface AMPARs could be explained by an increase in recycling back to the plasma membrane as well as by a blockade of endocytosis. This review article will focus on mechanisms that have been specifically implicated in regulating AMPAR endocytosis.

LTD is typically induced by stimulation of either NMDA receptors (NMDARs) or metabotropic glutamate receptors (mGluRs), resulting in the activation of numerous Ca^{2+}-dependent signaling cascades (Collingridge et al., 2010). The vast majority of dynamic protein-protein interactions in the regulation of AMPAR endocytosis have been defined in the context of NMDAR-dependent LTD in hippocampal neurons. While NMDAR- and mGluR-dependent forms of LTD are mechanistically similar, they differ in upstream signaling pathways, and consequently in some of the protein-protein interactions involved. However, there is insufficient evidence to completely define the distinct processes of mGluR- and NMDAR-dependent AMPAR endocytosis from the point of view of dynamic protein-protein interactions. While LTD is an important form of synaptic plasticity in the cerebellum as well as in forebrain neurons, hippocampal neurons have been more extensively investigated because at least until very recently, mechanistic cell biology studies have been better suited to cultured neurons than brain slice or *in vivo* preparations, and cerebellar Purkinje neurons are technically difficult to culture compared to hippocampal neurons. However, a number of protein-protein interactions that have been implicated in cerebellar LTD have been more fully defined as playing a role in AMPAR endocytosis in hippocampal neurons, and therefore it could be inferred that they are similarly involved in the cerebellum.

The mechanisms that underlie constitutive AMPAR endocytosis have much in common with activity-dependent endocytosis during LTD from the point of view of the protein-protein interactions involved. In fact, a number of protein-protein interactions that are either required for or restrict constitutive AMPAR endocytosis are up- or down-regulated in order to increase trafficking for LTD, and it is this concept that forms the core of this review. Nevertheless, while the majority of activity-dependent AMPAR endocytosis is thought to be clathrin and dynamin-dependent, some forms of constitutive AMPAR trafficking may proceed via clathrin and dynamin-independent mechanisms (Glebov et al., 2015), the details of which are beyond the scope of this review.

AMPAR subunits interact with a large (and still increasing) number of identified proteins, which facilitate and direct their trafficking between the synapse and the endosomal system. These accessory proteins in turn interact with other binding partners that integrate them into fundamental cell biological systems such as the actin cytoskeleton or the core endocytic machinery. The highly complex process of recruiting AMPARs to sites of

endocytosis, and facilitating their internalization requires the up- or down-regulation of several protein-protein interactions in response to intracellular signaling initiated by NMDAR or mGluR stimulation. While the primary focus of this review is the protein-protein interactions involved in endocytosis *per se*, other interactions that precede endocytosis must be regulated for endocytosis to proceed, so are also discussed here.

DISSOCIATION FROM PSD SCAFFOLDS

The PSD contains a multitude of scaffolding and signaling proteins involved in maintaining and regulating synaptic transmission (Feng and Zhang, 2009). PSD-95 functions as a "slot protein," defining a place for an AMPAR at the synapse, and it is thought that the number of PSD-95 molecules localized to the PSD plays an important role in maintaining the number of AMPARs at that synapse (Opazo et al., 2012; Won et al., 2017). AMPARs interact with the PDZ domains of PSD-95 via the C-terminal tail of transmembrane AMPAR regulatory proteins (TARPs), the most-studied family of AMPAR auxiliary subunit, of which Stargazin is the prototypical member (Chen et al., 2000; **Figure 1A**). The TARP—PSD-95 interaction reduces the lateral mobility of AMPARs at the synapse, and disrupting this interaction allows AMPARs to diffuse away from the synapse, still bound to TARPs (Bats et al., 2007). The TARP—PSD-95 interaction is dynamic and subject to regulation by phosphorylation of a number of serine residues in the TARP intracellular C-terminal domain via an indirect mechanism. Phosphorylation of the TARP C-terminal domain by CamKII inhibits its association with negatively charged phospholipids in the lipid bilayer, which in turn allows binding to PSD-95 and stabilization of receptors at the synapse (Sumioka et al., 2010). Dephosphorylation of these residues by the phosphatase PP1 (Tomita et al., 2005), downstream of NMDAR stimulation, favors association of the TARP intracellular domain with phospholipids, disrupting the TARP—PSD-95 interaction and consequently liberating the AMPAR from the confines of the PSD (Sumioka et al., 2010).

EARLY STAGES OF CLATHRIN-COATED PIT FORMATION

GluA2-AP2 Interaction

Following their dissociation from PSD scaffolds, it is thought that AMPARs diffuse from the synapse to EZs adjacent to the PSD (Lu et al., 2007). EZs have been defined by visualizing clusters of overexpressed fluorescently-tagged clathrin, and the structure of these sites with respect to the size or number of clathrin-coated pits (CCPs) present is unclear. One of the core elements of clathrin-mediated endocytosis (CME), and one of the first protein complexes to assemble at nascent CCPs, is the endocytic adaptor protein complex AP2, which functions to recruit and concentrate cargo at specific membrane domains. It clusters at $PI(4,5)P_2$-rich regions of the plasma membrane, and binds cargo proteins, numerous endocytic accessory proteins and clathrin (Traub, 2009; Kelly and Owen, 2011). The μ2 subunit of AP2 binds GluA2 and GluA3 subunits directly (**Figure 1C**), and this interaction is required for hippocampal LTD but not constitutive AMPAR endocytosis (Lee et al., 2002; Kastning et al., 2007). The precise cell biological mechanism of AP2 binding to GluA2 has not been revealed, but by analogy with other well-studied cargo proteins, presumably it functions to recruit GluA2-containing AMPARs to endocytic sites (Traub, 2009; Kelly and Owen, 2011). Since it is involved in NMDAR-dependent endocytosis and not constitutive trafficking (Lee et al., 2002), the GluA2-AP2 interaction must be strengthened by NMDAR stimulation, although a mechanism has not been explored biochemically. Nevertheless, it has been suggested that AP2 binds the Ca^{2+} sensing protein hippocalcin, forming a Ca^{2+}-dependent complex with AMPAR subunit GluA2 (Palmer et al., 2005). The AP2-hippocalcin interaction is required for LTD, suggesting that hippocalcin plays a role in recruiting AMPARs to endocytic sites in response to NMDAR-mediated Ca^{2+} signals.

TARP-AP2 Interaction

As well as binding GluA2 directly, AP2 also associates with the AMPAR complex via TARPs (Matsuda et al., 2013; **Figure 1C**). As discussed above, while TARPs dissociate from the PSD scaffold in response to plasticity stimuli, they remain associated with the AMPAR complex, and continue to play an important role in AMPAR trafficking. Stargazin binds the μ2 subunit of AP2 via a C-terminal region that includes or overlaps with the region involved in regulating its association with phospholipids and hence with PSD-95 via stargazin phosphorylation (Sumioka et al., 2010; Matsuda et al., 2013). There are nine serine residues in this critical C-terminal region of Stargazin, and a specific subset of serines have been shown to modulate the binding of Stargazin to AP2 in response to NMDAR stimulation. Both cerebellar LTD and hippocampal LTD are disrupted by mutation of these serine residues (Tomita et al., 2005; Nomura et al., 2012). While it has been shown that PP1 causes an overall dephosphorylation of Stargazin and CamKII is involved in an overall increase in phosphorylation (Tomita et al., 2005), mutagenesis data suggest that AP2 binding increases when a cluster of three serines is dephosphorylated (experimentally, mutated to alanines). Other protein interactions with the Stargazin C-tail depend on different patterns of phospho-null or phospho-mimetic mutations in this region (Matsuda et al., 2013). The details of the upstream signaling pathways that converge on Stargazin to define these specific patterns of phosphorylation are unclear. Interestingly, one of the species of phospholipid that the Stargazin C-tail associates with in a protein phosphorylation-dependent manner is $PI(4,5)P_2$, which is particularly concentrated at sites of endocytosis (Sumioka et al., 2010). Hence dephosphorylation of Stargazin may simultaneously promote association with AP2 and with $PI(4,5)P_2$ in the plasma membrane. While disrupting binding to AP2 inhibited the NMDAR-dependent trafficking of recombinant Stargazin to early endosomes, it is unclear which stage of endocytosis leading up to this point is affected (Matsuda et al., 2013). Since binding to μ2 subunit of AP2 is typically associated with cargo recruitment to endocytic sites in the early stages of CCP formation, this is the most likely function for

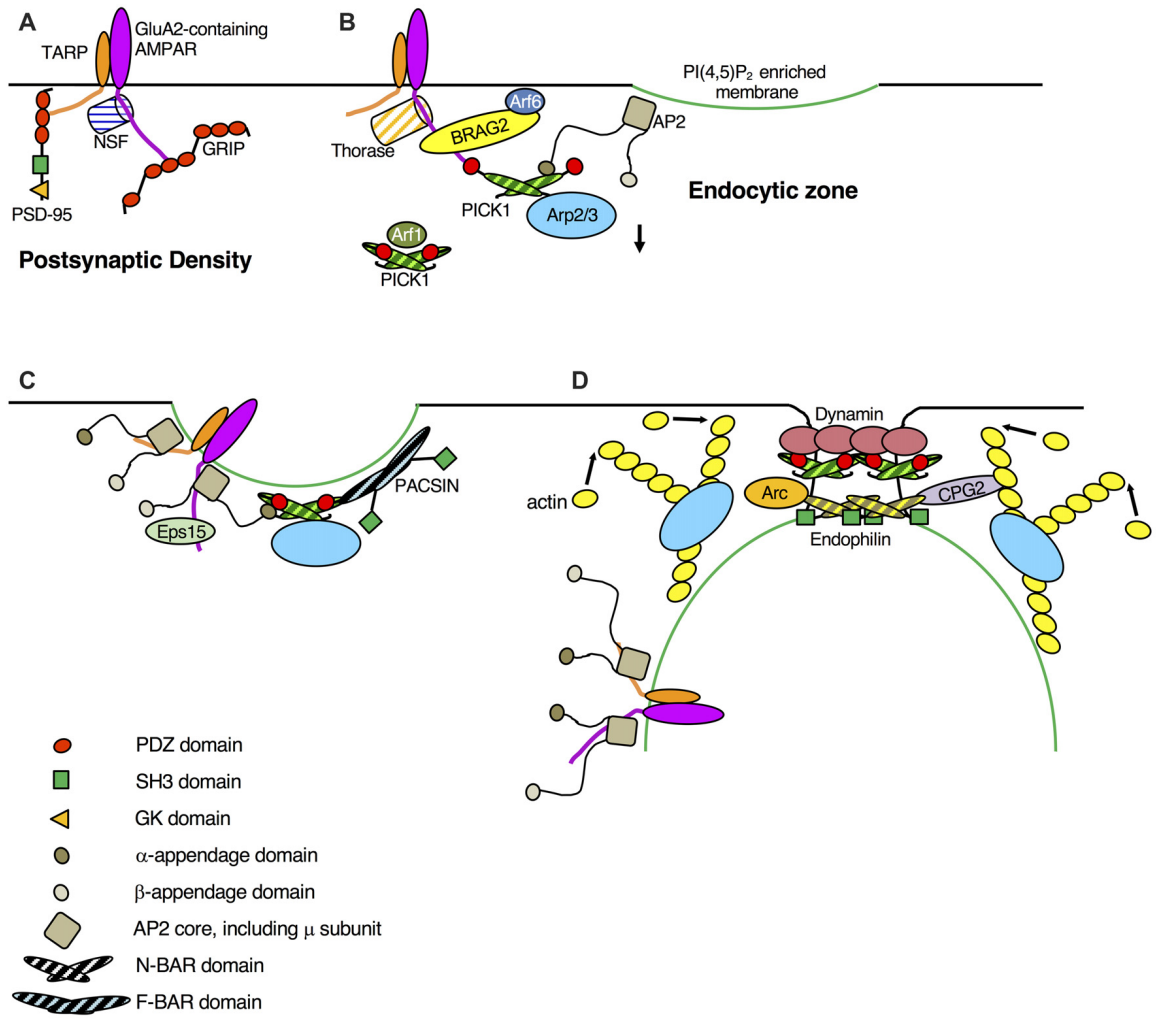

FIGURE 1 | Schematic showing dynamic protein-protein interactions in AMPA receptor (AMPAR) endocytosis. **(A)** GluA2-containing AMPARs at the synapse are bound to post-synaptic density-95 (PSD-95) via transmembrane AMPAR regulatory proteins (TARPs) and to GRIP via GluA2. NSF activity prevents protein interacting with C-Kinase 1 (PICK1) binding to GluA2. **(B)** As a result of long-term depression (LTD) induction (NMDA receptor (NMDAR) or metabotropic glutamate receptor (mGluR) stimulation), TARP dephosphorylation disrupts TARP-PSD-95, GluA2 S880 phosphorylation and Thorase activity disrupt GluA2-GRIP. Ca^{2+} directly enhances GluA2-PICK1 and disrupts GluA2-NSF, deactivation of Arf1 promotes PICK1-Arp2/3 (inactive). GluA2 Y876 dephosphorylation enhances GluA2-Brefeldin-Resistant Arf-G2 (BRAG2), which in turn activates Arf6, causing a local increase in $PI(4,5)P_2$ concentration, and consequent clustering of AP2. Calcineurin activity enhances AP2(α)-PICK1 to initiate AMPAR recruitment to clathrin-coated pits (CCPs). **(C)** TARP dephosphorylation enhances TARP-AP2(μ), and an unknown mechanism, possibly involving Hippocalcin, enhances GluA2-AP2(μ), both of which further promote AMPAR clustering at CCPs. AP2(α)-PICK1 interaction disrupts GluA2-PICK1. PACSIN phosphorylation enhances PICK1-PACSIN, which may stabilize curvature of the nascent CCP. Eps15 binds GluA1 in a ubiquitin-dependent manner. **(D)** As the complex geometry of the CCP develops, Bin-Amphiphysin-RVS (BAR) domain proteins stabilize the tight curvature of the CCP neck and recruit dynamin and other proteins to this structure. Calcineurin activity enhances PICK1-dynamin, activity-dependent increases in Arc and CPG2 expression enhance Endophilin-Arc and Endophilin-CPG2. CPG2 phosphorylation enhances CPG2-actin. Competition with Arp2/3 activators (e.g., N-WASP) disrupts PICK1-Arp2/3. Note that this schematic is limited to protein-protein interactions shown to be dynamically regulated in response to plasticity-inducing stimuli.

this interaction (**Figure 1C**). This leads to the question of why does μ2 subunit bind both GluA2 and Stargazin? Disrupting either of these interactions inhibits LTD, indicating that they are both important for activity-dependent AMPAR internalization (Lee et al., 2002; Matsuda et al., 2013). The number of TARPs that associate with an AMPAR complex has been suggested to vary (Greger et al., 2017). Perhaps the complement of TARPs associated with an AMPAR complex, and hence the number of μ2 binding sites, influences the speed or efficiency of AMPAR endocytosis? Moreover, while the vast majority of AMPARs contain GluA2 or GluA3 subunits, GluA1 homomers are thought to exist (Wenthold et al., 1996; Man, 2011). GluA1 does not bind μ2 (Kastning et al., 2007), hence the recruitment of these Ca^{2+}-permeable AMPARs to CCPs might depend on their TARP-μ2 interactions, allowing for a subtly distinct mode of regulation compared to GluA2-containing AMPARs, which may be critical for specific kinds of plasticity that involve Ca^{2+}-permeable AMPARs.

PICK1-AP2 Interaction

While the μ2 subunit is critical for cargo recruitment, the appendage domain of the α subunit of AP2 (α-adaptin) binds several endocytic accessory proteins including amphiphysin, which contains a Bin-Amphiphysin-RVS (BAR) domain that senses or contributes to membrane curvature at the neck of the CCP and functions to recruit the large GTPase dynamin to the CCP neck for fission of the endocytic vesicle. (Praefcke et al., 2004; Daumke et al., 2014; Suetsugu et al., 2014). A recent addition to the BAR domain proteins identified as an α-appendage interactor is protein interacting with C-Kinase 1 (PICK1; **Figure 1B**; Fiuza et al., 2017), which has a well-established role in decreasing the surface and synaptic levels of GluA2-containing AMPARs (Terashima et al., 2004). The PICK1 PDZ domain binds the C-terminal tail of AMPAR subunit GluA2, and disrupting this interaction with competing peptides or by mutagenesis inhibits both constitutive and NMDAR-stimulated AMPAR internalization and LTD in hippocampal neurons (Daw et al., 2000; Osten et al., 2000; Iwakura et al., 2001), as well as cerebellar LTD. While a basal level of PICK1 appears to be bound to GluA2 to promote constitutive internalization, the interaction is enhanced directly by Ca^{2+} ions following NMDAR stimulation (Hanley and Henley, 2005). A direct effect of Ca^{2+} on GluA2-PICK1 binding, without the need for additional enzymatic steps, allows a rapid response to NMDAR stimulation. PICK1 contains at least two Ca^{2+} binding sites, one of which, a short stretch of acidic amino acids at the N-terminus of PICK1, is responsible for mediating the NMDAR-stimulated increase in GluA2 binding. Mutagenesis revealed that the Ca^{2+}-binding property of PICK1 is necessary for NMDA-stimulated AMPAR internalization and LTD (Hanley and Henley, 2005; Citri et al., 2010).

PICK1 binds directly to AP2 with similar consensus motifs (FxDxF and DxF) to numerous other endocytic accessory proteins (Praefcke et al., 2004; Olesen et al., 2008; Fiuza et al., 2017). Mutating the critical aspartate residues to alanines in PICK1 disrupts AP2 binding and consequently inhibits both constitutive and NMDAR-dependent internalization of endogenous GluA2-containing AMPARs (Fiuza et al., 2017). While AP2-PICK1 binding is important for constitutive AMPAR internalization, NMDAR stimulation causes a marked increase in this interaction, which follows a slower time course compared to that of GluA2-PICK1, suggesting intermediate steps are involved in mediating the increase in binding, rather than a direct effect of Ca^{2+}. Indeed, the NMDAR-dependent increase in AP2-PICK1 binding requires activation of the Ca^{2+}-dependent phosphatase Calcineurin (Fiuza et al., 2017), which itself has a well-established role in NMDAR-dependent AMPAR internalization and LTD (Mulkey et al., 1994; Beattie et al., 2000). The substrate for Calcineurin in this mechanism is unknown. Furthermore, disrupting PICK1-AP2 binding blocks NMDAR-dependent recruitment of GluA2-containing AMPARs to clathrin clusters in neuronal dendrites, suggesting that PICK1 is involved in recruiting AMPARs to CCPs (**Figure 1B**). Mutagenesis of the PICK1 PDZ domain also blocks this trafficking event, indicating that AMPAR recruitment to endocytic sites also depends on PICK1 binding to GluA2 (Fiuza et al., 2017). However, α-adaptin and GluA2 binding to PICK1 are mutually exclusive, suggesting that the binding of both proteins simultaneously to PICK1 occurs only very transiently. Together, these observations indicate that PICK1 binds GluA2 immediately after NMDAR stimulation, followed by an increase in PICK1-AP2 binding, which consequently disrupts the interaction between PICK1 and GluA2 (Fiuza et al., 2017). While this suggests a mechanism for PICK1 in the recruitment of GluA2 to CCPs, the PICK1 interaction with α-adaptin is likely to be mechanistically distinct from the cargo recruitment function of the μ2 interactions. The α-appendage domains are found at the end of long flexible linker regions, which can reach out over a large area to bring in to the CCP accessory proteins required for inducing/sensing membrane curvature and recruiting dynamin (Praefcke et al., 2004). While PICK1 senses membrane curvature (Herlo et al., 2018) and binds dynamin (see following section), it also binds endocytic cargo. Hence, the PICK1—α-adaptin interaction may serve two functions; to enhance GluA2 clustering at CCPs because of the wide spatial sampling of the appendage domain, and to recruit a curvature-sensing regulator of dynamin.

GluA1-Eps15 Interaction

Eps15 is a well-characterized endocytic adaptor protein that binds to and promotes the endocytosis of ubiquitinated cargo (Polo et al., 2002). Eps15 interacts with GluA1, and this interaction is enhanced by ubiquitination of the GluA1 C-terminal domain by the E3 ligase Nedd4 (Lin and Man, 2014). While Eps15 was shown to be required for glutamate-induced AMPAR endocytosis, a role for the GluA1-Eps15 interaction *per se* in this trafficking event has not been demonstrated. Furthermore, a number of reports suggest that AMPAR subunit ubiquitination is regulated by ligand (AMPA) stimulation, but not by NMDAR stimulation or other models of synaptic plasticity (Schwarz et al., 2010; Widagdo et al., 2015).

GluA2-BRAG2 Interaction

The phospholipid composition of the plasma membrane is a critical determinant of AP2 clustering at nascent CCPs, since AP2 has high affinity for $PI(4,5)P_2$ (**Figures 1B,C**). Hence a mechanism to locally increase $PI(4,5)P_2$ concentration in the vicinity of AMPARs would promote AP2 binding to AMPAR subunits and associated proteins and hence facilitate endocytosis. Brefeldin-Resistant Arf-guanine nucleotide exchange factor 2 (BRAG2-GEF 2), a GEF for Arf6, binds directly to GluA2 at a site that includes Tyr 876 (Scholz et al., 2010; **Figure 1B**). Via this physical interaction, AMPAR stimulation increases BRAG2 GEF activity and consequently Arf6 activation in a mechanism that requires dephosphorylation of Y876. Arf6 is generally considered to function at the plasma membrane in recruiting lipid kinases to increase local concentration of $PI(4,5)P_2$ for CCP formation (D'Souza-Schorey and Chavrier, 2006). Hence, $PI(4,5)P_2$ levels might increase close to ligand-bound AMPARs, provided specific tyrosine phosphatases are activated to dephosphorylate Y876. However, such an effect

on plasma membrane phospholipids in the context of AMPAR trafficking has not been reported. This process is required for mGluR-dependent AMPAR internalisation and LTD (Scholz et al., 2010). NMDAR-dependent LTD also requires BRAG2, but it is likely that a subtly different mechanism is at play between the two modes of LTD induction. Studies from other labs report tyrosine dephosphorylation of GluA2 as part of the mechanism for mGluR-dependent LTD, which is thought to require activation of the tyrosine phosphatase STEP downstream of mGluR stimulation (Moult et al., 2006; Zhang et al., 2008). In contrast, NMDAR-dependent LTD is thought to require phosphorylation of Y876 (Ahmadian et al., 2004; Hayashi and Huganir, 2004; and see later section).

LATER STAGES OF CLATHRIN-COATED PIT FORMATION; BAR DOMAINS

A number of BAR domain proteins have been implicated in AMPAR endocytosis. Indeed, the first published evidence that LTD involves endocytosis was based on the use of a peptide corresponding to the amphiphysin SH3 domain to disrupt amphiphysin binding to dynamin, and hence inhibit dynamin recruitment to the CCP (Man et al., 2000). However, there appears to be no evidence to suggest that this interaction is regulated by NMDAR stimulation or other plasticity-inducing stimuli.

PICK1-Dynamin Interaction

The PICK1 BAR domain is proposed to have a similar degree of curvature as amphiphysin, it contains two AP2 α-appendage binding sites (the same as amphiphysin), and it also binds dynamin (**Figure 1D**; Praefcke et al., 2004; He et al., 2011; Karlsen et al., 2015; Fiuza et al., 2017). The PICK1-dynamin interaction shows a similar dependence on NMDAR stimulation and calcineurin activity as PICK1-AP2, raising the possibility that PICK1 binds dynamin only as a functional consequence of binding AP2. Nevertheless, in a reduced system of purified components, PICK1 binds dynamin directly and enhances dynamin polymerization (Fiuza et al., 2017). The similar degree of curvature of the PICK1 BAR domain to amphiphysin is consistent with a role in recruiting dynamin to the highly curved neck of the CCP and regulating its function there, although this has not been shown experimentally. It is unknown whether the PICK1 BAR domain functions to induce or stabilize membrane curvature, or simply sense and associate with membranes of a particular curvature to recruit dynamin to the neck of the CCP. It is also unclear whether PICK1 and amphiphysin play distinct or redundant roles in dynamin recruitment at the AMPAR-containing CCP. While amphiphysin binds the proline-rich domain of dynamin (Ferguson and De Camilli, 2012), PICK1 binds the GTPase domain (Fiuza et al., 2017), suggesting distinct roles in regulating dynamin function. Note that PICK1 does not appear to play a role in AMPAR endocytosis associated with down-scaling homeostatic plasticity (Anggono et al., 2011).

PACSIN-PICK1 Interaction

Another BAR domain protein shown to play a specific role in AMPAR endocytosis is PACSIN, also known as Syndapin. In contrast to the N-BAR domains of PICK1 or amphiphysin, PACSIN/Syndapin contains an F-BAR domain, which is elongated and has a preference for membranes with a larger radius of curvature (Qualmann et al., 2011). It is thought that F-BAR proteins are recruited to CCPs at an earlier stage of endocytosis compared to BAR or N-BAR proteins, in order to induce or stabilize the shallow curvature of the plasma membrane in the nascent CCP (Suetsugu et al., 2014). The precise temporal details of accessory protein recruitment to AMPAR-containing CCPs has not been specifically studied, however the recently-reported success at visualizing such events in neuronal dendrites with high temporal resolution suggests that progress in this direction will soon be made (Rosendale et al., 2017). PACSIN/Syndapin associates with AMPARs via an interaction with PICK1, and it has been suggested that phosphorylation of PACSIN/Syndapin at a cluster of three serines in the variable region between F-BAR and SH3 domains disrupts the interaction with PICK1 and reduces AMPAR internalization (Anggono et al., 2013). However, it has also been suggested that phosphorylation of the same three serines has more effect on recycling than on endocytosis of recombinant GluA2 (Widagdo et al., 2016). While knockdown of PACSIN/Syndapin expression reduces GluA2 endocytosis, indicating a critical role for the protein in this trafficking event, it is unclear whether any specific interaction with AMPARs or with AMPAR binding proteins is involved (Widagdo et al., 2016).

Arc-Endophilin-CPG2-Actin Interactions

Endophilin is another BAR domain protein that functions in a similar manner as amphiphysin, associating with the neck of CCPs to regulate dynamin recruitment (Ferguson and De Camilli, 2012). A specific role for endophilin in AMPAR endocytosis has been demonstrated by the discovery of a direct interaction between endophilin and the immediate early gene Arc/Arg3.1 (Chowdhury et al., 2006). Although activity-dependent regulation of this interaction has not been reported, Arc/Arg3.1 gene expression is regulated by neuronal activity, and therefore the interaction with endophilin would be upregulated under conditions of increased gene expression. While the precise function of this interaction in endocytosis is unclear, Arc/Arg3.1 is required for both LTD and for down-scaling homeostatic plasticity (Rial Verde et al., 2006; Shepherd et al., 2006). Endophilin also associates with CPG2, another protein whose expression is regulated by neuronal activity (Loebrich et al., 2016). CPG2 in turn associates with the actin cytoskeleton, and both the endophilin-CPG2 and CPG2-actin interactions are required for homeostatic down-scaling (Loebrich et al., 2013, 2016). Phosphorylation of CPG2 by PKA enhances its interaction with the actin cytoskeleton, and disrupting this phosphorylation event inhibits AMPAR internalization, suggesting a phosphorylation-dependent regulation of AMPAR endocytosis via a protein complex comprising actin/CPG2/endophilin (Loebrich et al., 2013).

THE ACTIN CYTOSKELETON

The role of the actin cytoskeleton in endocytosis is well-studied in the context of non-neuronal cells. Actin dynamics are proposed to generate forces that contribute to the changing geometry of the plasma membrane during CCP formation and to subsequent vesicle fission, and numerous proteins have been implicated in the regulation of this process (Kaksonen et al., 2006; Mooren et al., 2012). While it is likely that many of the same actin-binding protein players and consequent mechanisms are involved in regulating AMPAR endocytosis in neurons, there is little published evidence to support this directly. Nevertheless, it has been shown that the balance of actin polymerization and depolymerization is critical to AMPAR synaptic localization (Zhou et al., 2001).

PICK1-Arp2/3 Interaction

While a number of actin-binding proteins associate directly or indirectly with AMPARs, they have not been reliably assigned a role in endocytosis *per se*, and there are very few publications reporting that such interactions are regulated by plasticity stimuli. One example is PICK1, which binds directly to the actin-nucleating Arp2/3 complex (Rocca et al., 2008). This interaction is transiently enhanced by NMDAR stimulation and is required for NMDA-induced AMPAR internalization and LTD (Nakamura et al., 2011). The signaling mechanism that mediates this NMDAR-dependent increase in binding involves the small GTPase Arf1, which associates with PICK1 in its GTP-bound state and blocks the interaction with Arp2/3 (Rocca et al., 2013). NMDAR stimulation switches Arf1 from a GTP- to GDP-bound state via the Arf GAP GIT1, and GDP-bound Arf1 dissociates from PICK1, promoting binding to Arp2/3 (Rocca et al., 2013). PICK1 inhibits Arp2/3-mediated actin polymerization, suggesting a requirement for inhibition of this activity at an unknown stage of AMPAR endocytosis (Rocca et al., 2008). The precise spatial and temporal details of this inhibition of actin polymerization are likely to be critical and warrant further study. Interestingly, a role for PICK1 inhibition of Arp2/3 activity and modulation by Arf1 has also been suggested recently in a specific form of endocytosis in non-neuronal cells (Sathe et al., 2018). In this study, the authors suggest that PICK1 functions to recruit inactive Arp2/3 to the sites of endocytosis, in preparation for a subsequent burst of actin polymerization triggered by the small GTPase Cdc42 and BAR domain protein IRSp53. However, a report from another group suggested that PICK1 does not bind to Arp2/3, but instead is involved in vesicle motility via an as yet undefined myosin motor protein (Madasu et al., 2015). A role for such an interaction in AMPAR endocytosis was not suggested.

PROTEIN-PROTEIN INTERACTIONS THAT MODULATE AN UNDEFINED ASPECT OF AMPAR ENDOCYTOSIS

GluA2-GRIP Interaction

The GRIP family of multi-PDZ domain scaffold proteins plays multiple roles in AMPAR trafficking, including long-range trafficking via association with microtubule motor proteins, endosomal sorting, and stabilization at the synaptic membrane (Osten et al., 2000; Setou et al., 2002; Steiner et al., 2005). GRIP binds GluA2 at the same site as PICK1, hence the two interactions are mutually exclusive and dissociation from GRIP1 is likely necessary prior to binding PICK1 and consequent endocytosis. The GluA2-GRIP interaction is modulated by phosphorylation of GluA2 at Serine 880, which lies within the PDZ ligand (Chung et al., 2000), and also by the nearby Tyr 876 (Hayashi and Huganir, 2004). Both phosphorylation events can be stimulated by NMDAR activation (Kim et al., 2001; Hayashi and Huganir, 2004). PICK1 binding is unaffected by S880 and Y876 phosphorylation, therefore these signaling events cause a switch of GluA2 binding from GRIP to PICK1 binding. S880 phosphorylation has been shown to be a critical component of both hippocampal and cerebellar LTD (Kim et al., 2001; Chung et al., 2003). While protein kinase C is required for phosphorylating S880 in cerebellar LTD, the kinase for hippocampal LTD is unknown (Xia et al., 2000; Kim et al., 2001).

GluA2-Thorase and GluA2-NSF Interactions

A further mode of regulation of the GluA2-GRIP interaction is via the ATPase Thorase, whose activity is required for NMDAR-dependent GluA2 endocytosis and LTD (Zhang et al., 2011). Thorase binds both GluA2 and GRIP in an ATP-dependent manner, and its ATPase activity disrupts the GluA2-GRIP interaction to facilitate AMPAR endocytosis. Presumably the association of Thorase with the AMPAR-GRIP complex (or alternatively the enzymatic activity of Thorase) must itself be regulated by NMDAR activity, but such a mechanism has yet to be identified. Interestingly, a very similar, yet apparently independent mechanism regulates GluA2-PICK1 interactions. The ATPase NSF, well-characterized as a molecular chaperone for the SNARE complex, dissociates PICK1 from GluA2 in an ATP-dependent manner to limit AMPAR internalization (Hanley et al., 2002). Disrupting the GluA2-NSF interaction with competing peptides causes a rundown of AMPAR EPSCs that occludes subsequent expression of both hippocampal and cerebellar LTD (Luthi et al., 1999; Lee et al., 2002; Steinberg et al., 2004), suggesting that dissociation of this interaction is required for activity-dependent AMPAR internalization. In contrast to GluA2-Thorase, additional levels of modulation of the GluA2-NSF interaction have been identified. NSF binding to GluA2 is decreased in the presence of low-micromolar Ca^{2+}, suggesting that NMDAR-mediated Ca^{2+} influx reduces the NSF-dependent dissociation of PICK1 from GluA2 (Hanley, 2007). In addition, the identity of the SNAP protein cofactor is a critical determinant of NSF activity on this complex; α-SNAP stimulates, whereas β-SNAP inhibits GluA2-PICK1 dissociation by NSF (Hanley et al., 2002).

CONCLUDING REMARKS

I have reviewed what I believe to be the current state of knowledge about protein-protein interactions that are involved

in AMPAR endocytosis from the plasma membrane and are regulated in response to stimuli that induce long-term synaptic plasticity. There exists a wealth of knowledge about the orchestration of protein-protein interactions in general endocytosis mechanisms, many of which are likely to be involved in AMPAR endocytosis. The complex signaling pathways that are activated in response to the induction of synaptic plasticity are also well characterized, hence the potential for regulating already-known endocytic protein-protein interactions as a consequence of plasticity stimuli is significant and worthy of future investigation. Furthermore, it is emerging that the dysregulation of AMPAR endocytosis is a critical component of synaptic weakening associated with pathologies such as Alzheimer's, and therefore dynamic protein-protein interactions might become targets for therapeutic intervention.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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AMPA Receptor Trafficking for Postsynaptic Potentiation

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Long-term potentiation (LTP) of excitatory synaptic strength, which has long been considered a synaptic correlate for learning and memory, requires a fast recruitment of additional α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (AMPARs) to the postsynaptic sites. As cell biological concepts have been applied to the field and genetic manipulation and microscopic imaging technologies have been advanced, visualization of the trafficking of AMPARs to synapses for LTP has been investigated intensively over the last decade. Recycling endosomes have been reported as intracellular storage organelles to supply AMPARs for LTP through the endocytic recycling pathway. In addition, exocytic domains in the spine plasma membrane, where AMPARs are inserted from the intracellular compartment, and nanodomains, where diffusing AMPARs are trapped and immobilized inside synapses for LTP, have been described. Furthermore, cell surface lateral diffusion of AMPARs from extrasynaptic to synaptic sites has been reported as a key step for AMPAR location to the synaptic sites for LTP. This review article will discuss recent findings and views on the reservoir(s) of AMPARs and their trafficking for LTP expression by focusing on the exocytosis and lateral diffusion of AMPARs, and provide some future directions that need to be addressed in the field of LTP.

Keywords: AMPA receptors, long-term potentiation, postsynapse, exocytosis, lateral diffusion

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INTRODUCTION

Synapses are fundamental units of brain function and possess the remarkable ability to change their strength in function and structure through synaptic plasticity. Long-term potentiation (LTP), a well characterized form of synaptic plasticity that has long been considered a synaptic correlate for learning and memory, was discovered in the hippocampus in 1973 (Bliss and Lφmo, 1973). Prior to the discovery of LTP, Hebb's (1949) postulate that learning and memory involves synaptic strengthening elicited by the coordinated firing of pre- and postsynaptic cells was suggested. In addition, beginning with Ramón y Cajal (1852–1934), many neuroscientists have suggested that learning and memory should involve synaptic modifications (Malenka, 2003). A type of glutamate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor (AMPAR), has been investigated intensively as a key player in synaptic modifications involved in synaptic transmission, synaptic plasticity and, ultimately, learning and memory. LTP and long-term depression (LTD), another well characterized form of synaptic plasticity in the hippocampus, are expressed by long lasting changes of AMPAR-mediated synaptic responses. Exocytosis and endocytosis of AMPARs play critical roles in LTP and LTD, respectively, in aspects of both functional and structural plasticity of synapses

(Kessels and Malinow, 2009; Anggono and Huganir, 2012; Huganir and Nicoll, 2013). Indeed, learning induces LTP in the hippocampus (Whitlock et al., 2006). Learning alters AMPAR phosphorylation and synaptic delivery of AMPARs (Whitlock et al., 2006), which are readouts for LTP (Heynen et al., 2000; Lee et al., 2000; Malenka, 2003). Although studies of LTP were conducted originally through electrophysiological approaches, advances in the tools of molecular and cellular biology, biochemistry, state-of-the-art imaging and genetics have provided much more sophisticated information of AMPAR trafficking to synapses to support LTP mechanisms. This review article provides a brief introduction of AMPARs and LTP, followed by a focus on recent findings and views on AMPAR reservoir(s) for LTP by examining studies on the exocytosis and cell surface lateral diffusion of AMPARs during LTP.

AMPARs AND LTP

AMPARs are major ionotropic glutamate receptors that respond to physiological glutamate, a major excitatory neurotransmitter in the mammalian central nervous system. AMPARs have four subunits, GluA1–GluA4 encoded by *Gria1–Gria4* genes, and those subunits form hetero-tetramers composed of two dimers (Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994; Dingledine et al., 1999; Traynelis et al., 2010; Chater and Goda, 2014). The combination of each subunit forms a developmentally distinct receptor complex in the hippocampus (Wenthold et al., 1996; Zhu et al., 2000). Immature hippocampal neurons at early developmental stages express the GluA4 subunit, which complexes with the GluA2 subunit (Zhu et al., 2000). However, mature hippocampal neurons express two predominant combinations of AMPAR subunits, GluA1/GluA2 or GluA2/GluA3 heterotetrameric receptors (Wenthold et al., 1996). Regulation of the precise localization and number of AMPARs at the cell surface membrane is critical for most excitatory synaptic transmission at the steady state and also for long-term synaptic plasticity, such as LTP and LTD (Song and Huganir, 2002; Bredt and Nicoll, 2003).

The majority of studies on LTP have been performed on excitatory synapses between Schaffer collateral-commissural axons and CA1 pyramidal neuron dendrites in the hippocampus (Bear and Kirkwood, 1993; Kirkwood et al., 1993; Nicoll and Roche, 2013). While LTP is triggered rapidly by a brief high-frequency stimulation (HFS), it persists for days or even weeks *in vivo*. The "early phase" of LTP, which lasts approximately 60 min, requires the activation of *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) for its induction, together with subsequent Ca^{2+} influx and calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; Lisman, 1994; Lisman et al., 1997; Malenka and Nicoll, 1999). In addition, delivery of new AMPARs to the postsynaptic sites is believed to be responsible for LTP expression in its early phases. One distinguishable characteristic of the "late phase" of LTP, which lasts days or even weeks, from the "early phase" of LTP, is that the late phase requires gene transcription and new protein synthesis (Schuman et al., 2006; Reymann and Frey, 2007; Johnstone and Raymond, 2011; but also see Abbas et al., 2009; Villers et al., 2012).

Three major questions have been the focus of studies in the field of LTP. First, studies examined whether the increase in synaptic strength during LTP at Schaffer collateral-CA1 synapses is due primarily to presynaptic or postsynaptic modifications (Kullmann and Siegelbaum, 1995; Nicoll and Malenka, 1995; Emptage et al., 1999, 2003; Malenka and Nicoll, 1999; Ward et al., 2006; Kerchner and Nicoll, 2008; Enoki et al., 2009; Kullmann, 2012; Chater and Goda, 2014; Granger and Nicoll, 2014; Padamsey and Emptage, 2014). Second, studies have been conducted to determine which AMPAR subunits are responsible for LTP expression (Jia et al., 1996; Zamanillo et al., 1999; Hayashi et al., 2000; Shi et al., 2001; Granger et al., 2013; Granger and Nicoll, 2014; Diaz-Alonso et al., 2017; Zhou et al., 2018). Finally, studies have examined whether AMPARs are located to the synapse for LTP expression through exocytosis and/or lateral diffusion. The debate about whether the location of LTP expression at CA1 synapses is presynaptic or postsynaptic has lasted for more than two decades. Currently, most data, even those previously supporting a presynaptic change of increased release probability or decreased synaptic failure in LTP expression can be reconciled with postsynaptic changes by the "silent synapse" concept (Isaac et al., 1995, 1996; Liao et al., 1995, 1999; Durand et al., 1996; Gomperts et al., 1998; Nusser et al., 1998; Petralia et al., 1999; Takumi et al., 1999; Kerchner and Nicoll, 2008). It is now generally accepted that LTP expression at Schaffer collateral-CA1 synapses is mediated by AMPAR insertion into the synapse, supporting the postsynaptic view for LTP expression (Malenka and Nicoll, 1999; Shi et al., 1999; Hayashi et al., 2000; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Nicoll, 2003; Chater and Goda, 2014; Granger and Nicoll, 2014). Single channel conductance increases of AMPARs have been suggested to mediate LTP expression in the CA1 region of the hippocampus (Benke et al., 1998). However, a recent reevaluation of this study by the same group showed that insertion of AMPARs with high conductance can account for LTP expression (Benke and Traynelis, 2018). In addition to postsynaptically expressed LTP at Schaffer collateral-CA1 synapses, a distinct form of LTP at mossy fiber synapses, which is independent of NMDARs and expressed presynaptically unlike that at Schaffer collateral-CA1 synapses, has been well investigated and extensivley discussed (Nicoll and Malenka, 1995; Nicoll and Schmitz, 2005; Granger and Nicoll, 2014). AMPARs, particularly those containing the GluA1 subunit, have been suggested to play an important role in LTP expression at CA1 synapses in studies using knockout mice lacking GluA1 or GluA2 and electrophysiological recordings of hippocampal slice expressing tagged GluA1 or GluA2 (Jia et al., 1996; Zamanillo et al., 1999; Hayashi et al., 2000; Shi et al., 2001; Diaz-Alonso et al., 2017). The GluA1 subunit requirement for LTP has been investigated and supported by studies focusing on the cytoplasmic carboxy terminal (C-terminal) tail, which has been demonstrated to be involved in intracellular signaling through phosphorylation, palmitoylation or protein interactions (Barria et al., 1997;

Hayashi et al., 2000; Shi et al., 2001; Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006; Lin et al., 2009). However, the GluA1 C-terminal tail requirement for LTP was challenged by a report showing that LTP requires AMPAR trafficking, independent of subunit type (Granger et al., 2013). Interestingly, recent studies have demonstrated that the extracellular amino-terminal domain (ATD) of AMPARs governs their trafficking for synaptic plasticity dependent on the AMPAR subunit type (Diaz-Alonso et al., 2017; Watson et al., 2017). Further, the spatial resolution of AMPARs delivered into the synapse for LTP has been questioned whether it is through exocytosis directly from the intracellular pool to synaptic sites or through lateral mobility from the extrasynaptic plasma membrane or a combination of each (Lledo et al., 1998; Lu et al., 2001; Park et al., 2004; Kopec et al., 2006, 2007b; Yudowski et al., 2007; Jaskolski and Henley, 2009; Lin et al., 2009; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Opazo et al., 2010; Patterson et al., 2010; Cho et al., 2015; Penn et al., 2017; Temkin et al., 2017; Wu et al., 2017).

EXOCYTOSIS AND LATERAL MOBILITY OF AMPARs FOR LTP

Much evidence suggests that LTP expression is mediated by postsynaptic mechanisms (Lisman et al., 2012; Lu and Roche, 2012; Granger et al., 2013; Granger and Nicoll, 2014) and requires exocytosis at or near the postsynaptic membrane, which results in an increase in the number of AMPARs (Lledo et al., 1998; Lu et al., 2001; Park et al., 2004; Patterson et al., 2010; Ehlers, 2013; Wu et al., 2017). Initial studies showed that introducing various reagents that disrupt membrane fusion into the postsynaptic cells blocks LTP at Schaffer collateral-CA1 synapses (Lledo et al., 1998), implying that the exocytosis of intracellular vesicles harboring AMPARs is an essential step for LTP. A serial electron microscopy study demonstrated that endosomal compartments are located in dendrites and dendritic spines and serve as intracellular storehouses for the plasma membrane (Cooney et al., 2002). Some molecules that are retained on endosomal compartments can be delivered rapidly to the cell surface in response to stimuli (Lampson et al., 2001; Bryant et al., 2002; Cooney et al., 2002; Zeigerer et al., 2002; Govers et al., 2004; Guilherme et al., 2004). In addition, another study showed that synaptic activity that can induce LTP drives AMPARs to be endocytosed and reinserted to the plasma membrane (Ehlers, 2000). Together, these data suggest that endosomal organelles involved in endocytic recycling transport can serve as primary intracellular membrane compartments mobilized to the plasma membrane in response to LTP-inducing stimuli (Ehlers, 2013). Indeed, disrupting the transport of recycling endosomes to the plasma membrane using dominant negative forms of Rab11 (Ullrich et al., 1996; Zerial and Mcbride, 2001), syntaxin 13 (Prekeris et al., 1998), or Eps15 homology domain protein Rme1/EHD1 (Grant et al., 2001; Lin et al., 2001) blocked synaptic delivery of AMPARs during LTP (Park et al., 2004). Postsynaptic synaptotagmin-1 and synaptotagmin-7 were reported to mediate GluA1 exocytosis during LTP by acting as postsynaptic Ca^{2+}-sensors (Wu et al., 2017). Complexin, a regulator of SNARE-mediated neurotransmitter release in presynapses (Reim et al., 2001; Maximov et al., 2009), binds to SNARE complexes to mediate AMPAR exocytosis during LTP in postsynapses (Ahmad et al., 2012). Subsequently, postsynaptic SNARE proteins such as syntaxin 3, SNAP-47 and synaptobrevin-2, which are distinct from proteins involved in presynaptic neurotransmitter release, were reported to regulate AMPAR exocytosis during LTP (Jurado et al., 2013).

Visualization of activity-triggered exocytosis of AMPARs in dendrites and dendritic spines is possible using the pH-sensitive superecliptic pHluorin (SEP), whose fluorescence is quenched at low pH (Miesenbock et al., 1998). Indeed, using SEP-tagged AMPARs, the postsynaptic exocytosis of AMPARs during LTP has been visualized directly (Kopec et al., 2006, 2007a; Yudowski et al., 2007; Lin et al., 2009; Makino and Malinow, 2009; Petrini et al., 2009; Araki et al., 2010; Kennedy et al., 2010; Patterson et al., 2010; Cho et al., 2015). Glycine-induced LTP has been shown to be mediated by an accumulation and immobilization of SEP-GluA1s at synapses, due to both exocytosis and stabilization of GluA1s at the postsynaptic density (PSD; Petrini et al., 2009). Impairment of GluA1 recycling exocytosis with a dominant-negative mutant of Rab11 results in GluA1 being less mobile at synapses. In addition, the displacement of endocytic zones from the PSD by a point mutant of dynamin-3 unable to bind Homer1 (Lu et al., 2007) impairs glycine-induced LTP expression by blocking GluA1 recycling (Petrini et al., 2009). Taken together, these results suggest that the GluA1 endocytic recycling pool is crucial for maintaining a mobile population of surface GluA1s that can be mobilized to synapses for LTP. Bath application of glycine for inducing LTP increases the exocytic events of SEP-GluA1 in dendrites and dendritic spines (Yudowski et al., 2007; Cho et al., 2015). In a more localized activation using two-photon glutamate uncaging, which mimics single synaptic release with sufficient spatiotemporal resolution (Matsuzaki et al., 2004; Bagal et al., 2005; Harvey and Svoboda, 2007; Lee et al., 2009), SEP-GluA1 was observed to be exocytosed to dendrites and activated spines (Makino and Malinow, 2009; Patterson et al., 2010). The increase of AMPAR-mediated currents was observed in spines initially, and then in the dendrite following glutamate uncaging-evoked LTP (Makino and Malinow, 2009), consistent with GluA1 insertion directly to the spines.

Many studies have supported the idea that LTP triggers the exocytosis of AMPARs required for expression. In addition, several studies have demonstrated how the intracellular recycling endosome that stores AMPARs is mobilized to near or at the spine for LTP, and where AMPARs are exocytosed to the spine surface in relation to the PSD for LTP (Wang et al., 2008; Kennedy et al., 2010). The actin-based Ca^{2+}-sensitive motor protein myosin Vb has been reported to mediate the translocation of recycling endosomes harboring AMPARs into spines during LTP (Wang et al., 2008). Blockade of myosin Vb using RNA interference or chemical-genetic inhibition results in reduced LTP-induced SEP-GluA1 insertion and hippocampal slice LTP, indicating that the myosin Vb-mediated mobilization

of recycling endosomes is required for synaptic potentiation (Wang et al., 2008). Related actin-based myosin Va has also been reported to mediate the translocation of AMPARs to spines from the dendritic shaft during LTP (Correia et al., 2008). Neurons expressing a dominant-negative form of myosin Va or a short interfering RNA specific for myosin Va showed a blockade of synaptic delivery of GluA1 and LTP (Correia et al., 2008). Conversely, myosin Va mutant mice showed normal synaptic plasticity (Schnell and Nicoll, 2001), suggesting a potential compensation by other Class V myosins. In a recent report on another actin-dependent motor protein myosin IXa, myosin IXa$^{+/-}$ mice displayed impaired LTP (Folci et al., 2016), together indicating that myosin motor proteins play roles in AMPAR delivery during LTP. Using SEP-GluA1 and transferrin receptor (TfR), a classic recycling endosomal marker, GluA1 in TfR-positive recycling endosomes was shown to be exocytosed to spines adjacent to the PSD during glycine-induced LTP. Newly inserted SEP-GluA1 either quickly diffuses out of the spine or stays near the site of fusion in spines, whereas TfRs that co-exocytosed with SEP-GluA1 always diffused out of the spine immediately following the co-appearance of SEP-GluA1 (Kennedy et al., 2010). The exocytic events occurring adjacent to the PSD are mediated by syntaxin-4, which played a role in recycling endosome fusion to the spine plasma membrane. Disrupting syntaxin-4 blocks spine exocytosis and impairs LTP (Kennedy et al., 2010). The results of this study also suggest that different cargoes follow their own fate once they arrive at the spine surface. Although this study reported a requirement for syntaxin-4, but not for syntaxin-3 in LTP (Kennedy et al., 2010), other groups have demonstrated that LTP requires syntaxin-3, but not syntaxin-4 (Jurado et al., 2013; Arendt et al., 2015). Several explanations for these contradictory results have been extensively debated in the "Discussion" section of Jurado et al. (2013).

Recent work from Choquet and co-workers has provided a temporal profile of AMPAR trafficking for LTP expression by employing a novel approach that immobilizes surface AMPARs to prevent their diffusion on the cell surface (Penn et al., 2017). Biotin-tethered AMPAR subunit GluA1 or GluA2 can be expressed exogenously in cultured hippocampal neurons along with the endoplasmic-reticulum-retained biotin ligase (BirA-ER). In the presence of the biotin-binding protein NeutrAvidin, biotin-tethered AMPARs can be effectively crosslinked by NeutrAvidin, which reduces their surface diffusion as monitored by fluorescence recovery after photobleaching (FRAP; Penn et al., 2017). Using biotin-tethered GluA2 exogenously expressed in organotypic hippocampal slices prepared from GluA2-knockout mice, it was demonstrated that acute pre-treatment with NeutrAvidin to immobilize only pre-existing surface GluA2 results in a complete blockade of the short-term potentiation induced by a HFS LTP protocol. However, the hippocampal cells still express a detectable LTP, although small, indicating a contribution of exocytosis for LTP expression. Accordingly, the prevention of postsynaptic membrane fusion events by the intracellular application of tetanus toxin blocked HFS-induced LTP completely, but normal levels of short-term potentiation were still expressed, indicating a requirement of exocytosis for LTP expression (Penn et al., 2017).

It is very clear that LTP requires AMPAR exocytosis to sites adjacent to the PSD. These exocytosed receptors need to be relocated to synapses for synaptic potentiation (Makino and Malinow, 2009; Kennedy et al., 2010; Patterson et al., 2010). Interestingly, some populations of AMPARs may diffuse in and out between extrasynaptic and synaptic sites in order to tune synaptic transmission (Heine et al., 2008), and this receptor exchange organized on the surface membrane through lateral mobility is regulated dynamically by activity. Choquet and co-workers demonstrated that extrasynaptic surface AMPARs adjacent to the PSD arrive at synaptic sites through lateral diffusion upon LTP stimulation (Tardin et al., 2003; Opazo et al., 2010; Opazo and Choquet, 2011; Huganir and Nicoll, 2013; Chater and Goda, 2014; Constals et al., 2015; Compans et al., 2016; Penn et al., 2017). The lateral mobility of the surface GluA2 subunit inside nanodomains and/or outside of synapses was observed first using single-molecule fluorescence microscopy (Borgdorff and Choquet, 2002; Tardin et al., 2003). Lateral diffusion of surface GluA2 between extrasynaptic and synaptic sites is regulated by increased intracellular Ca^{2+}, glutamate application, and glycine-induced stimulation, suggesting that the lateral diffusion of AMPARs may act as an important controlling step for synaptic plasticity (Borgdorff and Choquet, 2002; Tardin et al., 2003). Taken together, these reports suggest that a pre-existing surface pool of AMPARs are a prompt source to reach synapses via lateral diffusion and are then trapped at synapses for short-term potentiation, while newly exocytosed AMPARs from the recycling endosome upon LTP stimulation are the major source for sustaining LTP expression (**Figure 1**). Future work should address the intriguing possibility that the prompt reservoir of AMPARs for LTP is an extra-nanodomain or a true extrasynaptic region.

STABILIZATION OF AMPARs AT SYNAPTIC SITES FOR LTP

Laterally diffusing surface AMPARs must be trapped and immobilized at nanodomains on synaptic sites for LTP stabilization. Stargazin, an AMPAR auxiliary protein (Tomita et al., 2005; Hafner et al., 2015), was reported as a key molecule involved in the trapping and stabilization of AMPARs at synaptic sites during LTP (Opazo et al., 2010). LTP-inducing stimulation to activate NMDARs resulting in Ca^{2+} influx triggers CaMKII activation. CaMKII-mediated phosphorylation of the C-terminal PDZ-binding domain in Stargazin creates a highly negatively charged C-terminal tail of Stargazin so that it repulses the negatively charged membrane lipid. The C-terminal tail of Stargazin then unfolds, which favors its binding to PSD-95, thereby increasing the synaptic trapping of AMPARs on the nanodomains (**Figure 1**; Opazo et al., 2010; Opazo and Choquet, 2011; Choquet and Triller, 2013).

Besides these intracellular mechanisms of AMPAR trapping and stabilization at synaptic sites, LTP stabilization might also involve trans-synaptic mechanisms involving the extracellular

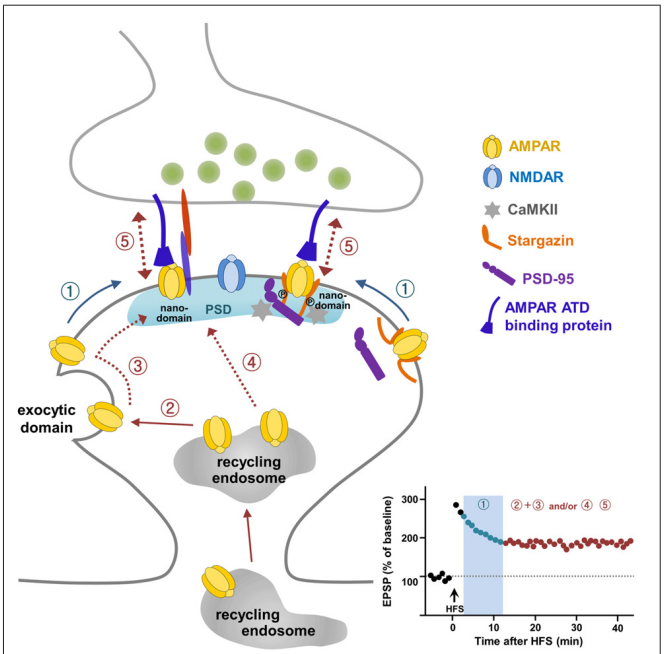

FIGURE 1 | A current model for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor (AMPAR) trafficking during long-term potentiation (LTP). Pre-existing extrasynaptic surface AMPARs are mobilized to the synaptic sites upon LTP stimulation via lateral diffusion (①) and are trapped in nanodomains within the synapse, which is required for short-term potentiation (① in LTP graph). AMPARs exocytosed from intracellular recycling endosomes to the exocytic domain adjacent to the postsynaptic density (PSD) replenish the extrasynaptic surface pool of AMPARs (②), and these exocytosed AMPARs are mobilized laterally to reach the synaptic site (③) and supply AMPARs for maintaining LTP expression. The direct delivery of a portion of AMPARs on recycling endosomes to the synaptic site during LTP (④) cannot be ruled out. AMPARs trapped at the synapse are immobilized through the regulation of Stargazin and probably through the subunit-specific regulation of the AMPAR amino-terminal domain (ATD) interacting protein (⑤).

ATD of AMPARs. Indeed, the involvement of extracellular domains of AMPARs in LTP has recently been reported (Diaz-Alonso et al., 2017; Watson et al., 2017). The extracellular ATDs of GluA1 and GluA2 exert a subunit-specific role in synaptic trafficking of AMPARs. The ATD of GluA1, but not GluA2, is required for surface GluA1 translocation to synapses (Diaz-Alonso et al., 2017). GluA1 without ATD exhibits increased mobility in synapses and failed to sustain LTP (Diaz-Alonso et al., 2017), indicating a requirement for ATD of GluA1 in LTP stabilization. Further, a concept of a trans-synaptic molecular nanocolumn, stretching from the presynaptic neurotransmitter release site to the postsynaptic receptor cluster, has been introduced (Savtchenko and Rusakov, 2014; Tang et al., 2016; Biederer et al., 2017). LTD-triggering stimuli reorganized nanocolumns through trans-synaptic nanocluster realignment whereas LTP-triggering stimuli reorganized only postsynaptic nanoclusters with no changes in presynaptic nanoclusters (Tang et al., 2016). It might be possible to observe presynaptic nanocluster reorganization if LTP could be stabilized, which can be mediated by trans-synaptic communications.

Super-resolution imaging technologies (Hell and Wichmann, 1994; Betzig, 1995; Betzig et al., 2006; Rust et al., 2006; Manley et al., 2008) with 10- to 100-nm spatial resolution, such as stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), universal point accumulation in nanoscale topography (u-PAINT), direct stochastic optical reconstruction microscopy (dSTORM) and electron microscopy have demonstrated AMPAR nanodomains inside synapses, with 1–3 of 80 nm clusters at each synapse and 20–25 AMPARs in each cluster (Nair et al., 2013), and trans-synaptic nanocolumns (Tang et al., 2016). Improvements in super-resolution imaging techniques and protein sensor development should allow greater manipulation and observation (Chater and Goda, 2014; Granger and Nicoll, 2014; Martineau et al., 2017). These improved methods will facilitate investigations as to whether new nanodomains, where AMPARs are trapped during LTP, and/or nanocoulmns are formed to mediate synaptic potentiation during LTP and whether AMPARs are trapped on pre-existing and/or newly formed nanodomains and/or nanocolumn, if generated, during LTP (Compans et al., 2016).

SUMMARY AND PERSPECTIVES

Over the past two decades, many laboratories have committed intensive effort to uncover mechanisms underlying AMPAR trafficking during LTP. These efforts, employing novel and advanced methods in electrophysiology, molecular and cellular biology, biochemistry, imaging and genetics provide a working model for how reserve pools of AMPARs are delivered to synapses for LTP (**Figure 1**). According to this model, pre-existing surface AMPARs are the first requirement for LTP expression. These pre-existing surface AMPARs are mobilized quickly to synaptic sites via lateral diffusion upon LTP stimulation and are trapped in microdomains within synapses (**Figure 1**①). To sustain the expression of LTP, more AMPARs need to be delivered to the synaptic sites. This occurs via exocytosis of AMPARs from the recycling endosome to extrasynaptic sites to replenish the surface pool of AMPARs (**Figure 1**②), which then diffuse laterally and are trapped at the synapses (**Figure 1**③). In addition, it cannot be ruled out that a portion of AMPARs are supplied to the synaptic sites via exocytosis directly from the recycling endosomes (**Figure 1**④). Although the study by Penn et al. (2017) has advanced our understanding of LTP expression, some questions still remain to be addressed. Future development of molecular and opto-genetic manipulations and imaging technologies with greater spatial and temporal resoultion, will help determine whether subunits of AMPARs interplay with each other to contribute differentially to LTP expression. In addition, it will be of interest to investigate the relationship between AMPAR surface mobility and LTD, and, further, to elucidate whether different learning paradigms such as fear conditioning, water maze, passive avoidance, or novel object recognition utilize specific AMPAR trafficking mechanisms. Indeed, the C-terminal tails of GluA1 and GluA2 have been reported to exert differential roles in spatial learning and memory

and contextual fear memory, respectively, suggesting a specific regulation of behavioral plasticity by AMPARs (Zhou et al., 2018).

The requirement of the C-terminal tail of GluA1 for LTP has been well accepted in the field since the sophisticated electrophysiology study of Shi et al. (2001). This work has resulted in many follow-up studies, whose main focus has been uncovering the nature of the molecules interacting with the C-terminal tails of AMPARs for the regulation of synaptic transmission and plasticity. However, the requirement of the C-terminal tail of GluA1 for LTP has been challenged (Granger et al., 2013), and subsequent studies have turned their attention toward the involvement of extracellular domains of AMPARs in LTP (Diaz-Alonso et al., 2017; Watson et al., 2017). Mobilization of surface AMPARs from extrasynaptic to synaptic sites is a well-recognized process for LTP expression. Stargazin has been identified as a regulator for LTP-triggered CaMKII-mediated trapping and immobilization of AMPARs diffusing in the membrane (Opazo et al., 2010). Analogous to the intracellular mechanism underlying immobilization of AMPARs at synapses by Stargazin, and given that studies highlight the importance of extracellular ATDs of AMPARs in LTP (Diaz-Alonso et al., 2017; Watson et al., 2017) and the reorganization of trans-synaptic nanocolumns by NMDAR activation (Tang et al., 2016), it is possible that trans-synaptic anchoring mechanisms, probably involving synaptic adhesion molecules, stabilize AMPARs by trapping them through their ATDs and preventing them from diffusing during LTP (**Figure 1⑤**). Some synaptic adhesion molecules may be good candidates for anchoring through direct or indirect interactions with the ATD of AMPARs in the cleft space (Shipman and Nicoll, 2012; Aoto et al., 2013; Anderson et al., 2015; Jang et al., 2016; Gulisano et al., 2017; Varbanov and Dityatev, 2017; Bhouri et al., 2018). Further studies of extracellular trans-synaptic ATD regulation will add more information about the specific mechanisms by identifying the molecules involved in AMPAR trafficking during LTP. The recent visualization and measurement of intracellular transport of newly synthesized AMPARs during LTP (Hangen et al., 2018) provides a possible way to investigate the intracellular dynamics and mechanisms of synaptic key molecules that link the transition from the early to late phase of LTP, which might be contributed by local protein synthesis in dendrites (Sutton and Schuman, 2005; Sutton et al., 2006). Together, these approaches will further expand our understanding of LTP and open a new era in studies into how LTP affects synaptic plasticity and ultimately learning and memory.

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The author confirms being the sole contributor of this work and approved it for publication.

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HYPOTHESIS AND THEORY
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Mechanisms and Role of Dendritic Membrane Trafficking for Long-Term Potentiation

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Long-term potentiation (LTP) of excitatory synapses is a major form of plasticity for learning and memory in the central nervous system. While the molecular mechanisms of LTP have been debated for decades, there is consensus that LTP induction activates membrane trafficking pathways within dendrites that are essential for synapse growth and strengthening. Current models suggest that key molecules for synaptic potentiation are sequestered within intracellular organelles, which are mobilized by synaptic activity to fuse with the plasma membrane following LTP induction. While the identity of the factors mobilized to the plasma membrane during LTP remain obscure, the field has narrowly focused on AMPA-type glutamate receptors. Here, we review recent literature and present new experimental data from our lab investigating whether AMPA receptors trafficked from intracellular organelles directly contribute to synaptic strengthening during LTP. We propose a modified model where membrane trafficking delivers distinct factors that are required to maintain synapse growth and AMPA receptor incorporation following LTP. Finally, we pose several fundamental questions that may guide further inquiry into the role of membrane trafficking for synaptic plasticity.

Keywords: long term potentiation, AMPA receptor, exocytosis, dendrite, membrane trafficking, recycling endosomes, dendritic spines, plasticity and learning

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INTRODUCTION

Information storage, learning, and adaptive behavior are thought to occur through use-dependent changes in the strength of synaptic connections. For example, long-term potentiation (LTP) of excitatory synapses is widely accepted as a critical form of plasticity for learning and memory throughout the brain (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Nicoll, 2017). While numerous pre- and postsynaptic LTP mechanisms have been described in diverse circuits, LTP has been most intensely investigated in pyramidal neurons of hippocampal region CA1. Here, multiple lines of evidence agree that LTP is predominantly mediated by increased function of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. Increased channel conductance, open probability, and receptor number have all been reported to be responsible for synaptic potentiation (Isaac et al., 1995; Liao et al., 1995; Roche et al., 1996; Barria et al., 1997; Benke et al., 1998; Derkach et al., 1999; Shi et al., 1999; Banke et al., 2000). Support for increased number of synaptic AMPA receptors during LTP primarily comes from biochemical measurements demonstrating the level of surface receptors increases following

LTP and from microscopy experiments directly visualizing tagged AMPA receptors as they cluster at postsynaptic sites following LTP induction (Shi et al., 1999; Heynen et al., 2000; Broutman and Baudry, 2001; Lu et al., 2001). Functional studies using peak-scaled non-stationary fluctuation analysis to estimate changes in receptor number and conductance following LTP are also consistent with insertion of AMPA receptors into the postsynaptic density (PSD) during LTP (Benke and Traynelis, 2018). While there is general agreement that AMPA receptors are recruited to the postsynaptic plasma membrane (PM) following LTP, the source of these receptors remains controversial. Two major pools of "extrasynaptic" receptors are available: those that are already laterally diffusing within the dendritic PM, and those that are housed in internal membrane-bound organelles. Thus, AMPA receptors could be added to the postsynaptic membrane by trapping diffusing surface receptors and/or through mobilizing receptors from internal stores. The latter mechanism requires that intracellular organelles housing AMPA receptors fuse near the postsynaptic membrane to deliver receptors to synapses undergoing plasticity. The early observation that LTP depends on membrane fusion provides tantalizing support for mobilization of receptors from intracellular pools. While there is strong evidence that AMPA receptors are mobilized to the PM during LTP, no study has definitively demonstrated this pool of receptors directly contributes to synapse potentiation. On the contrary, recent experiments support a major role for trapping laterally diffusing receptors at synaptic sites during LTP. Here we discuss literature supporting both sides of this issue and provide experimental data from our lab consistent with a model where membrane fusion delivers as-yet unidentified factors that stabilize AMPA receptors at synaptic sites following their initial incorporation by lateral diffusion.

Membrane Trafficking Is Essential for LTP

While the molecular mechanisms that govern LTP have been debated for decades, there is general consensus that membrane trafficking in the postsynaptic cell is essential. This was first reported by Lledo et al. (1998) who demonstrated that infusing postsynaptic neurons with factors that inhibit membrane fusion mediated by soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) family proteins, including a peptide that disrupts SNAP interactions, N-ethylmaleimide and botulinum neurotoxin B, blocked LTP. Intriguingly, none of these reagents affected the initial magnitude of synaptic potentiation that occurred following LTP induction, which likely arises from post-tetanic potentiation of neurotransmitter release and enhanced postsynaptic AMPA receptor function and/or number. However, synaptic responses gradually declined to baseline levels ∼20–30 min following LTP induction when membrane fusion was disrupted. These experiments provided the first evidence that membrane fusion in the postsynaptic cell is required for sustained synaptic potentiation during LTP. Given that the initial phase of LTP appeared normal when membrane fusion was blocked, these experiments also demonstrate that the trafficking requirement does not manifest until several minutes following LTP induction. Numerous subsequent studies using diverse LTP induction protocols and recording techniques have established postsynaptic membrane trafficking as a hallmark of LTP (Lu et al., 2001; Park et al., 2004, 2006; Kopec et al., 2007; Yang et al., 2008b).

Given the central importance of postsynaptic membrane fusion for LTP, a critical question is the identity of the organelle(s) undergoing fusion. There is a vast network of intracellular organelles present within neuronal dendrites and spines (Parton et al., 1992; Spacek and Harris, 1997; Cooney et al., 2002; Park et al., 2004, 2006; Rácz et al., 2004; Kennedy et al., 2010; Hanus et al., 2014; Esteves da Silva et al., 2015; Bowen et al., 2017; Hiester et al., 2017; Wu et al., 2017b). Among the organelles that could participate in rapid membrane remodeling at synapses, recycling endosomes (REs) stand out. REs are intracellular vesicles that regulate trafficking of protein cargoes to and from the PM (Maxfield and McGraw, 2004). In neurons, REs are distributed throughout the dendritic arbor and within a substantial fraction of dendritic spines. Importantly, REs are mobilized to fuse with the PM following LTP stimuli, resulting in the rapid delivery of resident RE cargo proteins to the dendritic surface (Park et al., 2006; Wang et al., 2008c; Kennedy et al., 2010; Keith et al., 2012; Roman-Vendrell et al., 2014; Woolfrey et al., 2015; Hiester et al., 2017). Importantly, disruption of postsynaptic RE function also disrupts functional LTP and accompanying morphological plasticity (Park et al., 2004, 2006; Brown et al., 2007; Wang et al., 2008c; Kennedy et al., 2010; Keith et al., 2012; Woolfrey et al., 2015). Activity triggered RE fusion occurs throughout neuronal dendrites, including within dendritic spine heads suggesting that the excitatory postsynaptic membrane could be rapidly remodeled via nearby RE fusion, although the precise location (i.e., spine head vs. dendritic shaft) of the RE fusion events relevant for LTP remains a controversial and open question (Spacek and Harris, 1997; Cooney et al., 2002; Rácz et al., 2004; Yudowski et al., 2007; Lin et al., 2009; Makino and Malinow, 2009; Kennedy et al., 2010; Patterson et al., 2010; Hiester et al., 2017; Wu et al., 2017b). Regardless of whether the LTP-relevant RE fusion events occur within or near activated spines, REs must be able to sense local activity in order to fuse near synapses undergoing LTP. While few studies have investigated the spatial relationship between activated synapses and RE fusion, Patterson et al., demonstrated that glutamate uncaging over individual dendritic spines triggers fusion of GluA1-containing vesicles both within the activated spine and in the nearby dendritic shaft (Patterson et al., 2010). This finding was supported by a subsequent study demonstrating that glutamate uncaging triggers RE fusion within activated spines, consistent with a role for RE fusion in synapse-specific GluA1 delivery events observed by Patterson et al. (Hiester et al., 2017). Whether spine RE fusion plays a direct role in LTP remains an open question, but at steady state, not all dendritic spines house REs raising the issue of whether spines lacking a resident RE are impaired for LTP. Intriguingly, LTP measured by long-lasting morphological spine growth following single spine glutamate uncaging was originally reported to occur in 55% of spines (and less frequently at larger spines) and functional LTP at presumed single synapses occurred at 65% of synapses tested, similar to the fraction of RE-containing spines, which has been reported at 25–50% depending on age and endosome classification criteria

(Petersen et al., 1998; Cooney et al., 2002; Matsuzaki et al., 2004; Park et al., 2006; Kennedy et al., 2010; Hiester et al., 2017). More refined local RE inactivation techniques will be required to begin addressing the spatial relationship between RE fusion and synapses undergoing LTP.

AMPA Receptors Localize to Dendritic REs and Are Mobilized to the Cell Surface by Synaptic Activity

Given that membrane fusion and RE function is essential for LTP, a central issue is the identity of the cargo delivered to synapses via RE fusion events. Because synaptic AMPA receptor content increases following LTP, many studies focused on determining whether RE fusion could be the major delivery route to the synapse. Indeed, an immunoelectron microscopy-based investigation of the ultrastructural localization of internal AMPA receptors identified a population of GluA2 that localizes to dendritic, but not spine endosomes, although peri-synaptic endocytic pits could be observed to contain GluA2 following NMDA receptor activation (Tao-Cheng et al., 2011). Using a sensitive antibody feeding assay to selectively label internalized pools of AMPA receptors, multiple studies have demonstrated localization of AMPA receptor subunits GluA1 and GluA2 to a large fraction of REs within dendritic shafts and spines (Ehlers, 2000; Park et al., 2004; Kennedy et al., 2010; Hiester et al., 2017). In these studies, the majority of internalized GluA1 co-localizes with RE marker proteins, supporting a major role for these organelles in AMPA receptor surface trafficking, though it is possible that constitutive trafficking of AMPA receptors occurs through a subset of REs, positive for the small GTPases Arf6 and TC10 (Zheng et al., 2015). Indeed, the molecular and functional heterogeneity of endosomes labeled with classical markers such as transferrin receptor or rab proteins deserves further investigation. For example, it remains unknown what mechanisms allow a subset of endosomes to be mobilized by synaptic activity to fuse with the PM. More direct support for regulated AMPA receptor surface delivery via REs came from experiments using an NMDA receptor-dependent chemical LTP (cLTP) stimulation. Because this form of stimulation globally activates many synaptic inputs, potentiation can be monitored by measuring the amplitude and frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) mediated by AMPA receptors and correlated with surface GluA1 levels measured by immunolabeling (Lu et al., 2001; Park et al., 2004). Following cLTP, surface GluA1 was elevated and mEPSC amplitude increased, providing a positive correlation between synapse potentiation and GluA1 delivery to the PM (Lu et al., 2001). Importantly, both potentiated mEPSC amplitude and increased surface GluA1 were blocked by tetanus neurotoxin (TeNT), which cleaves the vesicle associated membrane proteins (VAMPs) required for activity-dependent membrane fusion in axons and dendrites (Maletic-Savatic and Malinow, 1998; Lu et al., 2001). Subsequent studies utilizing similar cLTP stimuli also demonstrated that surface levels of endogenous (Ahmad et al., 2012; Jaafari et al., 2012, 2013; Jurado et al., 2013; Hiester et al., 2017; Wu et al., 2017a) and exogenously expressed (Passafaro et al., 2001; Park et al., 2004; Patterson et al., 2010) GluA1-containing AMPA receptors increase following stimulation. Many of these studies additionally demonstrated that the same SNARE machinery that is required for expression of LTP is also required for AMPA receptor surface delivery (Ahmad et al., 2012; Jurado et al., 2013; Wu et al., 2017a; Bin et al., 2018). Importantly, disrupting RE function also blocks regulated AMPA receptor surface delivery, synapse potentiation, and spine growth following LTP stimuli, supporting a model where REs are the primary organelles undergoing fusion for excitatory synaptic plasticity (Park et al., 2004, 2006; Brown et al., 2007; Wang et al., 2008c; Kennedy et al., 2010).

A complementary line of inquiry utilized longitudinal live-cell microscopy to directly visualize AMPA receptor trafficking during LTP. One of the most widely used techniques relies upon the pH sensitive green fluorescent protein superecliptic pHluorin (SEP), which is brightly fluorescent at neutral pH, but quenched within the acidic lumen of intracellular endosomes (Miesenbock et al., 1998). Numerous studies have used SEP-GluA1 to monitor activity-triggered AMPA receptor membrane insertion (Kopec et al., 2007; Yudowski et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Patterson et al., 2010). Following global cLTP stimulation, the frequency of SEP-GluA1 insertion events increases, indicating that internal stores of GluA1 are mobilized to the dendritic PM in an NMDA receptor-dependent manner (Yudowski et al., 2007). Further, activity-triggered SEP-GluA1 insertion events are inhibited by botulinum neurotoxins A and TeNT which cleave SNAP25 and VAMP family proteins respectively, providing another correlative link between functional LTP and AMPA receptor delivery to the PM (Makino and Malinow, 2009; Patterson et al., 2010). Similar global stimulation approaches and more refined single synapse glutamate uncaging techniques induce SEP-GluA1 insertion directly within dendritic spines (Kennedy et al., 2010; Patterson et al., 2010). Direct spine SEP-GluA1 delivery is the result of RE fusion, as demonstrated by dual color imaging of a RE marker protein along with SEP-GluA1 (Kennedy et al., 2010) (**Figures 1A,B**). Intriguingly, the timing of spine RE fusion is highly variable following the onset of stimulation. **Figure 1C** shows the timing of spine RE fusion before, during and following cLTP stimulation. While RE fusion can occur immediately following stimulation, many events in spines (and dendritic shafts) occur several minutes following stimulation (Kennedy et al., 2010). The broad timing of the events relative to the onset of stimulation raises the intriguing possibility that different subtypes of endosomes can differentially respond to activity to deliver distinct cargoes during different phases of plasticity. Alternatively, global stimulation paradigms where many synapses are simultaneously activated could deplete resources required for membrane fusion and therefore influence when and where the events occur. Indeed, the timing of spine RE fusion events was more tightly correlated with the onset of stimulation when individual synapses were activated using glutamate uncaging, but could still occur tens of seconds to minutes following stimulation (Patterson et al., 2010; Hiester et al., 2017).

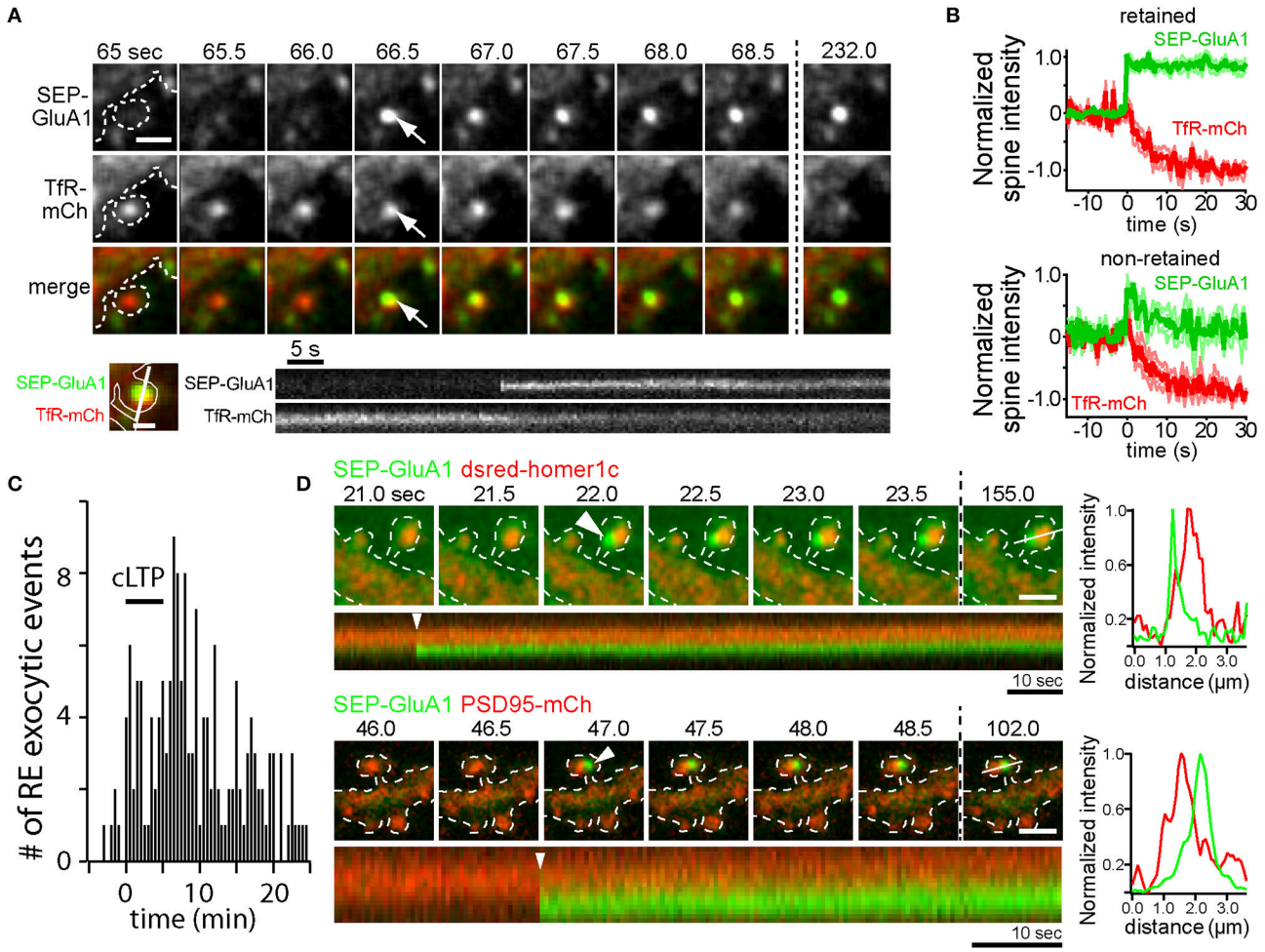

FIGURE 1 | AMPA receptors can be directly inserted into the spine plasma membrane adjacent to the PSD by RE fusion. **(A)** Time-lapse imaging of a hippocampal neuron coexpressing SEP-GluA1 (top row) and the RE marker protein TfR-mCh (middle row) following cLTP stimulation. Note the abrupt appearance of SEP- GluA1 fluorescence at the precise location of a spine RE (arrows). Scale bar, 1 μm. Below is a kymograph showing a single spine exocytic event of SEP-GluA1 (top) and TfR-mCh (bottom). Note that SEP-GluA1 is inserted and retained in the spine head, even as TfR-mCh from the same fusion event quickly diffuses away. Scale bar, 0.5 μm. **(B)** SEP-GluA1 exocytic events fall into two classes. Following RE fusion, SEP-GluA1 was either retained in spines (62% of total events, top panel) or quickly diffused away (38% of total events, bottom panel). In all cases, colocalized TfR-mCh signal (red traces) declined rapidly following the appearance of SEP-GluA1 (green traces). To average traces from multiple events, individual traces were aligned at the time of fusion, which was arbitrarily labeled $t = 0$ s. **(C)** The timing of RE fusion events within dendritic spines before, during and following cLTP stimulus (black bar) is plotted. Data in panels **(A–C)** were modified from Kennedy et al. (2010) and reprinted with permission from *Cell Press*. **(D)** AMPA receptors are inserted adjacent to the PSD. Time lapse imaging of hippocampal neurons coexpressing SEP-GluA1 and dsred-homer1c (top) or PSD95-mCh (bottom) following cLTP stimulation. Discrete SEP-GluA1 insertion events (arrowheads) occurred adjacent to, but not directly overlapping, the PSD. Scale bar, 1 μm. A kymograph measured along the line from the final time point is shown below each time series, and an intensity profile of the signal from each channel at the time of exocytosis ($t = 0$ s, arrowhead) is shown on the right demonstrating the two signals are optically resolvable.

Collectively, these studies point toward a mechanism whereby NMDA receptor activation during LTP drives Ca^{2+}-dependent fusion of intracellular REs, thus delivering GluA1-containing AMPA receptors to the cell surface. However, none of these studies demonstrate that newly delivered receptors play a direct role in potentiating synaptic responses. For example, the extent to which AMPA receptors recently trafficked to the cell surface stably incorporate into dendritic spines remains controversial with some studies demonstrating that SEP-GluA1 inserted into the dendritic shaft transiently enters spines but is not trapped (Yudowski et al., 2007; Makino and Malinow, 2009) and others demonstrating some degree of receptor trapping following direct insertion into spines (Kennedy et al., 2010; Patterson et al., 2010) (**Figures 1A,B**). In many of these studies SEP-GluA1 insertion events were relatively rare. For example, Patterson et al. demonstrate that newly inserted receptors contribute only 10–30% of the total accumulated spine SEP-GluA1 fluorescence following LTP induced by glutamate uncaging (Patterson et al., 2010). However, it should be noted that SEP-GluA1 experiments should be interpreted with caution. Data from our lab has shown that SEP-GluA1 localization to REs is substantially lower than that observed using more sensitive antibody feeding techniques to selectively quantify internal pools of endogenous GluA1 and GluA2 (Kennedy et al., 2010; Hiester et al., 2017). The reason

for this is unclear, but multiple studies have demonstrated that under basal conditions, N-terminally tagged GluA1 receptors do not efficiently integrate into synaptic sites (Díaz-Alonso et al., 2017; Watson et al., 2017). Thus, decreased recycling pools of SEP-GluA1 could arise from lack of agonist-induced internalization since they may not be activated under basal conditions. In any case, given the sparseness of endosomal SEP-GluA1, this approach likely underestimates the fraction of newly inserted endogenous receptors during LTP, making it difficult to determine when, where and whether newly inserted receptors could directly contribute to the LTP response. Furthermore, spine localization observed with traditional confocal microscopy does not necessarily prove that receptors contribute to synaptic function. For example, recent work from our lab and others have demonstrated that receptors in and adjacent to the PSD may not be functionally activated unless they are precisely positioned within sub-PSD nanodomains directly opposite sites of neurotransmitter release (MacGillavry et al., 2013; Tang et al., 2016; Biederer et al., 2017; Sinnen et al., 2017; Hruska et al., 2018). Indeed, we present new imaging experiments simultaneously visualizing PSD markers along with SEP-GluA1 spine insertion events. These events were rare due to the sparseness of detectable endosomal SEP-GluA1, but when they occured SEP-GluA1 remained optically resolvable from the PSD for at least several minutes following insertion (**Figure 1D**). While this observation demonstrates perisynaptic fusion of SEP-GluA1-containing endosomes, the fact that newly inserted receptors remain resolvable from the PSD should be interpreted with caution since movement into the PSD could be hindered by the N-terminal SEP tag through steric interference and/or disruption of N-terminal binding interactions (Díaz-Alonso et al., 2017; Watson et al., 2017). While SEP-GluA1 can be retained in perisynaptic regions within spines following membrane insertion, co-trafficking TfR-mCh reaching the surface in the same fusion event rapidly diffuses from the site of insertion, demonstrating a selective trapping mechanism for AMPA receptors (**Figures 1A,B,D**) (Kennedy et al., 2010). The molecular mechanisms responsible for spine trapping and the extent to which native receptors integrate into the PSD following surface delivery will require new approaches for labeling and tracking endogenous receptors (Wakayama et al., 2017).

Assessing the Role of Lateral Diffusion vs. Membrane Trafficking for AMPA Receptor Delivery During LTP

While it is generally agreed that diverse LTP stimuli trigger AMPA receptor delivery to the cell surface, whether newly delivered receptors directly contribute to the LTP response remains a fundamental question. Alternatively, fast lateral diffusion and trapping of receptors already present at the surface may be the primary driver of increased synaptic AMPA receptor number during LTP. Indeed, a pool of AMPA receptors laterally diffuses in the PM, where they frequently encounter synaptic sites (Borgdorff and Choquet, 2002; Bats et al., 2007; Ehlers et al., 2007; Petrini et al., 2009; Opazo et al., 2010). Given their fast activation and desensitization kinetics, a rapidly exchanging pool of receptors is thought to be required to sustain high-frequency neurotransmission (Heine et al., 2008a). Intriguingly, AMPA receptor surface diffusion is regulated by synaptic activity, which generally increases mobility (Tardin et al., 2003; Groc et al., 2004). For example Groc et al. (2004) demonstrate that neural stimulation increases mobility of extrasynaptic receptors, largely through liberating a pool of immobile receptors. This could result in an expanded pool of diffusing receptors for synaptic integration and potentiation. Diffusing AMPA receptors can be trapped at synaptic sites through interactions between transmembrane AMPA receptor regulatory proteins (TARPs) and synaptic scaffolding proteins (Borgdorff and Choquet, 2002; Ashby et al., 2006; Makino and Malinow, 2009). Accumulation of laterally diffusing AMPA receptors is regulated by synaptic activity (Ehlers et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009) in a manner that requires CaMKII phosphorylation of TARPs to promote anchoring of receptors to the postsynaptic scaffold protein PSD-95 (Hayashi et al., 2000; Schnell et al., 2002; Bats et al., 2007; Opazo et al., 2010). Activity-triggered trapping of laterally diffusing AMPA receptors occurs on rapid time scales (<1 min) (Petrini et al., 2009; Opazo et al., 2010), consistent with early synaptic potentiation that occurs within seconds to minutes following LTP induction. Thus, at least one mechanism has been described that could account for the rapid incorporation of extrasynaptic surface AMPA receptors into the postsynaptic membrane without a requirement for membrane trafficking.

To more directly assess the role of lateral diffusion vs. membrane trafficking for synaptic delivery of AMPA receptors, a recent study employed an acute crosslinking approach to prevent lateral diffusion of surface AMPA receptors prior to LTP induction (Penn et al., 2017). In this study, either neutravidin crosslinking of expressed, biotinylated GluA2-containing AMPA receptors, or antibody crosslinking of endogenous GluA2 subunits blocked the earliest phase of LTP that occurs within seconds to minutes following induction. This observation supports a model where the rapid, initial phase of synapse potentiation is driven by lateral diffusion of GluA2-containing receptors into the postsynaptic membrane. Interestingly, when receptors were crosslinked prior to LTP induction, postsynaptic responses slowly increased for tens of minutes following LTP induction. This gradual potentiation was blocked by TeNT, consistent with slow synaptic accumulation of newly inserted receptors that were not subject to pre-induction crosslinking. Importantly, the magnitude of the slow increase in synaptic responses was significantly smaller than the control LTP response, suggesting that receptors newly trafficked to the cell surface play a relatively minor role in the LTP response. Finally, inclusion of neutravidin to crosslink GluA2-containing AMPA receptors during the entire timeframe of the experiments blocked both the rapid and gradual phases of synaptic potentiation indicating that receptors newly trafficked to the PM also must laterally diffuse into the postsynaptic membrane. This observation is consistent with dendritic and peri-synaptic fusion of REs, whose AMPA receptor cargo would need to laterally diffuse into the PSD to contribute to synaptic function (**Figures 1C,D**). It should be noted that it is also possible that newly inserted AMPA receptors (or GluA2-lacking

receptors already present on the cell surface prior to LTP induction) could be blocked from entering functional domains within the PSD by pre-existing, crosslinked and immobilized GluA2-containing receptors. This interpretation could explain an apparent discrepancy between Penn et al. (2017), where GluA2-containing receptors were immobilized, and previous work demonstrating that GluA2-lacking receptors are initially responsible for synapse potentiation during initial stages (first ∼25 min) of LTP (Plant et al., 2006). Nevertheless, the results from Penn et al. (2017) are also consistent with a major role for lateral diffusion in the initial synaptic potentiation that occurs following LTP, leading to a model where activity-triggered postsynaptic vesicle fusion promotes stability of AMPA receptors already recruited to synapses by lateral diffusion. We sought to further test this model using a complementary approach where we directly visualized AMPA receptors following synaptic stimulation when regulated membrane fusion was blocked with the catalytic light chain of tetanus neurotoxin (TeNT). To assess the efficiency of TeNT in blocking regulated dendritic membrane fusion, we co-expressed TeNT along with TfR-SEP in dissociated hippocampal neurons. Numerous previous studies have shown that cLTP stimulation triggers robust fusion of TfR-containing REs with the PM, resulting in an overall increase in surface TfR-SEP signal (**Figure 2A**) (Park et al., 2006; Kennedy et al., 2010). Activity-triggered surface insertion of TfR-SEP was completely blocked in TeNT-expressing neurons, confirming the efficacy of TeNT in blocking regulated RE fusion during cLTP. We next tested the effects of TeNT on AMPA receptor surface delivery and synapse accumulation following cLTP. As in previous studies, we imaged live neurons expressing the AMPA receptor subunit GluA1 tagged extracellularly with superecliptic pHluorin (SEP), which allowed us to quantitatively track surface accumulation and retention of surface AMPA receptors at individual spines following LTP induction (Ashby et al., 2006; Kopec et al., 2007; Yudowski et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Patterson et al., 2010). In contrast to many previous studies, we imaged cells with minimal SEP-GluA1 expression levels where clear synaptic enrichment could be observed, resembling endogenous AMPA receptor distribution (and not simply an outline of the entire dendritic membrane). We measured total and spine-specific SEP-GluA1 signal during a 5 min baseline period and then exposed neurons to a cLTP stimulus. Quantification of total SEP signal was carried out several minutes following stimulation as others and we have observed rapid, stimulus dependent quenching of SEP-GluA1 signal specifically in the dendritic shaft during the cLTP stimulus, presumably due to the transient acidification of the endoplasmic reticulum that occurs upon NMDA receptor activation (**Supplementary Figure 1**) (Rathje et al., 2013). Under these conditions, we observed a modest, but significant elevation in total surface SEP-GluA1 following cLTP stimulation in control neurons (**Figure 2B**), consistent with previous studies (Petrini et al., 2009; Zhang et al., 2015). Interestingly, we observed a much more robust enrichment of SEP-GluA1 at dendritic spines (**Figures 2C,D**; **Video 1**). This increase mirrored spine growth measured with an mCh cell fill but is not simply a reflection of increased membrane surface area since we observed a robust enrichment of receptors within spines compared to the surrounding dendritic shaft (**Figure 2C**) (Lang et al., 2004; Matsuzaki et al., 2004; Kopec et al., 2006; Ehrlich et al., 2007; Yang et al., 2008a; Patterson et al., 2010). Increased spine SEP-GluA1 signal was frequently maintained for the duration of the imaging period (45 min post cLTP) (**Figures 2D–G**). To directly test the role of postsynaptic membrane fusion in contributing to spine GluA1 accumulation, we compared control neurons with neurons expressing the catalytic light chain of TeNT. While TeNT did not affect total basal surface levels of SEP-GluA1 prior to cLTP treatment, it completely blocked the activity-triggered increase we observed under control conditions, in agreement with previous studies demonstrating activity-triggered SEP-GluA1 trafficking from internal pools to the PM (**Figure 2B**) (Yudowski et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Patterson et al., 2010). Despite the fact that total SEP-GluA1 signal slightly decreased following stimulation in TeNT-expressing neurons, we still observed rapid activity-triggered accumulation of SEP-GluA1 signal in dendritic spines (**Figures 2C,D**). Surprisingly, SEP-GluA1 accumulation 10-15 min following cLTP induction was indistinguishable from controls, ruling out a major role for regulated membrane trafficking during the initial phase of AMPA receptor recruitment to synaptic sites (**Figure 2E**). Initial activity-triggered spine growth was also unperturbed by TeNT (**Figure 2D**), consistent with previous work (Yang et al., 2008a). Importantly, increased spine SEP-GluA1 signal was not maintained in a significant fraction of spines from TeNT-expressing neurons, returning to baseline, pre-stimulation levels ∼30 min following cLTP induction (**Figures 2C–G**, **Video 2**). Together these observations are consistent with membrane trafficking playing an essential role in maintaining receptors initially recruited to synaptic sites by lateral diffusion (Penn et al., 2017). While it is possible that some portion of the sustained synaptic SEP-GluA1 signal in control conditions is due to activity-triggered insertion of expressed receptors, we think that the previously reported internalization defects of SEP-GluA1 (Kennedy et al., 2010; Hiester et al., 2017) further support our interpretation that the majority of retained GluA1 comes from a pre-existing pool of surface receptors. Thus, we propose that the primary role of postsynaptic vesicle fusion during LTP is not to deliver new AMPA receptors to synapses, but to traffic unidentified factors that maintain accumulated synaptic receptors and stabilize spine growth (**Figure 3**). While there is abundant evidence that REs also deliver AMPA receptors to the PM, this pool may be more important for replenishing the "reserve pool" of extrasynaptic surface receptors critical for LTP (Granger et al., 2013).

Future Directions and Outstanding Questions

Recent work has begun to more critically interrogate the role of postsynaptic membrane trafficking during LTP and suggests that the role of activity-triggered membrane fusion extends beyond regulating trafficking of AMPA receptors. We speculate that intracellular vesicles, REs in particular, house a cocktail of important synaptic cargoes that can be mobilized to the

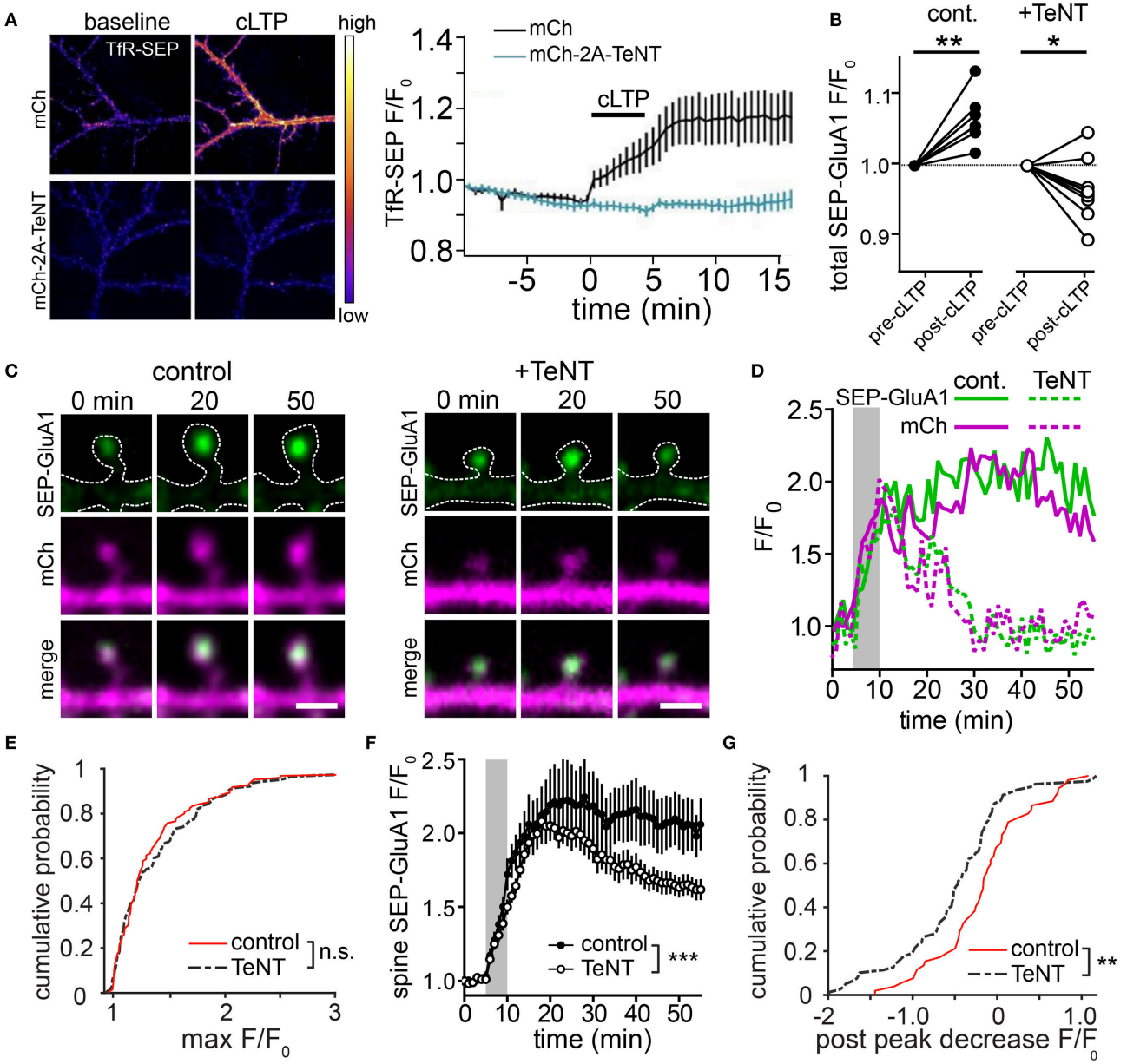

FIGURE 2 | Blocking postsynaptic exocytosis prevents stabilization of AMPA receptors initially recruited by activity in dendritic spines. **(A)** TeNT light chain blocks activity-triggered RE fusion and accumulation of surface TfR-SEP. Representative images of dissociated hippocampal neurons expressing TfR-SEP with mCherry (mCh, top panels) or mCh and TeNT (mCh-2A-TeNT, bottom panels). Images were taken pre- (left) and 15 min post (right) cLTP stimulation. The plot to the right shows TfR-SEP signal plotted as a function of time following cLTP (black bar). $N = 8$–10 neurons per condition. **(B)** Quantification of the normalized total dendritic SEP-GluA1 signal before and after cLTP stimulation for control ($n = 7$ neurons) and TeNT expressing ($n = 9$ neurons) neurons. $^{**}p < 0.001$, $^{*}p < 0.05$ (Paired two-tailed Student's *t*-test). **(C)** Representative examples of SEP-GluA1 spine accumulation from control (left) and TeNT expressing (right) neurons before (0 min), 20 and 50 min following cLTP stimulation. The top row shows the SEP-GluA1 signal, the middle row shows the mCh signal and the bottom row shows the merge of the two channels. The dotted line represents an outline of the cell morphology based on the mCh signal. Scale bar, 1 μm. **(D)** Traces showing the spine SEP-GluA1 and mCh signals in control (solid lines) and TeNT-expressing (dashed lines) neurons as a function of time for the spines shown in **(C)**. The gray box indicates the duration of the cLTP stimulus. **(E)** Cumulative probability of maximum SEP-GluA1 accumulation within randomly selected spines following cLTP stimulation for control neurons (red line, $n = 119$ spines from 6 neurons) and neurons expressing TeNT (black dashed line, $n = 138$ spines from 9 neurons). $p = 0.66$, Kolmogorov-Smirnov test. **(F)** Quantification of the SEP-GluA1 signal retention over time in selected dendritic spines for control (filled circles, $n = 52$ spines from 7 neurons) and TeNT expressing (open circles, $n = 87$ spines from 9 neurons) neurons. Only spines that acquired SEP signal >25% over baseline were selected for this analysis. The gray box indicates the duration of the cLTP stimulus. $^{***}p < 0.001$ (Two-way ANOVA, Bonferroni multiple comparisons test). **(G)** Shown is a cumulative probability plot of the decrease in spine SEP-GluA1 signal 50 min following cLTP stimulation in control neurons (red line) and neurons expressing TeNT (black dashed line). $p = 0.0082$, Kolmogorov-Smirnov test.

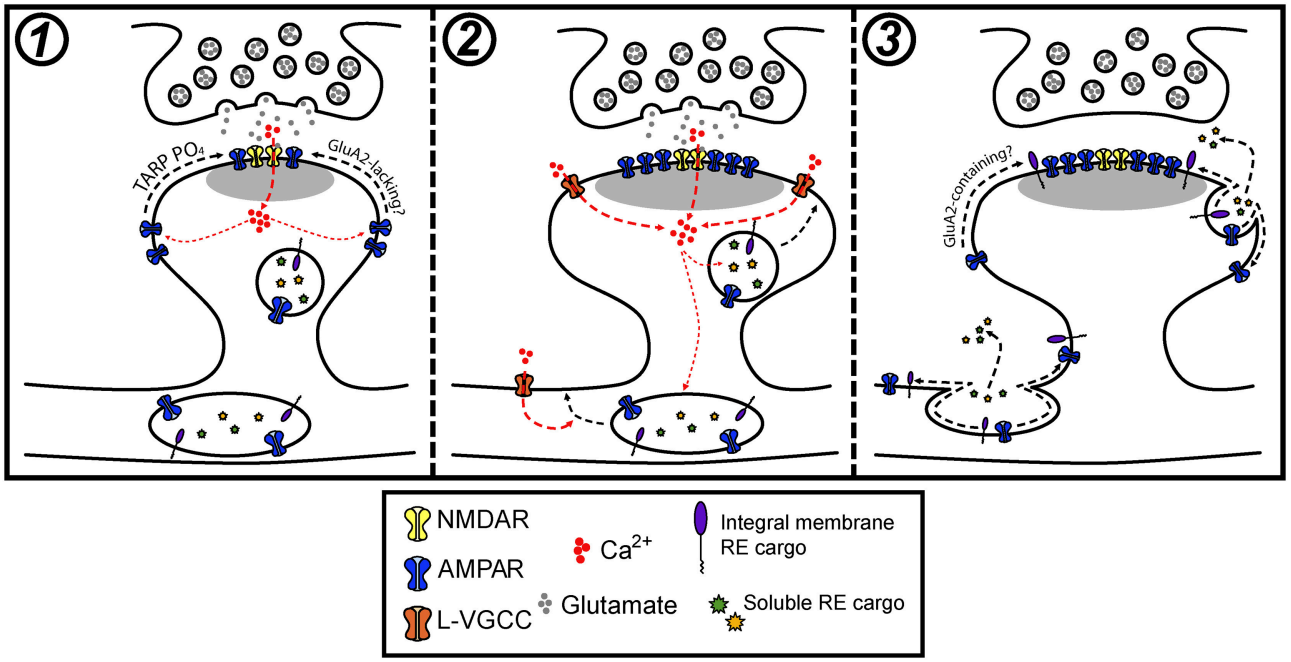

FIGURE 3 | Model for how postsynaptic membrane trafficking regulates AMPA receptor accumulation and stabilization during LTP. **(1)** The onset of an LTP stimulus promotes Ca^{2+} entry through NMDA receptors, which drives the initial trapping of pre-existing surface diffusing AMPA receptors at synapses. This can occur through activity-triggered phosphorylation of TARPs and subsequent binding to PSD scaffolds. **(2)** NMDA receptor and L-VGCC activation cooperatively promote the fusion of intracellular vesicles in dendrites and spines with the PM. **(3)** Activity-triggered fusion of postsynaptic vesicles delivers key synaptic cargoes, including AMPA receptors and other integral membrane proteins along with secreted soluble proteins to the PM. The delivery of one or a combination of these factors contributes to the stabilization of synaptic AMPA receptors that accumulated during the early stages of LTP.

neuronal PM in response to synaptic activation. We propose that activity-triggered delivery of these cargoes is critical for stabilizing synaptic AMPA receptors and spine growth during LTP (**Figure 3**). In this light, we think there are several fundamental gaps in our understanding of how membrane trafficking contributes to synaptic plasticity.

What Proteins Mediate AMPA Receptor Stability During LTP?

AMPA receptors interact with an expanding list of proteins, many of which can impact receptor function and interactions with PSD proteins (Schwenk et al., 2012, 2014; Garcia-Nafria et al., 2016). Several families of TARPs, are required for the synaptic incorporation of AMPA receptors (Hashimoto et al., 1999; Chen et al., 2000; Tomita et al., 2003; Wang et al., 2008a; for a detailed review see Jackson and Nicoll, 2011). In addition, several families of transmembrane cell adhesion molecules, several of which are implicated in LTP, interact with AMPA receptors, including cadherins (Bozdagi et al., 2000; Nuriya and Huganir, 2006; Saglietti et al., 2007; Heisler et al., 2014; Brigidi et al., 2015), integrins (Chan et al., 2006; Huang et al., 2006; Cingolani et al., 2008; Pozo et al., 2012), LRRTMs (de Wit et al., 2009; Soler-llavina et al., 2013; Bhouri et al., 2018), neuroligins (Heine et al., 2008b; Mondin et al., 2011; Aoto et al., 2013), SynDIGs (Kalashnikova et al., 2010; Chenaux et al., 2016; Matt et al., 2018), and IgsF11 (Jang et al., 2016). Activity-triggered delivery of these proteins to the postsynaptic membrane could provide a mechanism to retain laterally diffusing AMPA receptors during LTP or to stabilize receptors already recruited to and trapped in the PSD. Regulated trafficking of these molecules could also play a role in organizing trans-synaptic "nanocolumns" where postsynaptic receptors are precisely positioned opposite presynaptic neurotransmitter release sites (Tang et al., 2016). However, whether any of these proteins localize to REs or are trafficked to the PM in response to activity remains largely unknown, but a recent study demonstrating a major role for REs in forward trafficking through the biosynthetic secretory pathway suggests that a diverse array of integral membrane proteins and secreted factors could at least initially traffic through REs (Bowen et al., 2017). Interestingly, endosomal-mediated surface trafficking of N-cadherin is critical for neural migration (Kawauchi et al., 2010; Jossin and Cooper, 2011; Ye et al., 2014; Hara et al., 2016), indicating that N-cadherin function may be broadly regulated at the level of surface trafficking. N-cadherin stabilization on the cell surface may be further aided by palmitoylation and RE-dependent synaptic recruitment of the scaffolding protein δ-catenin (Brigidi et al., 2014, 2015). Moreover, surface levels of β3-integrin increase during homeostatic synaptic strengthening (Cingolani et al., 2008), highlighting the possibility that trafficking of important AMPA receptor interacting proteins may be coupled to synaptic activity. However, this does not appear to be the case for the canonical TARP stargazin, which does not appear to internalize with AMPA receptors following agonist-dependent endocytosis

(Tomita et al., 2004). Additionally, LRRTM 1 and 2 are required for both basal AMPA receptor transmission and LTP, suggesting these molecules may play a role in AMPAR receptor recruitment during synapse formation and plasticity (Bhouri et al., 2018). Whether LRRTMs are mobilized to the PM through regulated membrane trafficking mechanisms to modulate synaptic AMPA receptor stability during LTP remains unknown.

In addition to integral membrane proteins, peripheral membrane-associated proteins also associate with REs. For example, AKAP79/150, a key scaffold protein involved in coordinating postsynaptic kinase and phosphatase signaling, localizes to REs via palmitoylation and an N-terminal polybasic region and is delivered to dendritic spines following cLTP stimulation via a RE-dependent mechanism (Keith et al., 2012; Woolfrey et al., 2015). Thus activity-triggered RE fusion with the PM in dendrites and spines would be expected to alter the subcellular distribution of signaling complexes that could directly or indirectly modify AMPA receptor localization and/or function.

It is also possible that secreted signaling molecules may mediate AMPA receptor stability. An intriguing recent study identified a critical role for Wnt signaling during the early stages of LTP. This study demonstrated that a specific Wnt protein, Wnt7a/b, rapidly accumulates at synapses in response to cLTP stimulation, and that Wnt7a/b promotes the diffusional trapping of AMPA receptors through activation of postsynaptic frizzled-7 (Mcleod et al., 2018). Whether Wnt7a/b secretion occurs from pre- and/or postsynaptic neurons remains to be determined. Brain derived neurotrophic factor (BDNF) has also been reported to be secreted from dendrites and directly from activated spines, where it could act in an autocrine manner through local activation of TrkB to support spine growth associated with LTP (Tanaka et al., 2008; Harward et al., 2016). Finally, proteases that remodel the extracellular matrix have been implicated in synaptic plasticity (Wang et al., 2008b; Szepesi et al., 2014). In particular, matrix metalloproteinase-9 (MMP-9) has been demonstrated to be both necessary and sufficient for morphological and functional LTP (Nagy et al., 2006; Bozdagi et al., 2007; Wang et al., 2008b).

Intriguingly, MMP-9-dependent plasticity itself requires postsynaptic exocytosis. Wang et al. (2008b) demonstrated that both synaptic and structural plasticity induced by application of exogenous MMP9 was blocked by loading neurons with botulinum toxin B (which cleaves VAMP proteins required for regulated secretion), suggesting that critical activators or important substrates of MMP-9 undergo postsynaptic exocytosis during LTP. Related, Padamsey et al. (2017) demonstrated that neural activity triggers fusion of lysosomes with the postsynaptic membrane, and activates MMP-9 via secretion of the protease cathepsin-B. It is unclear how neural activity is coupled to lysosomal membrane fusion, but Ca^{2+} release from the lysosomes themselves appears to play a critical role in promoting fusion, suggesting that there may be mechanisms for lysosomal fusion that are distinct from other vesicle types (Padamsey et al., 2017). An additional study demonstrated that lysosomes are distributed throughout neuronal dendrites and that activation of NMDA receptors recruits lysosomes to dendritic spines, further supporting a role for lysosome-mediated postsynaptic trafficking during synaptic plasticity (Goo et al., 2017). Whether lysosomes regulate trafficking of additional cargoes and whether lysosomal fusion with the PM utilizes shared mechanisms with other types of vesicles remains to be seen. Lysosomes could also be recruited near synaptic sites in response to activity to fulfill their more canonical role in protein turnover during times of increased synaptic remodeling.

Finally, it is also possible that AMPA receptors themselves act as stabilizing factors. AMPA receptors at hippocampal synapses are tetramers, primarily composed of GluA1/2 or GluA2/3 subunit assemblies (Lu et al., 2009; Traynelis et al., 2010). Previous work has demonstrated that immediately following LTP induction, GluA2-lacking AMPA receptors (which are high-conductance, inwardly rectifying and Ca^{2+} permeable) are incorporated into synapses (Plant et al., 2006). GluA2-lacking receptors are replaced by GluA2-containing receptors, with full exchange occurring ∼25 min following LTP induction. This timescale is similar to the decay of excitatory postsynaptic potentials to baseline following LTP induction in the presence of various agents that block regulated membrane fusion reported in Lledo et al. (1998). Thus, it is possible that membrane fusion could deliver GluA2-containing receptors to synapses (or an unknown factor that promotes the exchange of GluA2-lacking for GluA2-containing AMPA receptors) in the minutes following LTP induction.

While the field has narrowly focused on AMPA receptors, identifying the full repertoire of synaptic proteins trafficked to the surface during LTP will be important for a comprehensive, mechanistic understanding of why postsynaptic membrane trafficking is essential for plasticity. One potential approach will be to perform quantitative proteomic measurements to identify those proteins whose surface localization is altered following global LTP stimuli and/or by blocking intracellular vesicle fusion with the PM. This approach was used to identify endosomal cargoes that contribute to cancer invasiveness and could potentially be applied to diverse forms of neuronal plasticity, although given the heterogeneity of neuronal and glial subtypes, whose surface proteome may behave differently, this approach may be challenging (Diaz-Vera et al., 2017). An alternative approach would be to specifically label proteins within organelles relevant for LTP to identify factors that are likely to traffic to the cell surface, or be secreted following synaptic stimulation (Hung et al., 2014). An advantage to this strategy is that it may be able to distinguish populations of proteins that are trafficked through different postsynaptic organelles (e.g., REs vs. lysosomes). In any case, defining the full spectrum of postsynaptic vesicular protein cargoes will be invaluable in understanding the relationship between membrane trafficking and spine growth/AMPA receptor stability during LTP.

How Is Synaptic Activity Coupled to Vesicle Fusion?

Strong synaptic activation associated with LTP drives robust fusion of intracellular vesicles with the postsynaptic membrane, but the precise mechanisms underlying how activity is coupled

to membrane fusion are only now emerging. Several studies have identified many of the fusion proteins that are required for expression of diverse forms of plasticity (Gerges et al., 2006; Lin et al., 2009; Araki et al., 2010; Kennedy et al., 2010; Ahmad et al., 2012; Jurado et al., 2013; Arendt et al., 2015; Wu et al., 2017a; Bin et al., 2018). Additionally, work from our lab demonstrated that L-type voltage-gated Ca^{2+} channels (L-VGCCs) play an important modulatory role during RE fusion by regulating whether REs partially or fully fuse with the PM, thus providing a potential mechanism for regulating the factors released to the PM or extracellular space during RE fusion events (Hiester et al., 2017) (**Figure 3**). Research aimed at identifying additional proteins that regulate postsynaptic fusion could help uncover similarly complex regulatory mechanisms. Further, it is possible that this avenue of research may also demonstrate a requirement for postsynaptic membrane fusion in other types of plasticity. Indeed, a recent study by Arendt et al. (2015) identified a requirement for SNARE-mediated membrane fusion during retinoic acid-induced homeostatic plasticity, suggesting that regulation of AMPA receptor stability through postsynaptic exocytosis may be broadly important for diverse forms of plasticity.

What Is the Role of Spine RE Fusion?

A lingering controversy regards the extent to which vesicle fusion occurs in dendritic spines. Multiple studies have demonstrated that endosomes can fuse with the dendritic spine plasma membrane (Kennedy et al., 2010; Patterson et al., 2010; Hiester et al., 2017). What remains unknown and controversial is the extent to which these fusion events could deliver AMPA receptors or other factors that could contribute to synaptic potentiation during LTP. Blocking regulated membrane trafficking with TeNT does not impede initial spine SEP-GluA1 accumulation or spine growth, but leads to loss of accumulated receptors and reduction in spine size several minutes following induction (**Figures 2C–G**). While this experiment does not specifically test the role of spine RE fusion (since TeNT blocks fusion in spines and the shaft), delivery of protein cargoes directly within dendritic spines is likely to have a much different functional outcome than in dendritic shafts, so it will be important to resolve this issue. Current strategies for directly visualizing AMPA receptor trafficking in live cells, (e.g., SEP-GluA1) likely underestimate the full extent of spine AMPA receptor insertion, underscoring the need for tools that will allow visualization of endogenous AMPA receptor trafficking (Kennedy et al., 2010; Hiester et al., 2017). One method that has been used in several studies relies upon differential antibody labeling to selectively visualize AMPARs that are inserted into the membrane after stimulation (Lu et al., 2001; Sun et al., 2005; Hiester et al., 2017; Werner et al., 2017). However, a major limitation of this technique is that it lacks the requisite temporal specificity to precisely identify when and where receptors are inserted into the membrane. An alternative approach would be to chemically label endogenous AMPA receptors in live cells (Wakayama et al., 2017), though it remains to be seen whether such a technique can be adapted to specifically monitor discrete receptor trafficking events. Genetically encoded affinity tags against endogenous excitatory and inhibitory synaptic scaffold and signaling proteins have been extremely valuable tools for labeling synaptic structures (Gross et al., 2013, 2016; Mora et al., 2013; Barcomb et al., 2015; Fossati et al., 2016; Kannan et al., 2016; Son et al., 2016; Spence et al., 2016; Uezu et al., 2016; Goodell et al., 2017; Lin et al., 2017; Sinnen et al., 2017; Walker et al., 2017). Similar reagents for labeling endogenous AMPA receptors would be valuable for addressing numerous basic questions concerning how endogenous receptors traffic. As with any tagging strategy, targeting intrabodies to benign epitopes within AMPA receptors will be critical. Likewise, new tools that would allow spatially-restricted inhibition of specific organelle function (e.g., REs and lysosomes) within different subcellular domains will be invaluable for unraveling precisely where, when and how membrane fusion relevant for plasticity occurs (Bourke et al., 2018).

CONCLUDING REMARKS

The neuronal postsynaptic membrane is a dynamic structure that undergoes major changes during synaptic plasticity. During LTP, activity-triggered recruitment of AMPA receptors is one of the most critical alterations at the synaptic membrane for enduring plasticity. While the field has made great progress in understanding many of the underlying mechanisms of AMPA receptor trafficking, our understanding of how AMPA receptor surface delivery contributes to plasticity is considerably less clear. Recent work challenges the assumption that activity-triggered delivery of AMPA receptors to the PM plays a direct role in LTP. However, this work highlights the importance of postsynaptic membrane fusion beyond merely delivering AMPA receptors, forcing the field to generate new models for how membrane trafficking contributes to synaptic plasticity. We propose that postsynaptic membrane fusion delivers diverse proteins to the dendritic PM, some of which may be critical for stabilizing synaptic AMPA receptors during LTP, thus reconciling seemingly contradictory results in the field. Identifying the complete cast of proteins delivered to the cell surface during plasticity and the intracellular organelles responsible will help to reshape our understanding of how membrane trafficking impacts synaptic function and plasticity.

METHODS

Cell Culture and Transfection

All animal procedures were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado School of Medicine. Dissociated hippocampal cultures were prepared from neonatal rat pups as previously described (Beaudoin et al., 2012) and grown on 18 mm poly-D-lysine (Sigma) coated coverslips in 12-well cell culture dishes in Neurobasal-A medium (Invitrogen) supplemented with B27 (Invitrogen) and Glutamax (Invitrogen) at an approximate density of 100,000 cells/well. Neurons were maintained at 37°C in a humidified incubator at 5% CO_2. All neurons were between DIV18 and DIV21 at the time of experiment.

Neurons were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations

and allowed to express plasmids for 48–72 h prior to experiments. For all experiments, neurons were transfected with a plasmid encoding the AMPA receptor subunit GluA1 tagged N-terminally with superecliptic pHluorin (SEP) (Kennedy et al., 2010). For control conditions, neurons were transfected with a plasmid encoding soluble mCh. For the tetanus toxin (TeNT) condition, neurons were transfected with a bicistronic plasmid encoding mCh fused to TeNT with a cleavable P2A peptide tag (mCh-P2A-TeNT) (Szymczak et al., 2004).

Image Acquisition and Data Analysis

Live cell imaging of dissociated neurons was carried out at 32°C on an Olympus IX71 equipped with a spinning disc scan head (Yokogawa). Excitation illumination was delivered from an acousto-optic tunable filter (AOTF) controlled laser launch (Andor). Images were acquired using a 60x Plan Apochromat 1.4 NA objective and collected on a 1024 × 1024 pixel Andor iXon EM-CCD camera. For all imaging experiments, the apical portion of the dendrtic arbor extending 25–100 μm from the cell soma was imaged. Data acquisition and analysis were performed with Metamorph (Molecular Devices) and ImageJ software. Some images were low pass filtered and interpolated for display. Only raw, unprocessed data were used for quantification.

To image activity-triggered SEP-GluA1 exocytosis and SEP-GluA1 translocation, transfected neurons were pretreated with tetrodotoxin (TTX, Tocris, 1–2 μM) for 1 h to inhibit evoked activity. Coverslips with cultured neurons were then placed in a live-cell imaging chamber (Ludin) and incubated in baseline ACSF solution containing (in mM): 130 NaCl, 5 KCl, 10 HEPES, 30 glucose, 1 $MgCl_2$, 2 $CaCl_2$, and 0.002 TTX (pH 7.4). To stimulate synaptic activity (cLTP stimulation), the baseline solution was exchanged for one that contained (in mM): 130 NaCl, 5 KCl, 10 HEPES, 30 glucose, 0 $MgCl_2$, 2 $CaCl_2$, 0 TTX, and 0.2 glycine. After cLTP stimulation, neurons were re-exposed to the baseline solution for the remainder of the imaging period.

To measure discrete SEP-GluA1 exocytosis events, single plane 2-color (SEP-GluA1, TfRmCh/dsred-homer1c/PSD95-mCh) images were acquired at 2 Hz. For measuring SEP-GluA1 surface delivery and spine morphology, 2-color (mCh, SEP-GluA1) 5 μM z-stacks were acquired every 1 min before, during and after bath stimulation. To quantify the rate of synaptic SEP-GluA1 accumulation circular ROIs were drawn over individual dendritic spine heads and the mean background-subtracted SEP-GluA1 signal was quantified in ImageJ. To measure the peak synaptic SEP-GluA1 accumulation in an unbiased manner (**Figure 1C**), ROIs were drawn over randomly selected dendritic spine heads using the mCh signal without regard to the amount of cLTP-induced SEP-GluA1 accumulation, and the average SEP-GluA1 signal between 10 and 15 mins post cLTP was calculated for each spine. To selectively measure the retainment of SEP-GluA1 after cLTP (**Figures 1D,E**), ROIs were drawn over dendritic spine heads that displayed at least a 25% increase in SEP-GluA1 accumulation over baseline. Data are plotted as the ratio of SEP-GluA1 fluorescence at any given time point over the SEP-GluA1 fluorescence at the start of the experiment (SEP-GluA1 F/F_0).

Statistical significance for experiments comparing the means of two populations was determined using a two-tailed unpaired Student's *t*-test. In cases where measurements of two populations were recorded over multiple time points, a two-way ANOVA with Bonferroni multiple comparison test was used or a two-sample Kolmogorov-Smirnov test.

AUTHOR CONTRIBUTIONS

BH, MB, AB, and MK contributed to data collection, analysis and interpretation. SS contributed to data analysis and interpretation. BH, and MK wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2018.00391/full#supplementary-material

Supplementary Figure 1 | ER acidification suppresses SEP-GluA1 signal during cLTP. **(A)** Method for masking the dendritic shaft for SEP-GluA1 signal analysis. A mask was generated so that the dendritic shaft signal could be selectively quantified. This was generated by subtracting a synapse mask, based on the SEP-GluA1 signal from a whole cell mask generated from the mCh cell fill (see methods below for details). Shown is the mCh cell fill (panel i), binary synapse mask based on the SEP-GluA1 signal (red, panel ii) and the SEP-GluA1 signal (gray scale, panel iii) with dendritic shaft mask overlayed (burgundy). Scale bar 10 μm. **(B)** Quantification of the spine (solid line) and shaft (dashed line) SEP-GluA1 signal as a function of time during and after cLTP (gray bar). Note the robust decrease in shaft signal, which slightly precedes synaptic accumulation. Error bars represent standard error of the mean.This decrease is likely due to ER acidification as outlined in Rathje et al. (2013).

Video 1 | SEP-GluA1 stably accumulates at dendritic spines in response to cLTP. Representative dendritic segment from a control neuron showing SEP-GluA1 (**Top**), mCh (**Middle**), and a merge of both channels (**Bottom**). The white dot in the bottom right indicates the duration of the cLTP stimulus. Asterisks mark dendritic spines displaying stable accumulation of SEP-GluA1 and spine growth. The duration of the video is 55 min with an acquisition rate of 1 image/min, and played back at 10 frames *per sec*.

Video 2 | SEP-GluA1 transiently accumulates at a subset of dendritic spines following cLTP when postsynaptic membrane fusion is blocked. Representative dendritic segment from a TeNT expressing neuron showing SEP-GluA1 (**Top**), mCh (**Middle**), and a merge of both channels (**Bottom**). The white dot in the bottom right indicates the duration of the cLTP stimulus. Asterisks mark dendritic spines displaying initial accumulation of SEP-GluA1 and spine growth that is not stably maintained throughout the experiment. The duration of the video is 55 min with an acquisition rate of 1 image/min, and played back at 10 frames *per sec*.

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REVIEW
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Visualization of Exo- and Endocytosis of AMPA Receptors During Hippocampal Synaptic Plasticity Around Postsynaptic-Like Membrane Formed on Glass Surface

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Regulation of exo- and endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPAR) plays a critical role in the expression of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) at excitatory central synapses. Enhanced AMPAR exocytosis or endocytosis has been suggested to contribute to LTP or LTD, respectively. However, several unsettled fundamental questions have remained about AMPAR exo- and endocytosis in the basal condition and during synaptic plasticity: (1) Does the size of each exo- or endocytosis event, and/or do the frequencies of these events change during LTP or LTD? If they change, what are the time courses of the respective changes? (2) Where does the exo- or endocytosis preferentially occur in each condition: inside or in the vicinity of postsynaptic membrane, or in the extrasynaptic membrane? (3) Do different types of AMPAR, such as GluA1 homo-tetramer, GluA1/2 hetero-tetramer and GluA2/3 hetero-tetramer, show distinct exo- and endocytosis changes? To address these questions, we developed new methods to observe individual events of AMPAR exo- or endocytosis with a high signal to noise (SN) ratio in a culture preparation using total internal reflection fluorescence microscopy (TIRFM). In these studies, hippocampal neurons were cultured on a neurexin (NRX)-coated glass coverslip, which induced formation of postsynaptic-like membrane (PSLM) directly on the glass surface. Then, a super-ecliptic pHluorin (SEP)-tagged AMPAR subunit such as GluA1 (GluA1-SEP) was expressed in neurons and its fluorescence changes during LTP induced by high frequency electrical field stimulation were observed with TIRFM, which showed different time courses of exocytosis changes of GluA1-, GluA2-, or GluA3-SEP in and around PSLM. In addition, a new method to detect individual endocytosis events of AMPAR was developed by combining TIFRM observation of GluA-SEP around PSLM with a rapid extracellular pH exchange method using a U-tube. Recent results on exo- and endocytosis changes of GluA-SEP during N-methyl-D-aspartate (NMDA)-induced

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA-type glutamate receptor; AZLM, active-zone-like membrane; BSA, Bovine serum albumin; Fc, Fragment crystallizable; LTP, Long-term potentiation; LTD, Long-term depression; LRRTM, Leucine rich repeat transmembrane; NLG, Neuroligin; NMDA, *N*-methyl-D-aspartate; NRX, Neurexin; PALM, Photo-activated localization microscopy; PSLM, Postsynaptic-like membrane; SEP, Super-ecliptic pHluorin; SN, Signal to noise; STORM, Stochastic optical reconstruction microscopy; SynCAM, Synaptic cell adhesion molecule; TIRFM, Total internal reflection fluorescence microscopy.

LTD suggested that suppression of AMPAR exocytosis rather than enhancement of AMPAR endocytosis primarily contributes to LTD expression, although the NMDA application transiently enhances clathrin-dependent endocytosis of GluA1-containing AMPAR.

Keywords: exocytosis, endocytosis, LTP, LTD, hippocampus, AMPA receptor, total internal reflection fluorescence microscopy, live-cell imaging

INTRODUCTION

Long-term potentiation (LTP) and long-term depression (LTD) at hippocampal glutamatergic synapses have been regarded as basic cellular mechanisms of learning and memory, and intensively studied (Malinow and Malenka, 2002; Kauer and Malenka, 2007; Collingridge et al., 2010; Huganir and Nicoll, 2013). Originally changes in functional properties of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPAR) were considered as molecular mechanisms of LTP or LTD. More recently, changes in the number of AMPAR on the postsynaptic membrane attracted much attention of synaptic physiologists as primary mechanisms of LTP or LTD expression (Malinow and Malenka, 2002; Kennedy and Ehlers, 2006; Derkach et al., 2007; Shepherd and Huganir, 2007; Huganir and Nicoll, 2013). AMPAR exocytosis, endocytosis, lateral movement on the plasma membrane and trapping in the postsynaptic membrane regulate the number of postsynaptic AMPARs (Malinow and Malenka, 2002; Shepherd and Huganir, 2007; Makino and Malinow, 2009; Opazo and Choquet, 2011; Huganir and Nicoll, 2013; Wu et al., 2017).

Enhancement of AMPAR exocytosis was suggested to contribute to LTP expression (Shi et al., 1999; Hayashi et al., 2000; Passafaro et al., 2001; Kopec et al., 2006; Park et al., 2006; Plant et al., 2006; Lin et al., 2009; Kennedy et al., 2010; Patterson et al., 2010; Huganir and Nicoll, 2013). Exocytosis was reported to occur around the postsynaptic membrane and in extra-synaptic membrane away from synapses (Kennedy et al., 2010). In the latter case, lateral movement of AMPAR and trapping of it on the postsynaptic membrane are necessary to accumulate AMPAR in the postsynaptic membrane (Opazo et al., 2010, 2012; Opazo and Choquet, 2011; Chen et al., 2015). However, the extent to which each pathway contributes to LTP expression remains an open question.

There are four types of AMPAR subunits GluA1–4 (Hollmann and Heinemann, 1994; Dingledine et al., 1999). In hippocampal glutamatergic synapses GluA1/GluA2 hetero-tetramer and GluA2/GluA3 hetero-tetramer are the main postsynaptic receptors (Dingledine et al., 1999). On the other hand, GluA1 homo-tetramer is present in some types of hippocampal neurons, and its involvement in synaptic plasticity has also been reported (Iino et al., 1990; Plant et al., 2006; Lu Y. et al., 2007; Sanderson et al., 2016). Thus, there may be specific regulatory mechanisms for each of these subtypes of AMPAR composed of different combinations of subunits, but this has not yet been precisely clarified.

Enhancement of AMPAR endocytosis has been suggested as a primary mechanism for LTD expression (Beattie et al., 2000; Lee et al., 2002; Ashby et al., 2004; Lin and Huganir, 2007; Fernández-Monreal et al., 2012). Both clathrin-dependent and -independent AMPAR endocytosis occur, and the contribution of the former to LTD induction has been reported (Glebov et al., 2015; Zheng et al., 2015). AMPAR endocytosis might occur not only in the extrasynaptic membrane but also in the vicinity of postsynaptic membrane (Blanpied et al., 2002; Kennedy and Ehlers, 2006; Lu J. et al., 2007; Tao-Cheng et al., 2011; Fujii et al., 2017, 2018). The involvement of GluA1 homo-tetramer in LTD induction has been suggested (Sanderson et al., 2016), although involvement of GluA2-lacking AMPAR in LTP or LTD has been debated (Passafaro et al., 2001; Plant et al., 2006; Adesnik and Nicoll, 2007; Gray et al., 2007; Lu Y. et al., 2007). AMPAR subtype changes might also take place during LTD.

LTP and LTD have been studied mainly by electrophysiological recording, immuno-cytological staining and biochemical assays combined with pharmacological or molecular biological manipulations and/or use of transgenic mice (Malinow and Malenka, 2002; Kauer and Malenka, 2007; Collingridge et al., 2010; Huganir and Nicoll, 2013). The use of live-cell imaging techniques in the analyses of LTP and LTD mechanisms has increased. Technical advancements have made it possible to detect individual events of exo- or endocytosis of AMPAR. Development of a pH-sensitive variant of green fluorescent protein called super-ecliptic pHluorin (SEP) enabled selective monitoring of proteins in neutral pH conditions, such as on the cell-surface, but not proteins inside intracellular organelles with acidic luminal solution (Miesenböck et al., 1998). SEP has been widely used in studies on AMPAR trafficking during LTP or LTD (Ashby et al., 2004; Lin and Huganir, 2007; Yudowski et al., 2007; Lin et al., 2009; Araki et al., 2010; Kennedy et al., 2010; Tanaka and Hirano, 2012; Rathje et al., 2013; Jullié et al., 2014; Tanaka et al., 2014; Fujii et al., 2017, 2018; Rosendale et al., 2017; Temkin et al., 2017; Wu et al., 2017). Total internal reflection fluorescence microscopy (TIRFM) provides very high signal/noise (SN) ratio images by limiting the depth of the visualization zone (Axelrod, 2001), and has also been used in live-cell imaging studies of AMPAR dynamics (Yudowski et al., 2007; Wang et al., 2008; Lin et al., 2009; Araki et al., 2010; Tanaka and Hirano, 2012; Jullié et al., 2014; Tanaka et al., 2014; Fujii et al., 2017, 2018; Rosendale et al., 2017). A rapid extracellular pH exchange method combined with the use of SEP made it possible to record individual endocytosed vesicles (Merrifield et al., 2005; Jullié et al., 2014; Rosendale et al., 2017).

Recently, we developed a new method to further improve the SN ratio and spatiotemporal resolution of live-cell imaging data of SEP-tagged AMPAR. We induced formation of postsynaptic-like membrane (PSLM) directly on the surface of a glass coverslip, and then studied the dynamics of GluA-SEP

around PSLM during LTP or LTD expression (Tanaka and Hirano, 2012; Tanaka et al., 2014; Fujii et al., 2017). In this review, I will briefly summarize recent results on AMPAR dynamics during synaptic plasticity obtained using GluA-SEP, PSLM and TIRFM.

FORMATION OF PSLM ON NEUREXIN-COATED GLASS

Several types of cell-adhesion molecules are found at synapses such as Neuroligin (NLG), Neurexin (NRX), Synaptic cell adhesion molecule (SynCAM), EphrinB, leucine rich repeat transmembrane (LRRTM) and N-Cadherin. Among them presynaptic membrane protein NRX and postsynaptic membrane protein NLG have been studied extensively (Levinson and El-Husseini, 2005; Dean and Dresbach, 2006; Craig and Kang, 2007; Südhof, 2008; Bukalo and Dityatev, 2012). Both of them have different subtypes and various splice variants. There are five NLG genes NLG 1–4 and NLG 4Y, and there are six NRX genes NRX 1α, 1β, 2α, 2β, 3α, 3β. NRXs undergo extensive alternative splicing, which could potentially generate >2,000 variants. Among these variants, splicing insertion of site 4 in β-NRX promotes GABAergic synapse formation, whereas β-NRX without site 4 insertion promotes glutamatergic synapse formation. It is also known that NLG 1 with splice insertion at site B promotes glutamatergic synapse formation, and that NLG 2 is primarily found at GABAergic synapses.

NLG expressed in non-neuronal cells co-cultured with neurons induces formation of presynaptic structures in axons, while NRX when similarly expressed induces formation of postsynaptic structures in dendrites (Scheiffele et al., 2000; Graf et al., 2004). Furthermore, NRX attached to beads induces clustering of postsynaptic proteins (Graf et al., 2004). These findings prompted us to test whether a glass coverslip coated with NRX could induce formation of postsynaptic structures on the glass surface. We considered that such postsynaptic structures formed directly on and parallel to the glass surface would be an ideal model of postsynaptic structure which could be used in live-cell fluorescence imaging experiments using TIRFM (**Figures 1**, **2**), because application of TIRFM to such structures would be efficient and effective. TIRFM can provide very high contrast fluorescence images by decreasing background signals. Excitation light reaches only about 100 nm above the glass surface in an inverted microscope equipped for TIRFM (**Figure 2**).

Glass coating with NRX was performed utilizing biotin-avidin interaction and an antibody which was described in detail elsewhere (Tanaka and Hirano, 2012; Tanaka et al., 2014; **Figure 1**). Briefly, glass coverslips were coated with bovine serum albumin (BSA) conjugated with biotin. Then, streptavidin, which binds to biotin, was overlaid. Next, the anti-fragment crystallizable (Fc) region of human immunoglobulin conjugated to biotin, which binds to streptavidin, was applied. Finally, NRX 1β without splicing insertion at site 4 fused to the FC region of human immunoglobulin was applied so that it was captured by the anti-Fc antibody.

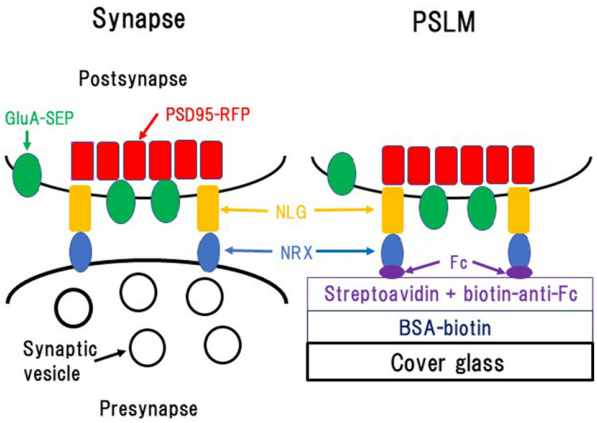

FIGURE 1 | Formation of postsynaptic-like membrane (PSLM). NRX, Neurexin; NLG, Neuroligin; BSA, Bovine serum albumin; Fc, fragment crystallizable of immunoglobulin. This figure is newly drawn based on our previous publications (Tanaka and Hirano, 2012; Tanaka et al., 2014), and copyright permission is not required.

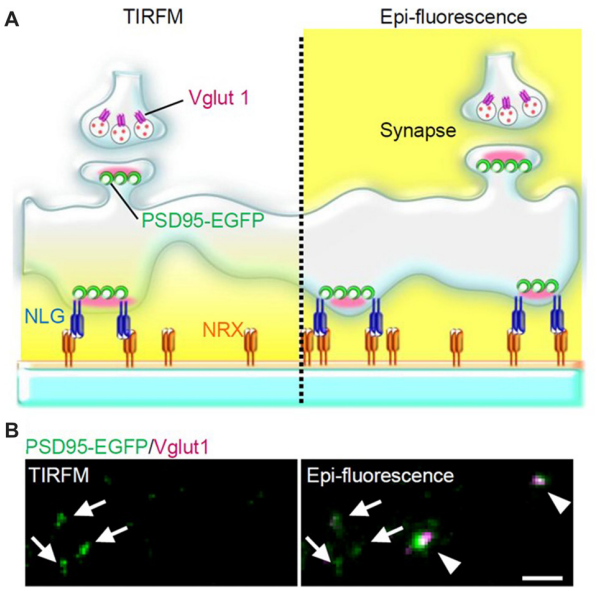

FIGURE 2 | PSLM and normal synapses observed with total internal reflection fluorescence microscopy (TIRFM) or with conventional epi-fluorescence. **(A)** Scheme of PSLM and a normal synapse on NRX-coated glass. Excitation light (yellow) reaches only PSLM and the lower parts of dendrites in TIRFM (left), whereas it covers the whole area under epi-fluorescence (right). At a normal synapse, postsynaptic PSD95 signal is apposed to presynaptic vglut1 signal. **(B)** PSD95-EGFP signal (green) and vglut1 signal (magenta) recorded with TIRFM (left) or with epi-fluorescence (right), respectively. Arrows indicate PSLMs that are clearly observed with TIRFM and are not accompanied by vglut1 signals, and arrowheads indicate normal synapses which are not clearly observed with TIRFM. These figure panels were first published in Tanaka et al. (2014), and copyright permission was obtained.

When hippocampal neurons overexpressing NLG 1 with splice insertion at site B were cultured on the NRX-coated coverslip, many punctate structures that contained PSD95, a marker protein of postsynaptic density,

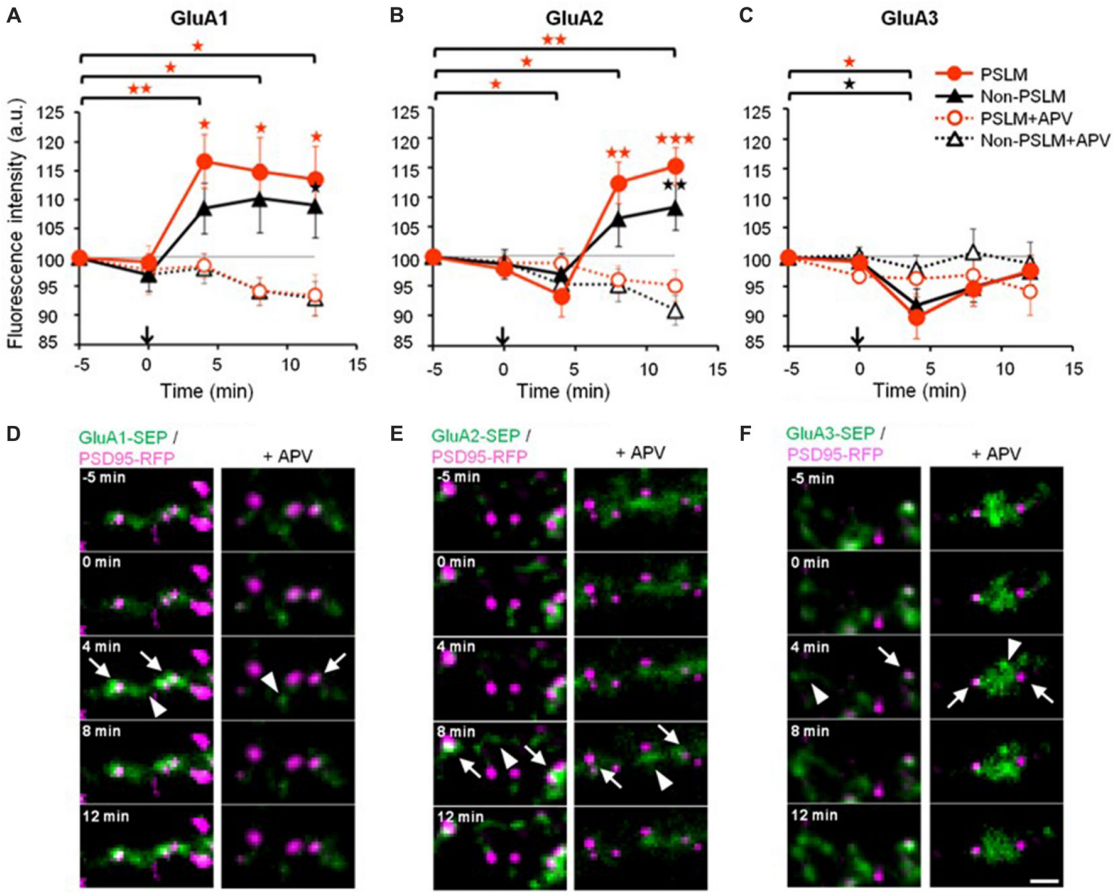

FIGURE 3 | Changes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPAR) subunit number by long-term potentiation (LTP)-inducing stimulation. **(A–C)** Averaged time courses of GluA1–3 fluorescence intensity in PSLM (red) and in non-PSLM (black) measured every 4 min before and after the field stimulation (arrows). Data in the presence of APV (+APV) are also shown (dotted lines). Error bars indicate SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. **(D–F)** GluA-super-ecliptic phluorin (SEP) signals (green) and PSD95-RFP signal (magenta) are shown. PSD95-RFP was recorded before the stimulation, and images of the two signals were overlaid. GluA-SEP signals in PSLM and non-PSLM are indicated by arrows and arrowheads, respectively. Scale bar, 2 μm. These figure panels were first published in Tanaka and Hirano (2012), and copyright permission is not necessary.

but that were not apposed to a presynaptic marker were observed with TIRFM (**Figure 2**). Such postsynaptic-like membrane (PSLM) exhibited accumulation of homer, another marker protein of postsynaptic density and AMPAR. We chose relatively large neurons possessing spines which presumably corresponded to pyramidal neurons for the experiments. Conditioning stimulation inducing either LTP or LTD increased or decreased the amount of AMPAR in PSLM, respectively (Tanaka and Hirano, 2012; Fujii et al., 2018).

EXOCYTOSIS REGULATION IN LTP AROUND PSLM

We reported that high frequency electrical field stimulation used to induce LTP increases the amount of SEP-tagged GluA subunit of AMPAR in and outside PSLM in a hippocampal culture preparation (**Figure 3**, Tanaka and Hirano, 2012). The increase is somewhat larger in PSLM. By the way, PSLM is not apposed to a presynaptic terminal releasing glutamate. We considered that glutamate released from nearby presynaptic terminals activates N-methyl-D-aspartate (NMDA) receptors and induces the changes in the amount of GluA-SEP in PSLM, because an antagonist of NMDA receptor APV suppresses the changes.

One possible factor contributing to the increase in the amount of GluA-SEP on the surface is enhancement of GluA-SEP exocytosis. Individual events of GluA-SEP exocytosis can be observed around PSLM by high frequency live-cell TIRFM imaging (**Figure 4**). LTP-inducing electrical stimulation increases the frequency of GluA-SEP exocytosis. We reported a transient (about 1 min) increase of GluA1-SEP exocytosis frequency around PSLM and a subsequent increase for several minutes outside PSLM. We also found that GluA1-SEP exocytosis does not occur in the center of PSLM, but rather it occurs in the periphery of PSLM or outside of PSLM. Exocytic domain adjacent to the postsynaptic membrane was previously reported (Kennedy et al., 2010).

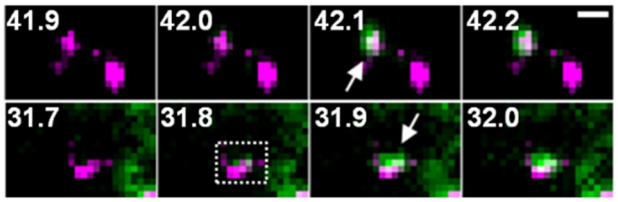

FIGURE 4 | Two examples of GluA1-SEP (green) exocytosis (arrows) shown together with PSD95-RFP (magenta). The numbers indicate time (seconds) after the field stimulation. These figure panels were first published in Tanaka and Hirano (2012), and copyright permission is not necessary.

Changes in the cell surface amounts and in the exocytosis frequencies of GluA2-SEP or GluA3-SEP also occur during LTP expression (Tanaka and Hirano, 2012). GluA2-SEP and GluA3-SEP show different time courses of these changes (**Figure 3**). Co-expression experiments of GluA1-SEP/GluA2, GluA1/GluA2-SEP or GluA2/GluA3-SEP were also performed. Based on the experimental results, we proposed the following scheme as a mechanism of the expression of LTP (**Figure 5**). (1) Exocytosis of GluA1 homo-tetramer occurs particularly in the periphery of PSLM immediately after the conditioning stimulation. (2) A few minutes after the conditioning stimulation, exocytosis of GluA1/GluA2 hetero-tetramer increases for several minutes outside PSLM. Some of the exocytosed GluA1/GluA2 hetero-tetramers may move into PSLM by diffusion on the plasma membrane. (3) From about 20 min after the conditioning stimulation exocytosis of GluA2/GluA3 gradually increases outside PSLM. The above scheme suggests that changes in the distribution of AMPAR subtypes are likely to occur during LTP. I presume that exo- and endocytosis of GluA1/GluA2 and GluA2/GluA3 hetero-tetramers are in equilibrium in a basal condition.

Regarding changes of AMPAR exocytosis during LTP expression, some unclear or conflicting observations have been reported about sites of exocytosis, AMPAR subtype specificity and precise time courses. Some previous studies reported different molecular regulation mechanisms between constitutive AMPAR exocytosis and regulated exocytosis during LTP induction (Ahmad et al., 2012; Temkin et al., 2017; Wu et al., 2017). Yudowski et al. (2007), Lin et al. (2009) and Makino and Malinow (2009) reported that the majority of GluA1 exocytosis occurred in extrasynaptic membrane, whereas Kennedy et al. (2010) found exocytic domains adjacent to postsynaptic density. The involvement of GluA2-lacking AMPAR such as GluA1 homo-tetramer in LTP has also been controversial (Passafaro et al., 2001; Plant et al., 2006; Adesnik and Nicoll, 2007; Gray et al., 2007; Lu Y. et al., 2007). Furthermore, there has been little precise information about how exocytosis of each subtype of AMPAR, such as GluA1/2 or GluA2/3 hetero-tetramer changes during LTP expression. Tanaka and Hirano (2012) provided some answers or information regarding these questions. However, I would like to note the following. First, PSLM is an artificial structure and may not necessarily express all normal functions of hippocampal glutamatergic postsynaptic membrane. Second, over-expressed GluA1-SEP might affect normal cellular processes. These points will be discussed later.

ENDO-AND EXOCYTOSIS REGULATION DURING LTD AROUND PSLM

NMDA application induces LTD in hippocampal culture preparations, which is accompanied by a decrease in amount of cell-surface AMPAR (Lee et al., 1998; Beattie et al., 2000; Collingridge et al., 2010; Fernández-Monreal et al., 2012). Enhancement of clathrin-dependent endocytosis has been considered to contribute to LTD expression (Glebov et al., 2015; Zheng et al., 2015). In order to obtain better understanding of the process of LTD expression, Fujii et al. (2017, 2018) used PSLM for analyses of AMPAR endocytosis during LTD. Individual endocytic events of cell surface molecules, including AMPAR, have been detected by the combination of a rapid extracellular pH change method with the use of SEP (Jullié et al., 2014; Rosendale et al., 2017). The extracellular pH change was performed with a θ tube in these studies. We used a U-tube instead of a θ tube for the extracellular pH exchange (Fujii et al., 2017, 2018; **Figure 6**). The use of a U-tube enables us to remove the applied solution from the experimental chamber, although the speed of solution exchange is somewhat slower than that of a method using a θ tube. Using the combination of rapid extracellular pH change and GluA1-SEP, individual events of GluA1-SEP endocytosis were observed around PSLM.

This combination method is also useful to precisely determine the amount of cell surface AMPAR, because some SEP fluorescence arises from molecules located in endoplasmic reticulum with relatively neutral luminal pH (Paroutis et al., 2004; Rathje et al., 2013). By subtracting fluorescence signals at pH 5.5 from those at pH 7.3, cell-surface signals can be isolated. This method allowed us to precisely analyze cell-surface amounts of GluA1-SEP and GluA2-SEP during LTD expression (Fujii et al., 2018). The thus estimated cell-surface amounts of both GluA1-SEP and GluA2-SEP gradually decrease after the NMDA application. The decrease of GluA1-SEP is sustained for more than 30 min, while that of GluA2-SEP tends to recover (Fujii et al., 2018).

The LTD-inducing NMDA application transiently increases the size of individual GluA1-SEP endocytic events and also the frequency of GluA1-SEP endocytosis for about 1 min. Interestingly this transiently enhanced large endocytosis is clathrin-dependent, whereas constitutive endocytosis of GluA1-SEP does not depend on clathrin (Fujii et al., 2017, 2018). This result is consistent with a previous study reporting that basal endocytosis of AMPAR does not depend on clathrin, but that the NMDA-induced endocytosis depends on clathrin (Glebov et al., 2015). Thus, there are at least two independent endocytosis pathways for AMPAR. We also reported that clathrin-dependent GluA1-SEP endocytosis induced by the NMDA application preferentially takes place in the periphery of PSLM, which is likely to correspond to the endocytic zone adjacent to the postsynaptic membrane (Blanpied et al., 2002; Lu J. et al., 2007).

Transient enhancement of GluA1-SEP endocytosis seems to be insufficient to explain slowly developing LTD

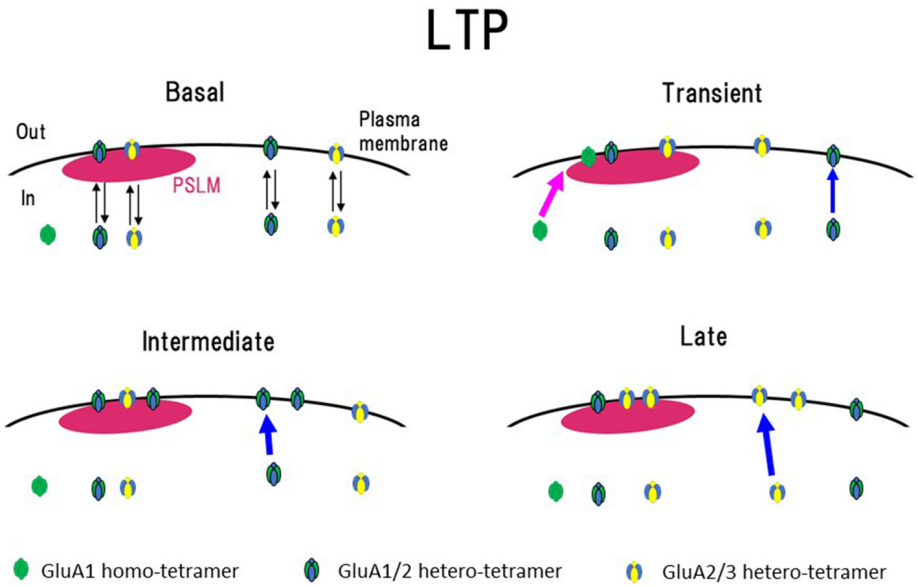

FIGURE 5 | Scheme of exocytosis changes of each AMPAR subtype during LTP expression. In a basal condition (Basal), exo- and endocytosis of GluA1/GluA2 and GluA2/GluA3 hetero-tetramer are in equilibrium. Soon after the high frequency electrical stimulation (Transient, around 1 min after the stimulation), exocytosis of GluA1 homo-tetramer occurs in the periphery of PSLM (red arrow), and exocytosis of GluA1/GluA2 increases outside PSLM (blue arrow). In the following period (Intermediate, about 3–10 min after the stimulation), exocytosis of GluA1/GluA2 increases outside PSLM (blue arrow). Finally (Late, about 20 min after the stimulation) exocytosis of GluA2/GluA3 increases outside PSLM (blue arrow). Some AMPAR exocytosed outside PSLM are likely to move into PSLM by lateral diffusion on the plasma membrane. This figure is newly drawn based on our previous publication (Tanaka and Hirano, 2012), and copyright permission is not required.

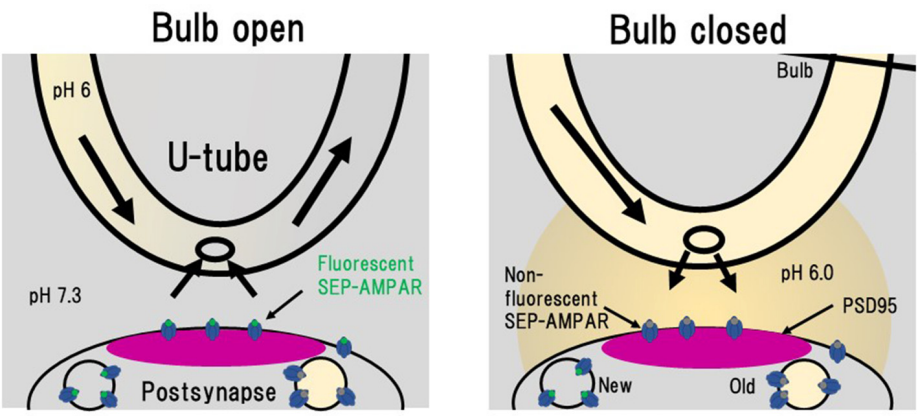

FIGURE 6 | Rapid extracellular pH exchange method using a U-tube and detection of individual AMPAR endocytosis. When a bulb on a U-tube is open (Bulb open), the pH 6.0 solution flows inside U-tube and the extracellular pH 7.3 solution is also soaked into the U-tube. When the bulb is closed (Bulb closed), the intra U-tube pH 6.0 solution leaks out to the extracellular solution. In this pH 6.0 condition, only SEP signals from intracellular vesicles with near-neutral luminal pH such as those immediately after endocytosis can be detected. This figure is newly drawn based on our previous publication (Fujii et al., 2017), and copyright permission is not required.

expression. Considering that the cell-surface amounts of molecules are regulated by the balance of endo- and exocytosis, we examined changes of GluA1-SEP exocytosis after the LTD-inducing NMDA application (Fujii et al., 2018). We found that after the NMDA application, GluA1-SEP exocytosis is transiently enhanced and then decreased. Taking all these results together, it was suggested that sustained suppression of AMPAR exocytosis, rather than enhanced endocytosis, plays a predominant role in LTD expression (**Figure 7**).

In addition, the cell-surface amount, and endo- and exocytosis of GluA2-SEP after the NMDA application were examined. We showed that GluA2-SEP exhibits different temporal profiles from those of GluA1-SEP, suggesting differential regulation of GluA2-SEP. However, how each type of AMPAR consisting of GluA1–3, such as GluA1/2 or

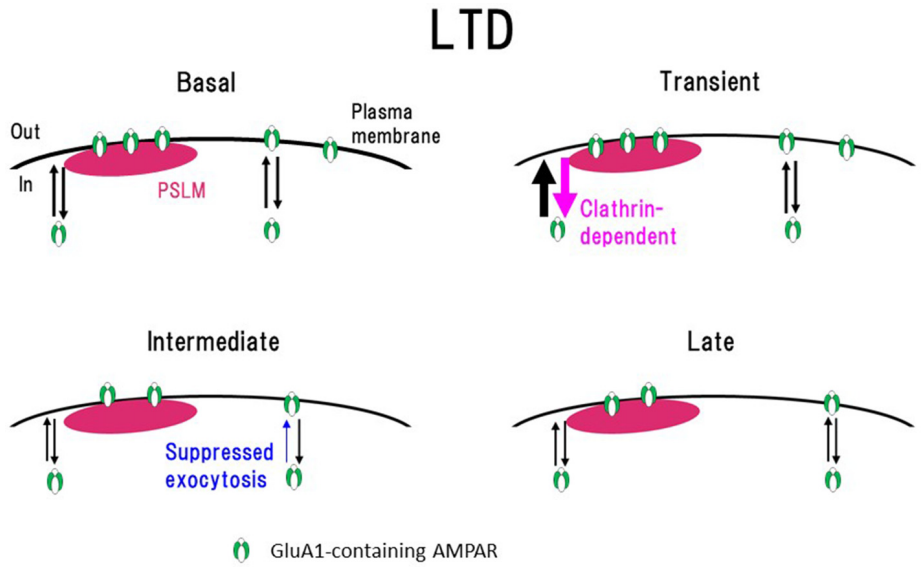

FIGURE 7 | Scheme of exo- and endocytosis changes of GluA1-containing AMPAR during long-term depression (LTD) expression. In a basal condition (Basal) exo- and endocytosis of GluA1-containing AMPAR are in equilibrium. Immediately after the N-methyl-D-aspartate (NMDA) application (Transient, around 1 min after the onset of NMDA application), clathrin-dependent endocytosis and exocytosis of AMPAR increase in the periphery of PSLM. In the following period (Intermediate, about 3–10 min), AMPAR exocytosis is suppressed. Finally (Late) exo- and endocytosis of AMPAR go into equilibrium in a low level. How each type of AMPAR changes during LTD remains to be clarified. This figure is newly drawn based on our previous publications (Fujii et al., 2017, 2018), and copyright permission is not required.

GluA2/3 hetero-tetramer, changes during LTD has not been reported. Transient and simultaneous enhancement of both exo- and endocytosis of GluA1 after the onset of NMDA application might contribute to substitutions of AMPAR subtypes. GluA2-containing AMPAR might be replaced by Ca^{2+} permeable AMPAR lacking GluA2 (Sanderson et al., 2016).

Importantly, similar changes of GluA1-SEP dynamics during LTD expression were observed in conventional synapses. Using oblique illumination, we studied the exo- and endocytosis changes of GluA1-SEP around conventional synapses in hippocampal neurons cultured on glass that was not coated with NRX (Fujii et al., 2018). Although, the SN ratio of fluorescence images of GluA1-SEP around synapses observed with oblique illumination was inferior to that obtained using PSLM and TIRFM, qualitatively similar results were obtained.

LATERAL MOVEMENT OF AMPAR ON THE PLASMA MEMBRANE

AMPAR moves around on the plasma membrane by lateral diffusion, and this movement has been studied by live-cell imaging of a fluorescent quantum dot attached to AMPAR (Bats et al., 2007; Groc et al., 2008). Diffusion is much faster in extrasynaptic membrane than in postsynaptic membrane. Notably, there is little movement of AMPAR in the postsynaptic membrane for a long time. Thus, AMPAR can be trapped at a postsynaptic membrane. Together with the balance of exo- and endocytosis, the efficiency of trapping of AMPAR at a postsynaptic membrane or the balance of coming-in and going-out of AMPAR to and from a postsynaptic membrane should influence the amount of AMPAR at a postsynaptic membrane (Opazo et al., 2010, 2012; Opazo and Choquet, 2011; Chen et al., 2015).

MERITS AND DEMERITS OF PSLM

High SN ratio images of fluorescent molecules can be obtained around PSLM with TIRFM through the reduction of background signals (**Figure 2**). Parallel formation of PSLM on the glass surface enables a simple interpretation of imaging data about how synaptic proteins are localized in and around PSLM. PSLM is also stable and does not move, whereas dendritic spines in which postsynaptic membrane is located occasionally move in culture and *in vivo* (Deng and Dunaevsky, 2005). In addition, PSLM can be found much more easily under TIRFM than conventional postsynaptic membranes (Tanaka et al., 2014). These points are significant merits of using PSLM for analyses of postsynaptic processes in a basal condition and during synaptic plasticity.

However, PSLM is certainly an artificial structure deficient in interaction with presynaptic structures, which could potentially affect some functions of postsynaptic membrane. Thus, certain care should be taken in interpretation of results obtained using PSLM. Nevertheless, PSLM retains essential properties of postsynaptic membrane, as evidenced by the accumulation of postsynaptic proteins such as PSD95 and homer, and dynamic changes of the amount of AMPAR relevant to the expression of LTP and LTD. Furthermore, it was demonstrated that exo- and endocytic changes of GluA1-SEP during LTD at conventional postsynaptic membranes were qualitatively similar to those observed at PSLM as explained above Fujii et al. (2018). Thus,

PSLM can be regarded as a useful experimental model and can provide guiding results and/or ideas that would be worth rigorously examining at conventional synapses.

FUTURE DIRECTIONS

There are many different types of synapses. Some are excitatory, and the others are inhibitory. There are also large differences in presynaptic transmitter release probability among synapses, which is likely to affect postsynaptic properties (Konnerth et al., 1990; Miyawaki and Hirano, 2011; Biederer et al., 2017). Synapses between a pair of neurons often change their characteristics during development (Pouzat and Hestrin, 1997; Yu and Goodrich, 2014). There are many types of synaptic adhesion molecules such as NLG, NRX, SynCAM, EphrinB, LRRTM and N-cadherin. In addition, there are different subtypes and different splice variants of NRX and NLG as explained above. They are differently distributed among synapses, and some of them are co-localized at a synapse. Combination of pre- and postsynaptic adhesion molecules are likely to play critical roles in determination of synaptic properties (Levinson and El-Husseini, 2005; Dean and Dresbach, 2006; Craig and Kang, 2007; Südhof, 2008; Bukalo and Dityatev, 2012). Changing a glass-coating presynaptic adhesion molecule, adding another coating molecule, or changing cultured neuronal type might reveal synapse-type specific postsynaptic properties. Such experiments might also provide useful information about determinant molecules for functional properties of a particular type of synapse.

Over-expression of AMPAR-SEP is also artificial manipulation which could affect normal cellular processes. Overexpression of a subunit of AMPAR such as GluA1 inevitably increases its relative amount, potentially affecting normal cellular processes. Indeed, we found that GluA2-SEP behaves somewhat differently depending on whether it is co-expressed with GluA1 (Tanaka and Hirano, 2012). I also note that SEP is not small in size and could affect AMPAR functions and regulations. One way to overcome these problems is to label endogenous AMPAR with a small fluorescent molecular probe (Wakayama et al., 2017).

Development of new fluorescent proteins such as pH-sensitive red fluorescent proteins will enable us to simultaneously monitor multiple proteins, and will certainly promote the analyses (Shen et al., 2014; Martineau et al., 2017). I would also like to note that application of super-resolution fluorescence imaging techniques, Stochastic optical reconstruction microscopy (STORM) and Photo-activated localization microscopy (PALM) in particular, were likely to match very well with analyses on PSLM, which is formed parallelly in a single focal plane (Dani et al., 2010; Maglione and Sigrist, 2013; Baddeley and Bewersdorf, 2018). STORM and PALM are used with TIRFM in most cases, and parallel formation of PSLM on the glass surface should facilitate image capture processes of STORM or PALM.

Another interesting extension of the culture method on the coated-glass surface is application to studies on presynaptic mechanisms. By coating glass surface with postsynaptic adhesion molecule, formation of presynaptic structure has been achieved (Funahashi et al., 2018). By this method, presynaptic active-zone-like membrane (AZLM) was formed on the glass surface coated with NLG, and single exocytosis event of a synaptic vesicle was visualized. Using an original experimental system, we also demonstrated fast diffusion of a synaptic vesicle protein synaptophysin tagged with SEP on the plasma membrane after membrane fusion of synaptic vesicle membrane, and also distinct distribution patterns of synchronous and asynchronous synaptic-vesicle release locations (Südhof, 2012; Kaeser and Regehr, 2014; Kavalali and Jorgensen, 2014; Maschi and Klyachko, 2017). Thus, the novel imaging preparations enabled by pre- and postsynaptic structure formation directly on the glass surface coated with a synaptic adhesion molecule combined with TIRFM, are expected to shed light on detailed molecular dynamics underlying synaptic transmission and plasticity.

AUTHOR CONTRIBUTIONS

TH wrote the manuscript.

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Brain-Derived Neurotrophic Factor (BDNF) Regulates Rab5-Positive Early Endosomes in Hippocampal Neurons to Induce Dendritic Branching

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Neurotrophin receptors use endosomal pathways for signaling in neurons. However, how neurotrophins regulate the endosomal system for proper signaling is unknown. Rabs are monomeric GTPases that act as molecular switches to regulate membrane trafficking by binding a wide range of effectors. Among the Rab GTPases, Rab5 is the key GTPase regulating early endosomes and is the first sorting organelle of endocytosed receptors. The objective of our work was to study the regulation of Rab5-positive endosomes by BDNF at different levels, including dynamic, activity and protein levels in hippocampal neurons. Short-term treatment with BDNF increased the colocalization of TrkB in dendrites and cell bodies, increasing the vesiculation of Rab5-positive endosomes. Consistently, BDNF increased the number and mobility of Rab5 endosomes in dendrites. Cell body fluorescence recovery after photobleaching of Rab-EGFP-expressing neurons suggested increased movement of Rab5 endosomes from dendrites to cell bodies. These results correlated with the BDNF-induced activation of Rab5 in dendrites, followed by increased activation of Rab5 in cell bodies. Long-term treatment of hippocampal neurons with BDNF increased the protein levels of Rab5 and Rab11 in an mTOR-dependent manner. While BDNF regulation of Rab5a levels occurred at both the transcriptional and translational levels, Rab11a levels were regulated at the translational level at the time points analyzed. Finally, expression of a dominant-negative mutant of Rab5 reduced the basal arborization of nontreated neurons, and although BDNF was partially able to rescue the effect of Rab5DN at the level of primary dendrites, BDNF-induced dendritic branching was largely reduced. Our findings indicate that BDNF regulates the Rab5-Rab11 endosomal system at different levels and that these processes are likely required for BDNF-induced dendritic branching.

Keywords: Neurotrophins, BDNF, TrkB, Rab5, Rab11, endosomes, dendritic branching, neuron

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a well-known neurotrophin that belongs to a small family of secreed proteins that includes nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) (Park and Poo, 2013). BDNF regulates many facets of the central neurons, including neuronal survival and differentiation, neuronal growth, synaptogenesis and plasticity and maintenance of neuronal circuits. BDNF is the most widely expressed neurotrophic factor in the brain and exerts its function by binding to the tropomyosin-related kinase receptor TrkB and the p75 neurotrophin receptor (p75). In addition, BDNF is secreted in an activity-dependent manner by autocrine and paracrine mechanisms (Bronfman et al., 2014).

The neuronal growth effects mediated by BDNF are mainly mediated by its tyrosine kinase receptor TrkB (Gonzalez et al., 2016). For example, BDNF binding to TrkB increases the branching of cortical and hippocampal neurons in dissociated cultures and organotypic slices (Horch and Katz, 2002). In addition, BDNF regulates the survival and migration of cortical neurons (Zhou et al., 2007; Zheng et al., 2008). These effects are induced by the activation of downstream signaling pathways after BDNF/TrkB receptor interaction. After binding BDNF, TrkB dimerizes and undergoes autophosphorylation at specific tyrosine residues of the intracellular domain. These phosphotyrosines are docking sites for adaptor proteins that lead to the activation of several signaling cascades including the mitogen-activated protein kinases (MAPKs), such as ERK1/2, ERK5 and p38, in addition to the phosphatidylinositol-3-kinase (PI3K)-Akt-mTOR pathway, phospholipase C-γ (PLC-γ) and the small GTPases of the Rho family Cdc42/Rac/RhoA (Huang and Reichardt, 2003; Minichiello, 2009).

Different lines of investigation have shown that internalization and postendocytic trafficking of Trk receptors determine their signaling properties and thus functional outcomes in neurons (Bronfman et al., 2014; Cosker and Segal, 2014). For example, Trk receptors ensure localized signaling responses to extracellular cues in axons (Ascano et al., 2012) and enhance downstream signaling to regulate neuronal differentiation (Zhang et al., 2000) and dendritic arborization (Lazo et al., 2013). Additionally, BDNF signals are retrogradely transported from dendrites to the soma to regulate gene expression (Cohen et al., 2011). Internalization of BDNF/TrkB is required for the sustained activation of PI3K and ERK signaling pathways and neurite outgrowth (Kumar et al., 2005; Zheng et al., 2008). Additionally, after internalization, endocytosed TrkB recruits microtubule-associated molecular motors such as dynein and neuronal kinesin KIF21B, which have both been described to contribute to the directionality of BDNF/TrkB endosomes in dendrites (Ghiretti et al., 2016; Ayloo et al., 2017).

The Rab monomeric GTPases are the main regulators of postendocytic trafficking of endocytic receptors. Rabs act as key regulators of vesicular trafficking by controlling the transport, anchoring and coupling of vesicles through effector binding. Among these effectors are the molecular motors and the SNARES, which generally join the Rabs in their GTP-bound state (Grosshans et al., 2006; Stenmark, 2009). In fact, Rabs are mediators of TrkB endosomal signaling (Zhou et al., 2012; Lazo et al., 2013; Sui et al., 2015). In the literature, more than 60 members of the GTPase Rab family have been described (Stenmark, 2009). Rab5, Rab7 and Rab11 are among the key GTPases known to be involved in BDNF/TrkB signaling (Zhou et al., 2012; Lazo et al., 2013).

After internalization, tyrosine kinase receptors (TRKs) enter the early or sorting endosomes, whose biology is regulated by Rab5 (Goh and Sorkin, 2013). Independent of the internalization mechanism of receptors, Rab5 tightly regulates the homotypic fusion of endosomes, forming the early or sorting endosome (Stenmark, 2009). There, receptors are sorted to the recycling pathway, which is regulated by Rab11, or to the late endocytic pathway regulated by Rab7 (Bronfman et al., 2014).

Studies from our laboratory and others have established that BDNF/TrkB regulates the activity and dynamics of Rab11-positive endosomes; in turn, Rab11 is required for BDNF-induced dendritic branching and local signaling in dendrites and synapses (Huang et al., 2013; Lazo et al., 2013; Song et al., 2015; Sui et al., 2015). Thus, transit through the early recycling pathway of TrkB receptors is a key step in BDNF signaling in neurons. However, whether BDNF/TrkB regulates Rab5 activity and dynamics in dendrites is unknown. Several lines of evidence indicate that Rab5-positive endosomes are required for proper neuronal morphology. Genetic experiments in Drosophila have shown that dynein and Rab5 are required for dendritic arborization in larvae (Satoh et al., 2008). On the other hand, Rab5 activity is regulated by TrkA in PC12 differentiation assays (Liu et al., 2007). Here, we first studied the short-term effects of BDNF treatment (5–30 min) on Rab5 dynamics and activity and then the long-term effects of BDNF treatment (4–24 h) on Rab5 and Rab11 protein and mRNA levels. We found that BDNF increases the number and dynamics of Rab5-positive endosomes in dendrites. Indeed, fluorescence recovery after photobleaching (FRAP) experiments showed that BDNF increases the recovery of Rab5-positive vesicles in the soma, which correlates with the increased activity of somatic Rab5, suggesting that BDNF increases the activation and movement of dendritic endosomes to cell bodies. Long-term treatment of hippocampal neurons with BDNF increased the protein levels of both Rab5 and Rab11 in an mTOR-dependent manner. In addition, BDNF also regulated mRNA levels of *rab5* (but not the mRNA levels of *rab11*). Both, Rab5 and Rab11 activity was required for proper morphological changes induced by long-term BDNF (48 h) treatment of neurons. Of note, in contrast to Rab11, reduced Rab5 activity impacted the basal levels of primary dendrites. BDNF was partially able to rescue this effect, but reduced Rab5 activity halted the full dendritic arborization induced by BDNF. Altogether, these results suggest that BDNF regulates the early recycling pathway at different levels to induce dendritic branching.

METHODOLOGY

All experiments were carried out in accordance with the approved guidelines of CONICYT (Chilean National

Commission for Scientific and Technological Research). The protocols used in this study were approved by the Biosecurity and Bioethical and Animal Welfare Committees of the P. Catholic University of Chile. Experiments involving vertebrates were approved by the Bioethical and Animal Welfare Committee of the P. Catholic University of Chile.

Materials

Minimum essential medium (MEM, 11700-077), Dulbecco's Modified Eagle's Medium (DMEM, 12800-017), Hank's balanced salt solution (HBSS, 14065-056), neurobasal medium (21103-049), OptiMEM (11058-021), Lipofectamine 2000 (11668-027), glutamine, B27 (17504-044), horse serum (HS, 16050-122), penicillin/streptomycin (15140-148), and trypsin (15090-046) were obtained from Invitrogen (Life Technologies, CA, US). Fetal bovine serum (FBS) HyClone (SH30071.03) was from GE Healthcare Life Science. Poly-L-lysine (P2636), AraC, Glutathione-Sepharose 4B (GE17-0756-01) and isopropyl β-D-thiogalactoside (IPTG, I6758) were from Sigma (MO, US). BDNF was purchased from Alomone Labs (Jerusalem, Israel). TrkB-Fc was acquired from R&D Systems (688TK, MN, US). Anti-βIII tubulin antibody, mouse anti-Flag (F3165), Mowiol 4-88 and the inhibitor actinomycin D (A1410) were purchased from Sigma (St. Louis, MO, US). Protease-free bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch (West Grove, PA, US). A MAP2 antibody was purchased from Upstate-Millipore (Billerica, MA). Protein-phosphatase inhibitors were from Thermo Fisher Scientific. The inhibitors cycloheximide (239763) and rapamycin (553210) were purchased from Calbiochem (Darmstadt, Germany). Mouse anti-glutathione-S-transferase (GST) (Ab92) and mouse anti-Rab5 (ab18211) were purchased from Abcam. Rabbit anti-Rab11 (715300) was purchased from Invitrogen. The Flag-TrkB plasmid was a gift of Dr. Francis Lee (Weill Cornell University, NY, US), EGFP-Rab5 and EGFP-Rab5DN were gifts of Dr. Victor Faundez (Emory University, GA, US), EGFP- Rab11DN was a gift of Dr. Rejji Kuruvilla (John Hopkins University, MD, US), and pGEX-GST-Rabaptin5 was donated by Dr. Vicente Torres (University of Chile, Chile).

Hippocampal Neuron Primary Culture

Embryonic hippocampal neurons from rats of either sex (embryonic days 17-19) were dissected as described previously (Shimada et al., 1998; Fan et al., 2004) in HBSS. After disaggregation, neurons were resuspended in MEM supplemented with 10% HS, 20% D-glucose, and 0.5 mM glutamine and were seeded on coverslips or plastic plates coated with poly-L-lysine (1 mg/ml). For morphological experiments, 7000 cells/cm^2 were seeded on coverslips. For protein or mRNA experiments, 15000 cells/cm^2 were seeded on plastic plates. After 4 h, the culture medium was replaced with neurobasal medium supplemented with 2% B27 and 1X glutamax. Proliferation of nonneuronal cells was limited using cytosine arabinoside at 3 days *in vitro* (DIV). The animals were obtained from the animal facilities of Pontificia Universidad Católica de Chile and euthanatized under deep anesthesia according to the bioethical protocols of our institution.

Analysis of the Levels of Messenger RNA (mRNA) in Hippocampal Neurons After BDNF Stimulation

Hippocampal neurons at 9 DIV were incubated for 90 min in neurobasal media for depletion of endogenous trophic factors and then were treated with 50 ng/mL BDNF for 4 or 12 h. Total RNA was extracted from primary neurons by using TRIzol and purified using the RNeasy kit (Qiagen, Hilden, Alemania) according to the manufacturer's instructions. cDNA was prepared by reverse transcription of 1 μg of total RNA with random primers using Maloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). The resulting cDNAs were amplified by using Brilliant II SYBR Green qPCR (Stratagene) with an Mx3000P thermocycler (Stratagene). All mRNA expression data were normalized to *β-actin*, *tbp* and *pjk-1* expression in the corresponding sample (Santos and Duarte, 2008). Finally, $2^{-\Delta\Delta Ct}$ analysis was performed. Oligonucleotide sequences for the primers used are shown in **Table 1**.

Western Blot Analyses

To study Rab5a and Rab11a protein levels, neurons were depleted with neurobasal media in the presence or absence of 5 μM actinomycin D (for Rab5a) or 25 μM cycloheximide (for Rab5a and Rab11a) with 50 ng/mL BDNF for 24 h or were treated in the presence or absence of 200 nM rapamycin for 60 min and then stimulated for 4 and 12 h with 50 ng/mL BDNF in the presence or absence of the drug. Next, cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% SDS and 1% Triton X-100) containing protease and phosphatase inhibitors. Standard SDS gel electrophoresis and Western blotting procedures were used to analyze the cell extracts using anti-Rab5a (1:1000), anti-Rab11a (1:1000) and anti-β-III tubulin (1:1000) antibodies.

TABLE 1 | Primers used to evaluate the mRNA levels of Rab5a, Arc, β-actin, TBP, and PGK-1.

Gene	Primer (5′-3′)
rab5a	F:GGCTAATCGAGGAGCAACAA
	R:ACAAAGCGAAGCACCAGACT
arc	F:GGAGGGAGGTCTTCTACCGT
	R:CTACAGAGACAGTGTGGCGG
β-actin	F:CCCGCGAGTACAACCTTCT
	R:CGTCATCCATGGCGAACT
tbp	F:CTGTTTCATGGTGCGTGACGAT
	R:AAGCCCTGAGCATAAGGTGGAA
pgk-1	F:TGCTGGGCAAGGATGTTCTGTT
	R:ACATGAAAGCGGAGGTTCTCCA
rab11a	F:AAAGTTACCCTGCTGCCTGG
	R:CTGCCAGGAAAGGAGACTGG

F, forward primer; R, reverse primer.

Immunoendocytosis of Flag-TrkB and Colocalization

Neurons were transfected with Flag-TrkB and EGFP-Rab5 using Lipofectamine 2000 and the manufacturer's instructions when cultures were at 7 DIV. Forty-eight hours later, neurons were incubated for 90 min in neurobasal media for depletion of endogenous trophic factors and treated with mouse anti-Flag antibodies conjugated to an Alexa Fluor 555 fluorochrome (20 μg/mL). After 30 min, the cells were washed with PBS at 37°C and stimulated with 50 ng/mL BDNF for 5 or 15 min, fixed and compared with noninternalized controls (cells not treated with BDNF). Images of neurons were acquired using confocal microscopy, processed with deconvolution algorithms, and then colocalization of Flag-TrkB with EGFP-Rab5 was analyzed by calculating Manders correlation index (M1) (Bolte and Cordelières, 2006).

Live-Cell Imaging of EGFP-Rab5

Neurons were transfected with EGFP-Rab5 as described above. After 24 h, the cells were depleted with neurobasal media during 180 min. Then, the cells were transferred to a Tyrode media (124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM D-glucose and 25 mM HEPES, pH 7.4). Live-cell imaging was performed on a Nikon Eclipse C2 confocal microscope equipped with a live-cell temperature controller (LCI cu-501) and digital camera connected to a computer with Software NIS-Elements C. Images of a single neuron transfected with EGFP-Rab5 were acquired using a 60X objective at intervals of 7.3 s for 5 min to establish the basal level of distribution and dynamic. After 5 min, neurons were stimulated with 50 ng/mL BDNF, allowing 3 min for diffusion of the ligand, and we started an additional 30 min of capture.

Quantification of the number of endosomes-like vesicles containing EGFP-Rab5 in dendrites was performed by comparing the fraction of total dendritic Rab5 that was found in structures larger than 0.2 μm^2. Images of the video were segmented with ImageJ, and the number of endosome-like vesicles was quantitated in 30-μm segments of primary dendrites.

Analysis of the mobility of Rab5-positive endosomes was performed by comparing the distribution of fluorescence in the same dendrite at different time points (0, 5, 15, 30 min). We quantified the number of particles moving more than 5 μm as a mobile fraction in nonstimulated neurons (control) and in neurons treated with BDNF for 5-30 min.

Live-Cell Imaging and Fluorescence Recovery After Photobleaching (FRAP) of EGFP-Rab5

The neurons were transfected with EGFP-Rab5 at 8 DIV. After 24 h, the cells were depleted from B27 for 180 min in neurobasal media. Then, the cells were transferred to a Tyrode media supplemented with TrkB/Fc (200 nM), and live-cell imaging was performed on a Nikon Eclipse C2 confocal microscope equipped with a live-cell temperature controller (LCI cu-501). Images of a single neuron transfected with EGFP-Rab5 were acquired using a 60X objective at intervals of 4 s for 5 min each at 5, 15 and 30 min to establish the basal level. After a brief wash with Tyrode media, neurons were stimulated with 50 ng/mL BDNF, allowing 3 min for diffusion of the ligand, and we started an additional 30 min of capture for intervals of 4 s for 300 s each at 5, 15, and 30 min. For the FRAP assay, a prebleach image was acquired at 2% laser power, after which a selected area was bleached at 100% laser power with 10 successive bleach scans separated by 1 s, assisted by the microscope software. Postbleach recovery images were acquired every 7.3 s for 300 s. Postacquisition image processing was performed using ImageJ. Adjustment and analyses were performed on the videos as brightness/contrast adjustments to all pixels in the images and as manual tracking of objects across multiples frames, respectively (Snapp et al., 2003). To quantify the percentage of endosome-like vesicles in the cell bodies of neurons transfected with EGFP-Rab5, first a threshold of the photobleached zone was applied. Prior to bleaching, a quantification was performed using the same selected region of interest (ROI). Then, the number of vesicles that recovered fluorescence associated with EGFP-Rab5 was quantified at 0, 5, 15, and 30 min after photobleaching.

Microscopy Detection and Quantification of Active Rab5 in Dendrites and Cell Bodies

The fusion protein Rab5BD-GST was produced in BL21 E. coli, transformed with a pGEX-GST-Rabaptin5 plasmid and stimulated for 4 h with IPTG. The Rab5BD-GST protein was purified from bacteria lysate using glutathione-Sepharose beads. For use as a probe, the protein was eluted in a solution of reduced glutathione. Similar methods have been described previously for other GTPases such as GST-FIP3 (Lazo et al., 2013). To test the protein as a probe, hippocampal neurons at 8 DIV were transfected with EGFP-Rab5DN, EGFP-Rab5CA or EGFP; in addition, nontransfected neurons were stimulated with 50 ng/mL BDNF for 5 or 30 min, fixed with paraformaldehyde (PFA), permeabilized and blocked in 3% fish gelatin in incubation buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA, 0.25 M sucrose and 0.2% Triton X-100, pH 7.2) for 45 min. The neurons were then incubated overnight with ∼10 μg/mL Rab5BD-GST in incubation buffer at 4°C. After 2 brief washes in HBSS, the neurons were fixed again in PFA, washed in PBS and then a standard immunofluorescence assay with rabbit anti-GST (1:500) and mouse anti-MAP2 (1:1000) was performed.

The neurons to be quantified were selected based on the MAP2 labeling to avoid the specific selection of a neuron with high or low levels of Rab5BD-GST. Three primary dendrites and the cell body were identified, and the integrated intensity was measured (intensity of the signal standardized by the area) per cell body and associated dendrites. The background was calculated from images of neurons treated with GST, and this baseline was calculated for and subtracted from each dataset.

Stimulation and Measurement of Dendritic Arborization Induced by BDNF

Hippocampal neurons (7 DIV) were transduced with EGFP, EGFP-Rab5DN or EGFP-Rab11DN adenoviruses and stimulated with 50 ng/mL BDNF in culture medium. After 48 h, dendritic arborization was analyzed by Sholl analysis (Sholl, 1953) and by

counting the number of branching points as described previously (Lazo et al., 2013). For analysis of dendritic branching, the neurons were immunostained with anti-MAP2. Dendrites were visualized by confocal microscopy using a Zeiss Axiovert 2000 inverted microscope equipped with a laser scanning module and Pascal 5 software (Carl Zeiss). Images were acquired using a 63X objective at 1024 X 1024-pixel resolution along the z-axis of whole cells. Z-stacks were integrated, and the images were segmented to obtain binary images. Ten concentric circles with increasing diameters (10 μm each step) were traced around the cell body, and the number of intersections between dendrites and circles was counted and plotted for each diameter. The adenovirus vector work was performed under biosafety level 2 conditions using a Labculture Class II, Type A2 cabinet (ESCO, Singapore). Analysis was performed using the ImageJ program.

Statistics

For statistical analysis, the GraphPad Prism 7 program was used. Multiple comparisons were performed with ANOVA with Bonferroni's posttest. To determine if two sets of data were significantly different from each other the Student's *t*-test was applied.

RESULTS

BDNF Increases the Colocalization of TrkB With Rab5-Positive Endosomes, Increasing its Vesiculation and Mobility in Dendrites

Neurotrophins use the early endosomal route, regulated by the Rab5 monomeric GTPase, to signal and regulate different physiological processes (Deinhardt et al., 2006; Ascano et al., 2012; Lazo et al., 2013). However, to date, there are no studies addressing the functional relationship of Rab5-positive endosomes with BDNF signaling. To address this issue, we analyzed the dynamics and activity of Rab5 endosomes upon short-term administration (5–30 min) of BDNF. First, we studied whether TrkB and Rab5 colocalize after BDNF treatment by cotransfecting hippocampal neurons with EGFP-Rab5 and Flag-TrkB tagged on its NH2 domain. As reported previously for the colocalization of Rab11 and TrkB, we performed immunoendocytosis by labeling the surface expression of TrkB in the absence or presence of BDNF (Lazo et al., 2013). We observed that on neurons that were not stimulated with BDNF, the TrkB receptors were dispersed to the periphery of the cell bodies and dendrites in large patches (**Figure 1A**). In addition, EGFP-Rab5 was concentrated in the cell body, although it was possible to identify some Rab5-positive endosomes in the dendrites (**Figure 1A**). After stimulation with BDNF, the EGFP-Rab5 distribution was more vesiculated, and there was an apparent increase in the presence of EGFP-Rab5 in dendrites. TrkB distribution also appeared more vesiculated after 15 min of BDNF treatment (**Figure 1A**). In addition, BDNF increased the colocalization of TrkB-positive endosomes with EGFP-Rab5 endosomes in cell bodies in a time-dependent manner (**Figure 1B**), as well as in dendrites (**Figure 1C**). Interestingly, after 5 min of BDNF treatment, the colocalization of TrkB and Rab5 in dendrites was already the same as at 15 min of stimulation. However, in the soma, the colocalization was increased after 15 min of treatment compared to that at 5 min (**Figures 1B,C**). These results suggest that BDNF increases TrkB and Rab5 colocalization and changes the dynamics of Rab5 in the dendrites and somas of hippocampal neurons.

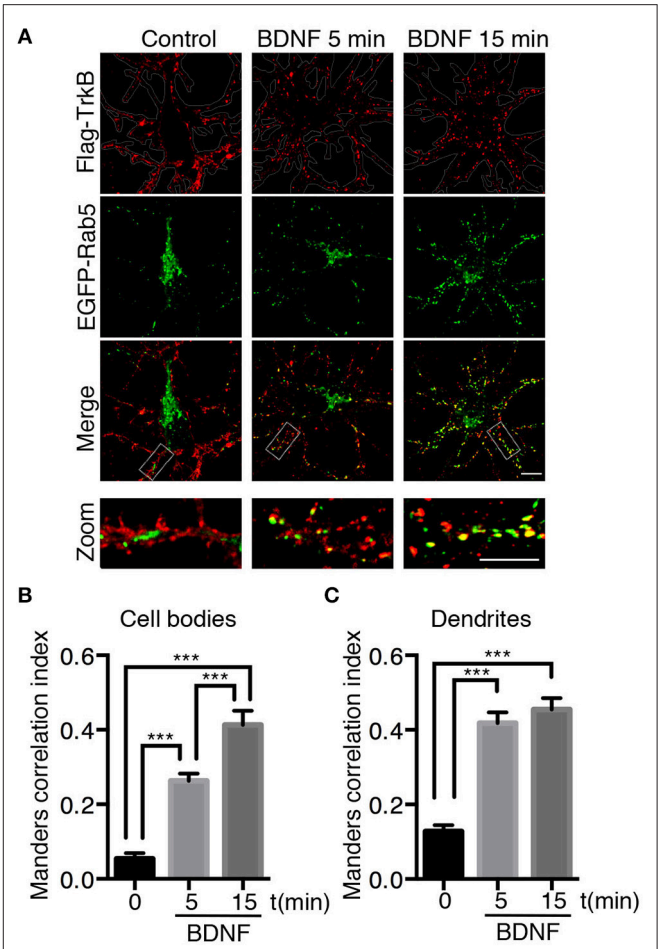

FIGURE 1 | Endocytosed TrkB receptors colocalize with Rab5 after BDNF stimulation in dendrites and cell bodies of hippocampal neurons. Hippocampal neurons (7 DIV) were cotransfected with Flag-TrkB and EGFP-Rab5 (green). After 48 h, neurons were incubated with anti-Flag antibodies (red) at 4°C for 30 min. TrkB internalization was stimulated with BDNF at 37°C for 5 or 15 min and then the neurons were fixed and observed by confocal microscopy. **(A)** In nonstimulated neurons, TrkB receptors are localized in the plasma membrane, and Rab5 is located within the neurons (time 0). After BDNF stimulation, TrkB receptors show an intracellular distribution and colocalize with Rab5. **(B)** Quantification of colocalization of TrkB-Flag with EGFP-Rab5 in cell bodies using Manders correlation index. **(C)** Quantification of colocalization of Flag-TrkB with EGFP-Rab5 in primary dendrites using Manders correlation index. $N = 30$ neurons from 3 different experiments. The results are expressed as the mean ± SEM. $^{***}p < 0.001$ by ANOVA with Bonferroni's posttest.

To further study whether BDNF regulates Rab5-positive endosomes, we studied the dynamics of EGFP-Rab5 in transfected hippocampal neurons by time-lapse microscopy of living cells before and after 5 min of BDNF stimulation. We found an increase in the number of Rab5-positive endosomes (**Figures 2A,B**), defined as dark vesicles using a threshold analysis in ImageJ, without changing the total EGFP-Rab5-associated fluorescence (**Figure 2C**). Additionally, the mobility

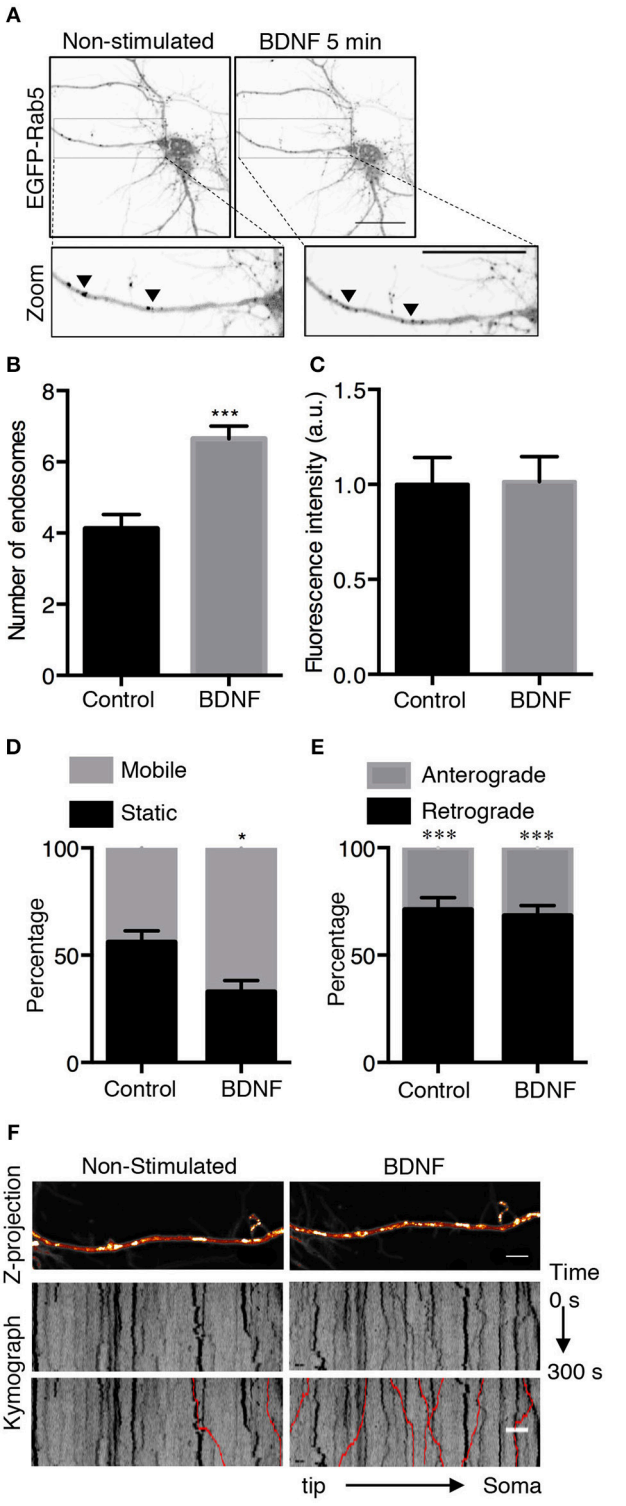

FIGURE 2 | BDNF increases the number and mobility of Rab5-positive endosomes in dendrites. **(A)** Representative image illustrating changes in the number of Rab5-positive endosomes in nonstimulated conditions (control) and after 5 min of BDNF treatment (BDNF) in the same neuron. EGFP-Rab5 endosomes are observed as dark spots within dendrites. A zoomed-in image of the dendrite is shown in the lower part, indicating that BDNF increases the number of Rab5-positive endosomes in dendrites (arrowheads). Scale bar, 10 μm. **(B)** Quantification of the number of endosomes in 30 μm^2 of dendrites in nonstimulated neurons (control) and after BDNF treatment (5 min). Endosomes were segmented by a fluorescence threshold and then quantified using ImageJ software. A total of 40 dendrites from 6 neurons were included in the study from 3 independent experiments. **(C)** Quantification of the fluorescence intensity of EGFP-Rab5 in the dendrites of neurons in the nonstimulated and BDNF conditions. **(D)** Quantification of mobile and static particles in dendrites expressed as a percentage based on the total number of particles per condition. Endosomes that traveled 5 μm or more after 300 s of recording were considered mobile endosomes. **(E)** Quantification of anterograde and retrograde mobile particles in dendrites expressed as a percentage of the total number of particles in each condition. **(F)** Representative image of a Z-projection of dendrites in the nonstimulated and BDNF conditions, showing the change in the mobile fraction of EGFP-Rab5. In the lower part is the kymograph of each neurite during the 300 s recording. In red are the endosomes considered to be mobile vesicles. Scale bar, 5 μm. The results are expressed as the mean ± SEM. *$p < 0.5$ or ***$p < 0.001$ by Student's *t*-test.

of Rab5 after BDNF treatment was increased, measured as endosomes that moved more than 5 μM in a time lapse of 300 s, shown as red lines in the kymograph (**Figures 2D,F**). Interestingly, the movement of EGFP-Rab5 is biased to the retrograde direction, as reported before in the literature (Kollins et al., 2009; Ayloo et al., 2017), a process that was not changed with the addition of BDNF (**Figure 2E**).

BDNF Increases the Recovery of Vesicular Rab5 After Photobleaching in the Cell Body, a Process That Correlates With Increased Rab5 Activity

To better understand the effect of BDNF on the mobility of EGFP-Rab5 endosomes in dendrites, we performed FRAP assays of hippocampal neurons stimulated with BDNF for 5 min. When a dendrite was photobleached, two populations of endosomes where observed: static (white and cyan arrowheads) and mobile (yellow arrowheads) (**Figures 3A,B** and **Supplementary Video 1**). Immediately after photobleaching, there was a recovery of cytoplasm-associated fluorescence, as shown with the blue arrow (**Figure 3A**). Then, an endosome derived from the static endosome, shown as the cyan arrowhead (blue arrow), recovered the fluorescence of the static photobleached endosome (yellow and white arrowheads in **Figure 3A**). Consistently, when the soma-associated fluorescence was bleached, it was possible to observe vesicles moving retrogradely toward the soma, as shown in the panels of **Figure 4** and **Supplementary Videos 2**, **3**. Altogether, these results suggest that by increasing the number and mobility of Rab5-positive endosomes, as shown in **Figure 2**, BDNF increases the retrograde transport of Rab5-positive endosomes to the soma. To study this possibility, we utilized FRAP assays of the complete cell bodies, including the initial segments of the dendrites, and studied the recovery of EGFP-Rab5 fluorescence in the soma of the cell bodies of cells treated with or without BDNF for 30 min. We noticed that there were two components in the EGFP-Rab5-associated fluorescence that were recovered after

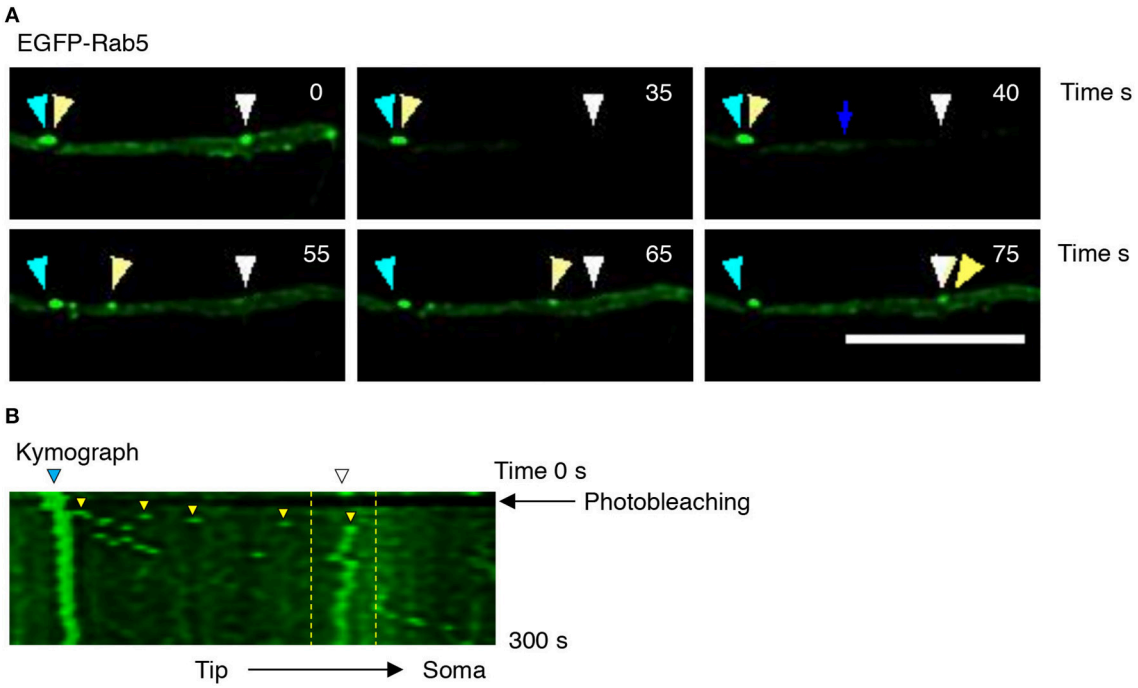

FIGURE 3 | Evaluation of EGFP-Rab5 movement by time-lapse microscopy after photobleaching in hippocampal neurons treated with BDNF. **(A)** Representative image of FRAP performed in primary dendrites of hippocampal neurons treated with BDNF. The recovery was evaluated during 300 s. Images were taken every 7.3 s. Representative individual frame of time-lapse performed in a neuron expressing EGFP-Rab5. The image shows both cytosolic-associated EGFP-Rab5 and vesicular-associated EGFP-Rab5 fluorescence (green) in dendrites prior to FRAP (0 s), during FRAP (35 s), and after photobleaching (until 75 s). The blue arrow shows nonvesicular (or soluble) EGFP-Rab5 fluorescence recovery. The white arrowhead indicates a static endosome that is photobleached (35 s), which is recovered after 40 s by recruiting a mobile endosome, which is indicated with the yellow arrowhead. The cyan arrowhead shows a static endosome that generates the endosome labeled with a yellow arrowhead. Scale bar, 10 μm. **(B)** Kymograph of the endosome movement event shown in **(A)**. In between the yellow lines is located the endosome recovered **(**indicated by the white arrowhead in **A)** by a retrograde-transported endosome, which is indicated by the yellow arrowhead in **(A)**. The black arrow indicates the moment in which the photobleaching was performed (10 s).

photobleaching. One accounted for the fluorescence associated with cytoplasmic EGFP-Rab5, and the other accounted for the fluorescence of EGFP-Rab5 associated with vesicles (**Figure 3A**, blue arrow and yellow arrowhead). When we quantified the FRAP in the cell body of EGFP-Rab5-transfected neurons, we did not observe changes in the kinetics of fluorescence recovery in neurons treated with BDNF compared to control neurons (**Figure 5B**). However, we observed that after BDNF stimulation, the recovery of Rab5-positive endosomes was faster than that in the control condition (**Supplementary Videos 4**, **5**). Therefore, we applied a threshold to each image obtained after photobleaching, as indicated in **Figure 5A**, and quantified the fluorescence associated with EGFP-Rab5-positive vesicles. For these experiments, we considered the initial number of vesicles before the photobleaching as 100% of particles and then quantified the number of visible vesicles in the cell body after 5, 15, and 30 min of BDNF stimulation. We found that BDNF increased the number of vesicles in a time-dependent manner compared to the number observed in nontreated neurons (**Figure 5A**, yellow box in zoom, and **Figure 5C**). In addition, we repeated this protocol in neurons expressing EGFP-Rab11, and we observed that BDNF did not increase the recovery of EGFP-Rab11 fluorescence in the cell bodies (**Figure 5D** and **Supplementary Figure 1**). Because EGFP-Rab11 fluorescence in the cell bodies appeared to be less vesicular and less defined than EGFP-Rab5 fluorescence, we were unable to quantify discrete Rab11 endosomes (**Supplementary Figure 1**). Our experiments suggest that BDNF increases the transport of Rab5 endosomes toward the cell body.

To assess whether the increased TrkB/Rab5 colocalization and Rab5 mobility in dendrites and somas correlate with increased Rab5 activity after BDNF stimulation, we studied the distribution of active Rab5 *in situ* using the GST-fused with the Rab5 binding domain of Rabaptin5 (Rab5BD-GST), which specifically recognizes the GTP-bound active form of Rab5 (Wu et al., 2014). Using Rab5BD-GST as a probe of active-endogenous Rab5 (Rab5-GTP), followed by staining with an antibody against GST, we found that the treatment of neurons with BDNF for 5 and 30 min increased the amount of Rab5-GTP in the cell bodies and dendrites of hippocampal neurons in a time-dependent manner, with no changes in the levels of endogenous Rab5 measured by Western blotting (**Figures 6A–C,E**). In cell bodies, there was a significant increase in Rab5 activity after 30 min of BDNF stimulation that was not due to increased levels of Rab5 protein by BDNF treatment (**Figures 6B,E**). However, in dendrites, we observed increased levels of Rab5-GTP after

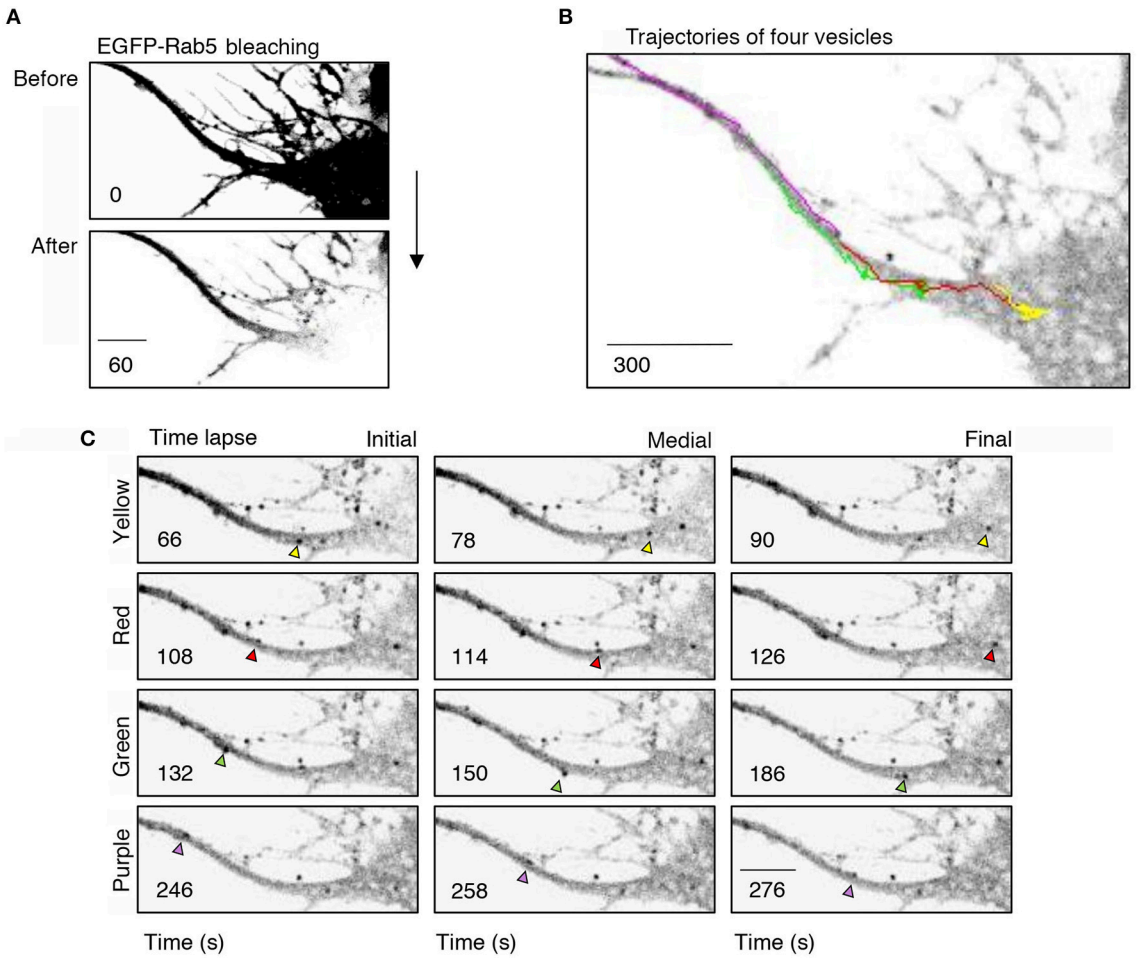

FIGURE 4 | Retrograde transport of EGFP-Rab5 endosomes from dendrites to the cell body. **(A)** Cell body and primary dendrite of a hippocampal neuron before (upper panel) and after photobleaching (lower panel). **(B)** Representative image of a time-lapse recording of EGFP-Rab5-associated fluorescence after photobleaching indicating the trajectories of four vesicles positive for EGFP-Rab5 performed in a primary dendrite and soma of a hippocampal neuron stimulated with BDNF. **(C)** Representative image of the initial, medial and final point of the trajectories shown in **(B)**. The vesicles whose trajectories were labeled in **(B)** are indicated with arrowheads of the same color of the trajectory. The numbers inside the panels indicate the seconds after photobleaching. Scale bar, 10 μm.

only 5 min of BDNF stimulation (**Figure 6D**), similar to the results for TrkB and Rab5 colocalization in dendrites and cell bodies (**Figure 1**). As negative and positive controls for this experiment, we used hippocampal neurons transfected with a Rab5 dominant-negative (Rab5DN) or a Rab5 constitutively active (Rab5CA) mutant, respectively. Neurons expressing Rab5DN displayed significantly lower Rab5BD-GST labeling than neurons expressing Rab5CA (**Figure 6D**). Altogether, these results suggest that BDNF increases the activity of Rab5 in the soma and dendrites of hippocampal neurons in a spatial- and time-dependent manner.

Long-Term Treatment of Hippocampal Neurons With BDNF Results in Increased Protein Levels of Rab5 and Rab11

BDNF signaling increases protein levels by increasing transcription in a CREB-dependent manner downstream of PLC-gamma and ERK1/2 or translation in a mTOR-dependent manner downstream of PI3K and ERK1/2 signaling (Gonzalez et al., 2016). Therefore, we studied the effect of long-term administration of BDNF (4–24 h) on Rab5 and Rab11 protein levels. First, we studied whether the administration of BDNF for 4 or 12 h regulated the levels of the *rab5a* and *rab11a* genes. We found that BDNF increased the levels of *rab5a* in a time-dependent manner; an approximately 4-fold increase in *rab5a* was observed after 4 h of BDNF treatment (**Supplementary Figure 2A**), whereas after 12 h of BDNF stimulation, the levels of *rab5a* decreased to approximately 0.5-fold over the levels of the control (**Supplementary Figure 2B**). Conversely, the level of *rab11a* was unchanged by BDNF treatment at any of the time points studied (**Supplementary Figures 2A,B**). In this context, we first evaluated whether BDNF increases the protein levels of Rab5 after 24 h of BDNF treatment. We found that BDNF increased the level of Rab5a in approximately 20% of hippocampal neurons

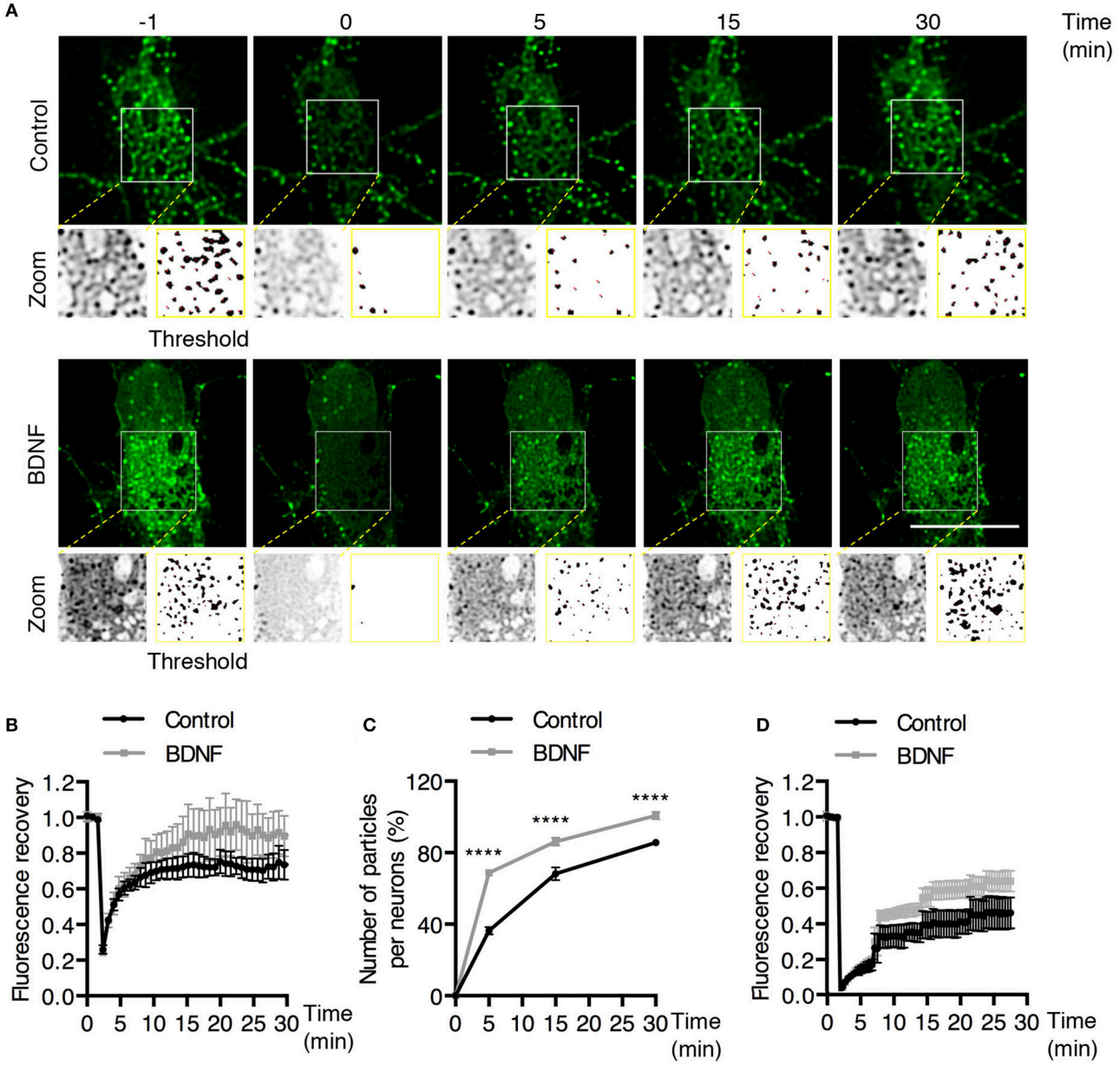

FIGURE 5 | BDNF increases the recovery of Rab5-positive endosomes after photobleaching. **(A)** FRAP was performed in cell bodies of hippocampal neurons (time 0), and recovery was evaluated after 5, 15, and 30 min of photobleaching. Representative individual frame of a recording performed in a neuron expressing EGFP-Rab5. Recording was performed under control conditions or after BDNF treatment. The image shows endosome-like Rab5 particles in the cell body prior to FRAP (−1 min), during FRAP (0 min) and after FRAP (5, 15, 30 min). The white square indicated in the picture shows a zoomed-in image. On the right, a threshold image of the zoomed image (left) shows the endosomes quantified in **(C)**. **(B)** Time course of fluorescence recovery after FRAP of EGFP-Rab5-associated fluorescence in the photobleached soma. Black squares indicate the control neurons (n = 7) and gray squares the BDNF-stimulated neurons ($n = 7$). **(C)** The graph indicates the percentage (100% fluorescence was established at 1 min before FRAP) of endosome-like particles quantified (at $t = 0$, 5, 15, and 30 min) after FRAP. The black squares indicate the values obtained for the control neurons ($n = 7$), and gray squares indicate stimulated BDNF neurons ($n = 7$). **(D)** Time course of fluorescence recovery after FRAP of EGFP-Rab11-associated fluorescence in the photobleached soma. Black squares indicate the control neurons ($n = 5$) and gray squares the BDNF-stimulated neurons ($n = 6$). Four independent experiments were performed. Scale bar, 10 μm. The results are expressed as the mean ± SEM. ****$p < 0.0001$ by two-way ANOVA with Bonferroni's posttest.

(**Figure 7A**) in a transcription- and translation-dependent manner, which is consistent with the results presented in **Figures 7B,C**, showing that actinomycin D and cycloheximide reduced the levels of Rab5 after BDNF treatment. Since BDNF increases protein translation in an mTOR-dependent manner (Takei et al., 2004), we evaluated whether the increase in Rab5a protein levels was sensitive to rapamycin, an mTOR pathway inhibitor (Schratt et al., 2004). We observed that 4 h of BDNF

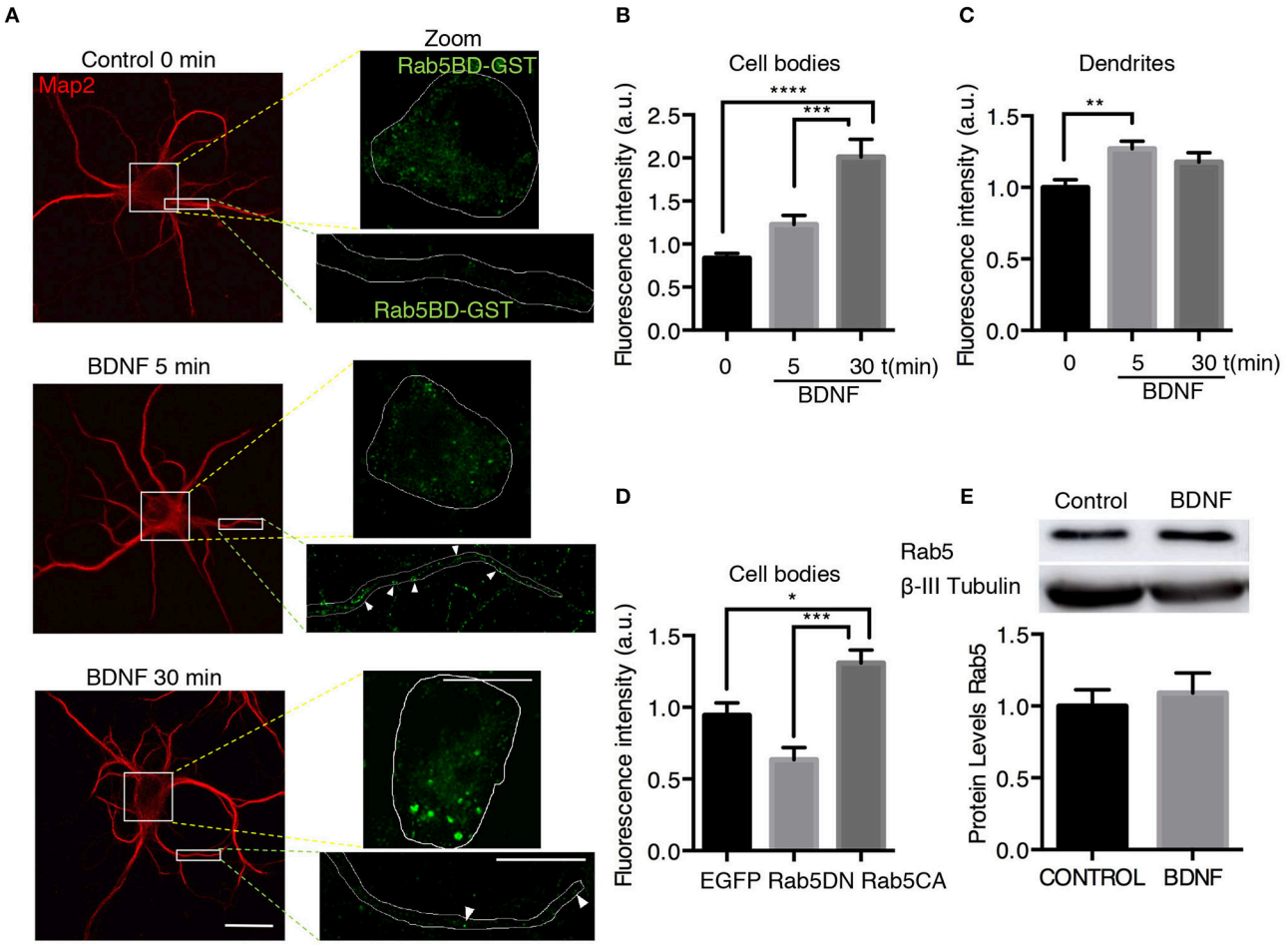

FIGURE 6 | BDNF increases the activity of Rab5. **(A)** Neurons were nonstimulated (control time 0 min) or stimulated with BDNF for 5 min (BDNF 5 min) and 30 min (BDNF 30 min). Representative image of MAP2 immunostaining (red) and Rab5BD-GST (green). The white box indicates the region shown in the amplified photos of the cell body in the left panel and the dendrite shown in the right panel. Scale bar, 10 μm. **(B)** Quantification of fluorescence intensity in the cell body. **(C)** Quantification of fluorescence intensity in primary dendrites of control neurons and neurons treated for 5 and 30 min with BDNF. The results for the soma and dendrites were normalized to the values of control neurons (time 0 min). A total of 28–29 cell bodies and 66–87 dendrites were included from 4 independent experiments. **(D)** Quantification of fluorescence intensity in the cell bodies of 7 DIV neurons transfected with EGFP, EGFP-Rab5DN, or EGFP-Rab5CA. The results are expressed as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ by ANOVA with Bonferroni's posttest. **(E)** The Rab5a and β-III tubulin levels of hippocampal neurons (9 DIV) stimulated with BDNF for 30 min. Lower part, quantification of the Rab5 levels of three independent experiments. The results are expressed as the mean ± SEM by Student's *t*-test.

treatment did not affect the protein levels of Rab5; however, the presence of rapamycin decreased the increase in Rab5 protein levels caused by 12 h of BDNF treatment to the basal level (**Figures 7D,E**). These results indicate that BDNF regulates Rab5 protein levels by increasing or stabilizing its mRNA and by increasing its translation in an mTOR-dependent manner.

We also analyzed the protein levels of Rab11 upon BDNF treatment. Similar, to the findings for Rab5, BDNF increased the level of Rab11 after 24 h of treatment; this effect was abolished by cycloheximide (**Figures 8A,B**). In contrast, 4 h of BDNF treatment did not affect the protein level of Rab11 (**Figure 8C**), while 12 h of BDNF treatment increased the level of Rab11 to values similar to 24 h of treatment (**Figure 8D**). In addition, the BDNF effect on the Rab11 protein levels (12 h treatment) was diminished by rapamycin (**Figure 8D**). All together, these results indicate that BDNF regulated the levels of both the Rab5 and Rab11 GTPases at the translational level in an mTor-dependent manner.

Long-Term Treatment of Hippocampal Neurons With BDNF Results in Increased Dendritic Branching That Is Impaired by Reducing the Activity of the Rab5 and Rab11 Proteins

It is well known that BDNF induces an increase in dendritic branching both *in vivo* and *in vitro* (Gonzalez et al., 2016). To evaluate whether Rab5 activity is required for BDNF-induced

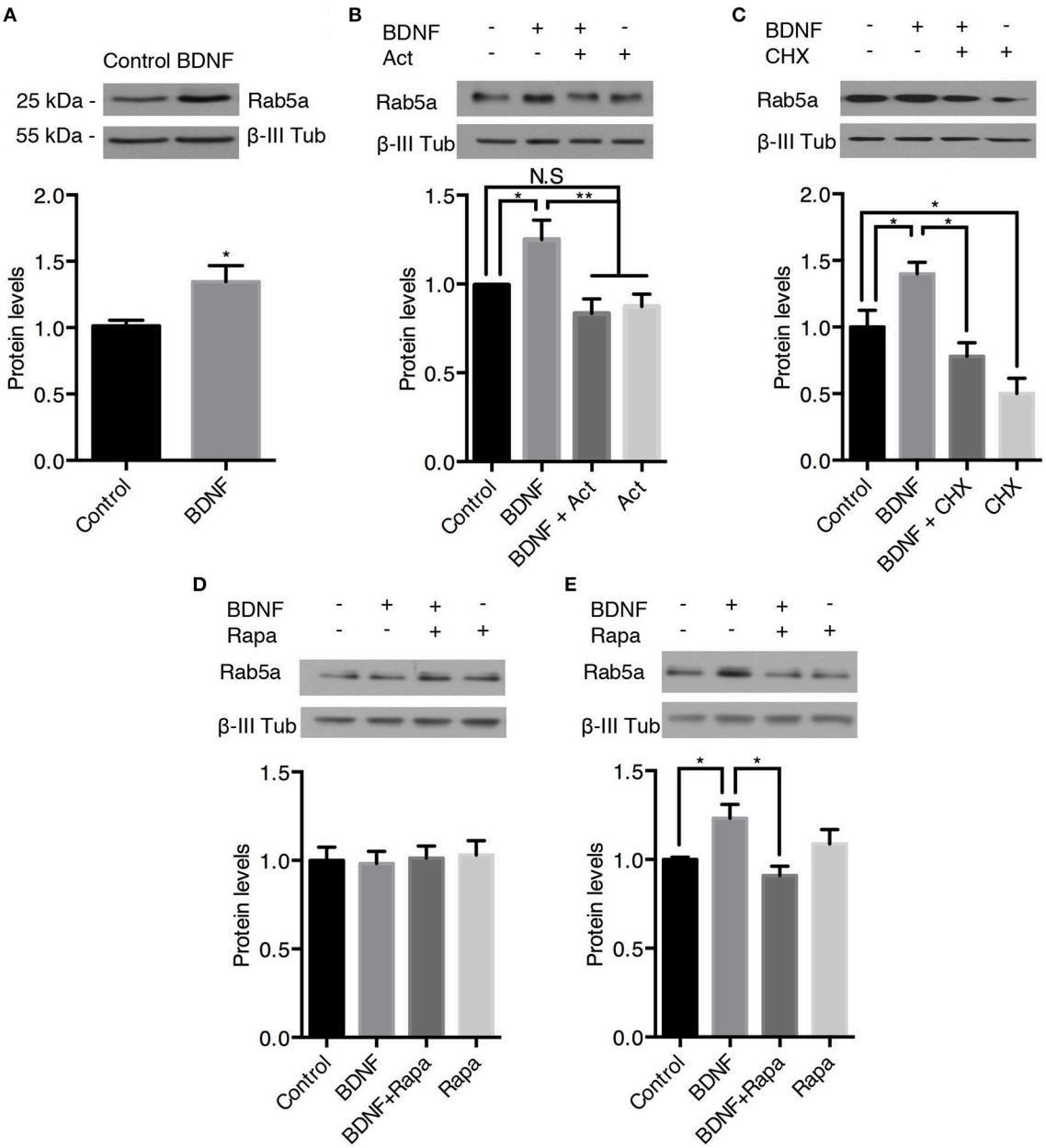

FIGURE 7 | BDNF increases the protein levels of Rab5a. The Rab5a and ß-III tubulin levels in hippocampal neurons (9 DIV) stimulated with BDNF for 24 h **(A)** in the presence or absence of Actinomycin D **(B)** or cycloheximide (CHX) **(C)**. Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent 5 independent experiments. The results are expressed as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$ by ANOVA with Bonferroni's posttest. **(D,E)** Ra5a levels after 4 h **(D)** or 12 h **(E)** of treatment with rapamycin (Rapa) in the presence or absence of BDNF. Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent four independent experiments. The results are expressed as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$ by ANOVA with Bonferroni's posttest.

dendritic branching in hippocampal neurons, we stimulated neurons expressing EGFP or the dominant-negative mutant of Rab5 (EGFP-Rab5DN) with BDNF for 48 h. We found that the expression of EGFP-Rab5DN produced a change in the morphology of the somato-dendritic arbor in comparison with neurons that only expressed EGFP (**Figure 9A**). Using Sholl analysis and the quantification of branching points, we found that the expression of Rab5DN reduces the branching points compared to the control condition (**Figures 9A–C**). Although neurons expressing Rab5DN responded to BDNF by increasing the number of primary dendrites, they were not able to respond to the same extent as neurons expressing EGFP and treated with BDNF, which showed an increase in branching points in addition to an increase in the number

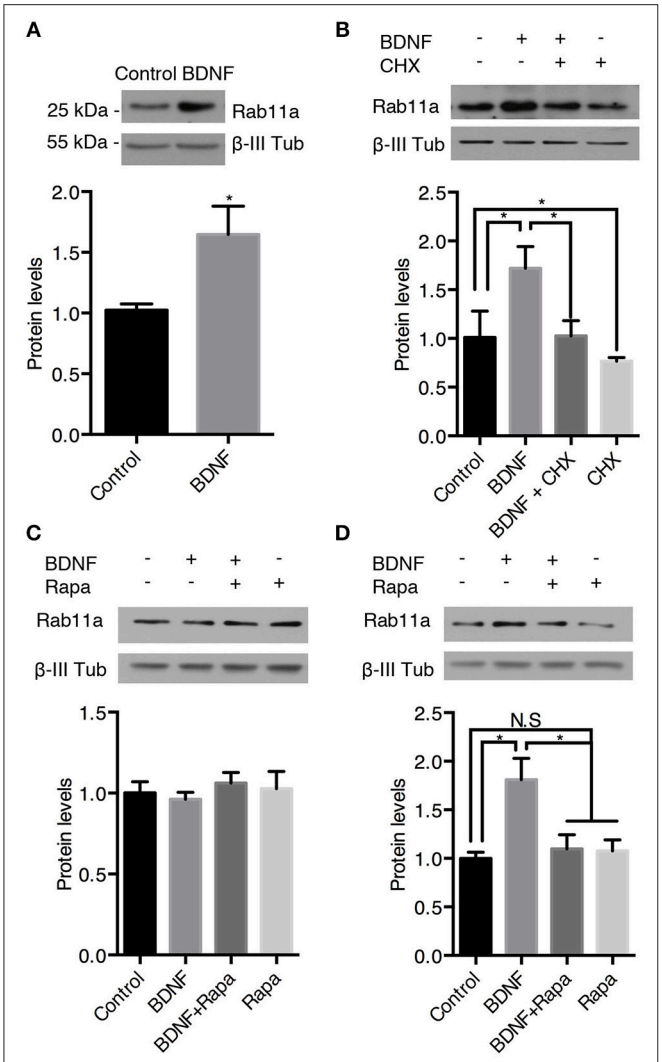

FIGURE 8 | BDNF increases the protein levels of Rab11a. The Rab11a and ß-III tubulin levels in hippocampal neurons (9 DIV) stimulated with BDNF for 24 h **(A)** in the presence or absence of cycloheximide (CHX) **(B)**. Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent 5 independent experiments. The results are expressed as the mean ± SEM. $*p < 0.05$; $**p < 0.01$ by ANOVA with Bonferroni's posttest. **(C,D)** Ra11a levels after 4 h **(C)** or 12 h **(D)** of treatment with rapamycin (Rapa) in the presence or absence of BDNF. Bottom panel, densitometric quantification of the Ra11a levels normalized to the ß-III tubulin levels. The data represent 4 independent experiments. The results are expressed as the mean ± SEM. $*p < 0.05$ by ANOVA with Bonferroni's posttest.

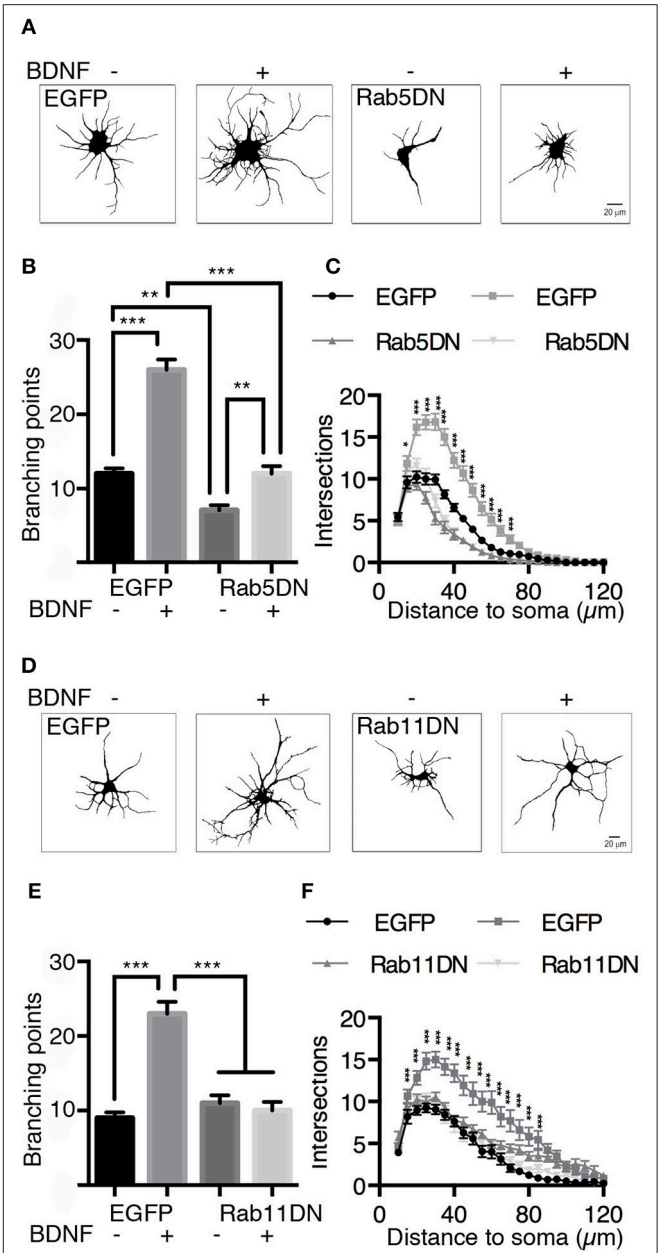

FIGURE 9 | The expression of dominant-negative mutants of Rab5 (Rab5DN) decreases BDNF-induced dendritic branching in hippocampal neurons. **(A)** Representative image of hippocampal neurons (7 DIV) transduced with adenovirus EGFP (in A at the left) or with a dominant-negative mutant of Rab5 fused to EGFP (Rab5DN in **A** at the right) and stimulated with BDNF for 48 h. After fixation, the neurons were labeled with anti-MAP2, observed by fluorescence microscopy and subjected to morphometric analysis. **(B)** Sholl analysis of the arborization profiles of neurons expressing EGFP and Rab5DN-EGFP in the presence or absence of BDNF. **(C)** Quantification of the branching points of neurons that overexpressed Rab5DN or EGFP and exposed to BDNF. $N = 27–34$ neurons from 3 different experiments. **(D)** Representative image of hippocampal neurons (7 DIV) that were transduced with adenovirus EGFP (in **D** at the left) or a dominant-negative mutant of Rab11 fused to EGFP (Rab11DN in **A** at the right) and stimulated with BDNF for 48 h. After fixation, the neurons were labeled with anti-MAP2, observed by fluorescence microscopy and subjected to morphometric analysis. **(E)** Sholl

(Continued)

of primary dendrites (**Figure 9C**). These results are somehow different from those observed when neurons express a dominant-negative mutant for Rab11 (Rab11DN). Similar to our previous observations (Lazo et al., 2013), neurons expressing Rab11DN have a similar number of dendrites to neurons expressing EGFP. However, they did not respond to BDNF (**Figures 9D–F**). These results indicate that Rab5 activity is required for the maintenance of dendritic arbors *in vitro,* and although EGFP-Rab5-expressing neurons responded to BDNF by increasing

FIGURE 9 | analysis of the arborization profiles of neurons expressing EGFP and Rab11DN-EGFP in the presence or absence of BDNF. **(F)** Quantification of the branching points of neurons that overexpressed Rab11DN or EGFP exposed to BDNF. $N = 16–19$ neurons from 3 different experiments. The results are expressed as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by two-way ANOVA with Bonferroni's posttest.

the number of primary dendrites, Rab5 activity is required to observe the effect of BDNF on the branching of higher level dendrites.

DISCUSSION

Several lines of evidence have consistently shown that the internalization and transit of Trk receptors through the endocytic pathway are required for proper signaling and neuronal function (Bronfman et al., 2014; Cosker and Segal, 2014). The mechanism by which neurotrophin receptors use the endosomal pathway for signaling in neurons is well documented. For example, "signaling endosomes" containing ligand-bound neurotrophin receptors have been extensively described for axon-to-nucleus communication in peripheral neurons (Bronfman et al., 2003; Delcroix et al., 2003; Harrington et al., 2011). Additionally, BDNF signaling endosomes have been described to have a role in dendrite-to-nucleus communication in central neurons (Cohen et al., 2011). However, how neurotrophins regulate the endosomal system for proper signaling is just beginning to be understood (Cosker and Segal, 2014). Rabs are monomeric GTPases that act as molecular switches to regulate membrane trafficking. They achieve this function by binding a wide range of effectors that include SNAREs, signaling molecules and molecular motors. Among the Rab GTPases, Rab5 is the key GTPase regulating early endosomes and the first endocytic station of endocytosed receptors (Stenmark, 2009). Of note, several lines of evidence have shown that there is crosstalk between Rab5 activity and tyrosine kinase signaling receptors (Chiariello et al., 1999; Jozic et al., 2012; Ong et al., 2014). Our aim was to study the regulation of the Rab5-positive endosomes in relation to BDNF and at different levels, including dynamics, activity and protein levels in hippocampal neurons. We found that BDNF increased the colocalization of TrkB in dendrites and cell bodies, increasing the vesiculation of Rab5-positive endosomes in the somatodendritic compartment. These findings correlated with the increased mobility of Rab5 endosomes in dendrites and increased the movement of Rab5 endosomes from dendrites to the cell body. Consistently, BDNF induced an early activation of Rab5 in dendrites (5 min) followed by increased activation of Rab5 in cell bodies (30 min). Long-term treatment of hippocampal neurons with BDNF (12–24 h) increased the protein levels of Rab5 and Rab11 in an mTOR-dependent manner. Finally, expression of a dominant-negative mutant of Rab5 reduced the basal arborization of nontreated neurons and BDNF-induced arborization. We propose that BDNF increases the activity of Rab5 in dendrites to foster local dendritic growth and to increase BDNF signaling propagation to the cell soma.

We have previously shown that BDNF/TrkB increases the activity of Rab11 in dendrites of hippocampal neurons by increasing local recycling and thus signaling of BDNF (Lazo et al., 2013). Rab5 regulates the fusion of endocytosed vesicles to form early endosomes where receptors are sorted to the recycling pathway that is regulated by Rab11. Here, we show that BDNF signaling also regulates the activity of Rab5, suggesting that BDNF in dendrites increases the activity of both GTPases to increase the early recycling pathways for local signaling. One intriguing aspect of our research, however, is that the mobility of both endosomes was oppositely regulated by BDNF. While BDNF decreases the mobility of Rab11 to allow local recycling (Lazo et al., 2013), it increases both the number and mobility of Rab5-positive vesicles in dendrites (current study). The movement of Rab5 vesicles increased in both the anterograde and retrograde directions. However, Rab5 movements were biased to the retrograde direction, consistent with a study indicating that 60% of microtubules are oriented with the minus end toward the soma in mammalian cells (Ayloo et al., 2017). It is possible that while anterograde movement of Rab5-positive vesicles is required for dendritic growth, retrograde movement of Rab5 resulted in increased levels of Rab5-positive vesicles in the cell body. Consistently, we showed by live-cell microscopy that Rab5-positive endosomes moved from primary dendrites to the cell body. While performing live-cell microscopy of dendritic EGFP-Rab5 transfected neurons after photobleaching, we observed that Rab5-associated fluorescence recovered in the same place, in addition to the observed mobile vesicles (**Figure 3**), suggesting that we monitored both stationary and mobile early endosomes. Altogether, our research suggests that BDNF defines a different population of Rab5 early endosomes that sort components to the recycling pathway for local recycling, and another population engages in long-distance trafficking to the soma or to distal dendrites. It is possible that a coordinated action of actin-based motors regulates local trafficking of signaling receptors since both Rab5 and Rab11 interact with myosin proteins to coordinate local membrane trafficking (Schafer et al., 2014; Sui et al., 2015; Masters et al., 2017).

Different lines of evidence have shown that both dynein and neuronal kinesin KIF21B engage TrkB-BDNF for long-distance trafficking in dendrites (Ghiretti et al., 2016; Ayloo et al., 2017). On the other hand, active Rab5 has been described to bind the Hook-interacting protein complex, which interacts with dynein and dynactin to regulate the retrograde transport of axonal proteins in neurons (Guo et al., 2016). Additionally, there is evidence that dynein and dynactin contribute to 85–98% of long-inward translocation of Rab5 early endosomes in HeLa cells (Flores-Rodriguez et al., 2011). Of note, dynein-mediated transport of Rab5-positive early endosomes is required for dendritic branching in *Drosophila melanogaster* dopamine neurons (Satoh et al., 2008). These results are consistent with our findings showing that Rab5 activity is required for the stability of dendrites and BDNF-mediated dendritic branching in hippocampal neurons (**Figure 7**). Altogether, these results suggest that microtubule-associated molecular motors, most likely dynein, drive the long-distance movement of Rab5 endosomes from dendrites to the soma in response to BDNF,

which is a process required for dendritic branching. This process might be important in dendrite-to-nucleus communication as suggested by the results showing that dendritic BDNF increases expression of the immediate early genes c-fos and Arc in the cell bodies of both hippocampal and striatal neurons (Cohen et al., 2011; Liot et al., 2013).

We also observed that BDNF increases Rab5 vesiculation and the number of Rab5-positive endosomes in dendrites, a process that correlates with increased vesicles containing active Rab5. Fusion and fission events are required for proper early endosome function and sorting of endocytosed receptors and ligands (Skjeldal et al., 2012). Rab5 regulates these process by regulating fusion of newly endocytosed receptors to form the early or sorting endosomes; from there, fission events allow sorting into the endocytic pathways (Driskell et al., 2007). It is possible that BDNF increases fusion and fission events to increase the vesiculation of Rab5 in dendrites, or increases the recruitment of cytosolic inactive Rab5 to newly formed or preexisting endosomes (**Figure 10**). However, direct evidence of these phenomena remain to be analyzed by a more refined technique such as fluorescence resonance energy transfer as performed by Verboogen to visualize SNARE trafficking and fusion (Verboogen et al., 2017).

Like other Rab proteins, Rab5 activity and localization is regulated by GEFs, GAPs and different effectors (Zerial and McBride, 2001; Stenmark, 2009). Different extracellular cues have been described to regulate Rab5 activity. For instance, in PC12 cells, NGF activation of TrkA recruits RabGAP5, which inactivates Rab5, producing a delay in the maturation of signaling endosomes and prolonging signaling and neurite outgrowth in PC12 cells (Liu et al., 2007). Consistently, expression of a dominant-negative Rab7 in PC12 cells enhances NGF-mediated signaling (Saxena et al., 2005) while it abolishes axonal retrograde transport of TrkB-positive endosomes in motor neurons (Deinhardt et al., 2006). On the other hand, in cortical neurons, semaphorin 3A increases the activity of Rab5 in axons to promote growth cone collapse (Wu et al., 2014), suggesting that the activation of Rab5 might induce different outcomes depending on the extracellular cues, the signaling pathways activated and the neuronal processes that are regulated. We observed that BDNF increases Rab5 activity and that these processes are required for BDNF-dependent dendritic arborization, suggesting that in hippocampal neurons, BDNF-mediated activation of Rab5 is required for proper signaling, contrary to the results observed in PC12 cells (Liu et al., 2007). One question that arises is how BDNF regulates the activity of Rab5. There are no antecedents that could lead us to hypothesize a direct effect of TrkB signaling on Rab5 activity. However, we could speculate that by phosphorylating Rab5, BDNF modulates its interaction with GEFs, thus increasing its activation. In support of this speculation is the fact that different kinases including ERK1, a BDNF/TrkB downstream kinase, phosphorylate Rab5 (Chiariello et al., 1999), and Rin1, a GEF for Rab5, has been associated with other RTKs to increase Rab5 activity (Hunker et al., 2006). The activation of Rab5 should be a tightly regulated process, and we observed that the activation of Rab5 induced by BDNF is time dependent without promoting sustained activation. Sustained activation of Rab5 in axons disrupts retrograde axonal trafficking of NGF signals in basal forebrain cholinergic neurons, suggesting that Rab5 activity

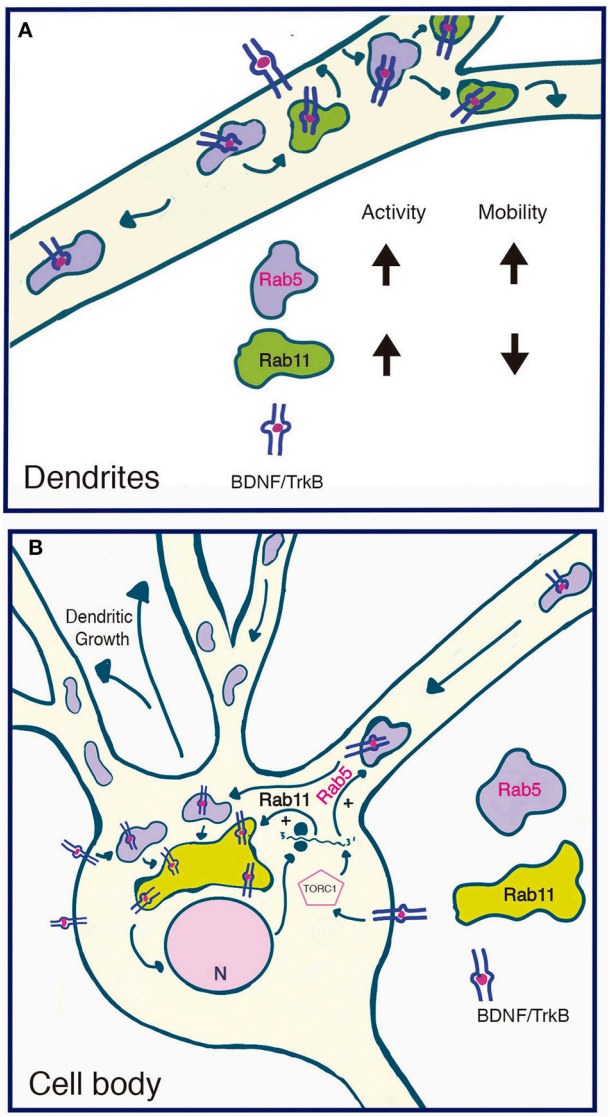

FIGURE 10 | Model summarizing the functional relationship between the early recycling pathway and BDNF/TrkB signaling. **(A)** In dendrites, BDNF increases the activity and reduces the mobility of Rab11-endosomes, fostering the local recycling of TrkB in dendrites and increasing local BDNF signaling (Lazo et al., 2013). On the other hand, BDNF transiently increases the activity of Rab5, a process that increases the number and mobility of Rab5 endosomes in dendrites. Since Rab5 early endosomes are upstream of Rab11 recycling endosomes, increased activity of Rab5 may, on the one hand, foster the Rab11-dependent recycling of TrkB in dendrites and, on the other hand, increase the transport of Rab5 endosomes to the soma. **(B)** In the soma, increased activity of both Rab5 (this paper) and Rab11 (Lazo et al., 2013) by BDNF may increase the cell body recycling of TrkB, increasing the long-lasting signaling of BDNF required for dendritic growth. Increased activity of the TORC1 complex by BDNF increased the protein levels of Rab5 and Rab11 acting as a positive feedback loop that contributes to BDNF-induced dendritic branching.

must be tightly regulated for proper neuronal function (Xu et al., 2016). Overactivation of Rab5 could be deleterious for neurons. Indeed, we have observed that expression of Rab5CA induces neurodegeneration in hippocampal cultures (data not shown), a phenomenon that it is not observed when hippocampal neurons are transduced with Rab11CA (Lazo et al., 2013).

Our results indicate that both Rab5 and Rab11 activity are required for BDNF-induced dendritic branching, indicating that the transit and correct endosomal sorting of BDNF receptors are required for proper signaling. For example, retrolinkin, a receptor that tethers vesicles, interacts with endophilin A1, a protein involved in generating endocytic necks, which is recruited to the early endosomal compartment in response to BDNF (Burk et al., 2017). Both proteins are required for BDNF early endocytic trafficking and spatiotemporal regulation of BDNF-induced ERK activation (Fu et al., 2011).

Finally, we found that BDNF increases both the mRNA and proteins levels of Rab5a in an mTOR-dependent manner. The activation of mTOR kinase has been described as a key signaling pathway regulating the translation of proteins mediated by BDNF (Leal et al., 2014). Specificity is achieved because BDNF, in addition to regulating translation, induces a specific miRNA-dependent repression (specific miRNA downregulation) and stabilizes the Dicer-TRBP complex, increasing global maturation of miRNA (Ruiz et al., 2014). The fact that the protein levels of Rab5 and Rab11 are upregulated by BDNF in an mTOR-dependent manner suggests that the specific growth program initiated by BDNF acts as a positive feedback loop to increase BDNF- and Rab5-Rab11-dependent dendritic growth. Indeed, mTOR activation is required for dendritic arborization of central neurons (Jaworski et al., 2005) and, consistently, both Rab5 and Rab11 activity is required for BDNF-induced neuronal growth.

Our results and those of others allow us to propose a model (**Figure 10**) addressing the functional role between the early recycling pathway regulated by Rab5-Rab11 and BDNF/TrkB signaling in neurons. We propose that BDNF is able to regulate the endosomal system by regulating the activity of Rab5 and Rab11 in a time- and space-dependent manner. This process allows both increased local signaling in dendrites and increased signaling in cell bodies. While this model predicts two different populations of recycling endosomes (dendritic Rab11 endosomes vs. the perinuclear cell body Rab11 recycling endosome), the early endosomal pathway might be coordinating dendritic and cell body signaling.

Altogether, our results suggest that Rabs are key proteins that regulate BDNF signaling, and further research is required to better understand the mechanism that leads to BDNF-mediated activation of Rab5 and Rab11 and how this process is coordinated with molecular motors for both local and long-distance signaling of BDNF.

AUTHOR CONTRIBUTIONS

GM-A performed and designed experiments and drafted the manuscript. AG performed and designed experiments and drafted part of section Materials and Methods and Figure Legends. NS performed experiments and FB supervised the experimental design and drafted the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2018.00493/full#supplementary-material

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frontiers in Cellular Neuroscience

REVIEW
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Reciprocal Regulation of KCC2 Trafficking and Synaptic Activity

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The main inhibitory neurotransmitter receptors in the adult central nervous system (CNS) are type A γ-aminobutyric acid receptors ($GABA_A$Rs) and glycine receptors (GlyRs). Synaptic responses mediated by GlyR and $GABA_A$R display a hyperpolarizing shift during development. This shift relies mainly on the developmental up-regulation of the K^+-Cl^- co-transporter KCC2 responsible for the extrusion of Cl^-. In mature neurons, altered KCC2 function—mainly through increased endocytosis—leads to the re-emergence of depolarizing GABAergic and glycinergic signaling, which promotes hyperexcitability and pathological activities. Identifying signaling pathways and molecular partners that control KCC2 surface stability thus represents a key step in the development of novel therapeutic strategies. Here, we present our current knowledge on the cellular and molecular mechanisms governing the plasma membrane turnover rate of the transporter under resting conditions and in response to synaptic activity. We also discuss the notion that KCC2 lateral diffusion is one of the first parameters modulating the transporter membrane stability, allowing for rapid adaptation of Cl^- transport to changes in neuronal activity.

Keywords: $GABA_A$R, chloride homeostasis, membrane turnover, lateral diffusion, clustering

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INTRODUCTION

Excitatory and inhibitory neurotransmission depend on the electrochemical ion gradients across the plasma membrane. The activation of postsynaptic ionotropic glutamate receptors leads to an influx of positively charged ions and thereby generates a depolarizing, excitatory postsynaptic potential (EPSP). In contrast, the net effect of activation of ionotropic anion permeable channels, such as type A γ-aminobutyric acid receptors ($GABA_A$Rs) or glycine receptors (GlyRs), depends on the gradient of anions across the plasma membrane, predominantly chloride (Cl^-) and bicarbonate (HCO_3^-; Bormann et al., 1987; Kaila and Voipio, 1987). The chloride gradient is mainly established by two secondary active transporters: the K^+-Cl^- cotransporter KCC2 that extrudes chloride out of the neuron using the potassium gradient (generated by the Na^+/K^+ ATPase), and the Na^+-K^+-Cl^- cotransporter NKCC1 which usually transports chloride into the neuron based on transmembrane sodium and potassium gradients also generated by the Na^+/K^+ ATPase (**Figure 1**). Hence, the balance of expression and activity of these transporters influence intracellular chloride concentration ($[Cl^-]_i$) and the efficacy and polarity of GABAergic and glycinergic transmission. In immature neurons, where NKCC1 expression

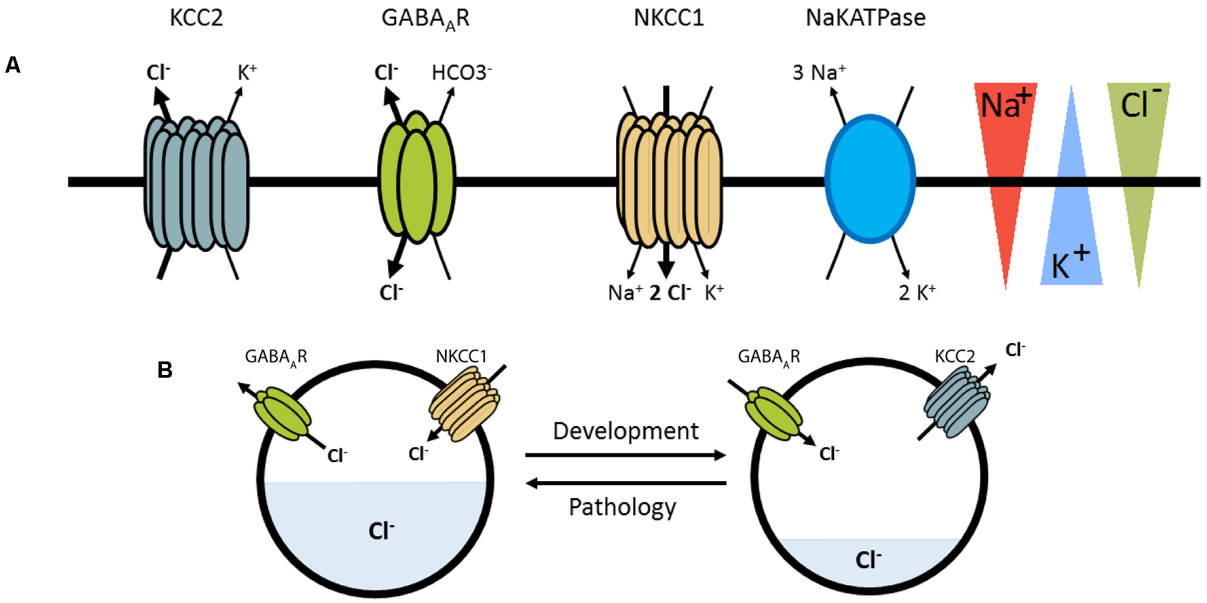

FIGURE 1 | Pivotal role of KCC2 and NKCC1 in the regulation of $[Cl]_i$. **(A)** KCC2 and NKCC1 transport chloride across the plasma membrane according to the Na^+ and K^+ gradients imposed by the Na^+K^+ ATPase. γ-aminobutyric acid receptor ($GABA_AR$) are primarily permeable to chloride ions and thus GABAergic transmission depends on KCC2 and NKCC1 regulation. **(B)** Depending on pathological conditions or developmental stages, GABAergic transmission may be depolarizing, correlated with an increased NKCC1/KCC2 functional ratio.

predominates, high $[Cl^-]_i$ is associated with depolarizing responses to GABA and glycine reflecting Cl^- efflux. In contrast, an increased expression of KCC2 in mature neurons lowers $[Cl^-]_i$ leading to an influx of Cl^- ions and hyperpolarizing responses upon $GABA_AR$/GlyR activation.

In addition to its role in maintaining low $[Cl^-]_i$, KCC2 regulates the formation (Li et al., 2007), functional maintenance and plasticity (Gauvain et al., 2011; Fiumelli et al., 2013; Chevy et al., 2015; Llano et al., 2015) of glutamatergic synapses. Consistent with its key role in regulating inhibitory and excitatory neurotransmission, alterations in KCC2 expression and function have emerged as a common mechanism underlying pathological activity in a variety of neurological and psychiatric disorders (Medina et al., 2014; Kahle and Delpire, 2016; Moore et al., 2017; Wang et al., 2018). Understanding the mechanisms regulating KCC2 expression and function is therefore crucial to develop novel and efficient therapeutic strategies. Here, we will review the cellular and molecular mechanisms controlling KCC2 turnover and describe how these mechanisms are rapidly tuned when neuronal activity is challenged.

KCC2 STRUCTURE AND REGULATORY SEQUENCES

KCC2 is one of nine members of the cation-chloride co-transporter (CCC) family encoded by the genes *Slc12a1-9*. KCC2 is a glycoprotein of 120 kDa with a predicted structure of 12 transmembrane segments (TMs), six extracellular loops flanked by a short intracellular amino terminal domain (NTD; amino acids 1–103) and a long intracellular carboxy-terminal domain (CTD; last 500 amino acids; Hartmann and Nothwang, 2015; **Figure 2**).

Two different KCC2 isoforms, KCC2a and KCC2b, are produced by use of alternative promoters of the *Slc12a5* gene encoding KCC2 (Uvarov et al., 2007). The NTD of KCC2a is 23 amino acids longer than the KCC2b one (Uvarov et al., 2007) and contains a putative SPAK (STE20/SPS1-related, proline alanine-rich kinase) and OSR1 kinase (Oxydative stress response 1) interaction site (de Los Heros et al., 2014; **Table 1**). Both isoforms show similar ion transport properties when expressed in human embryonic kidney (HEK) 293 cells and cultured hippocampal and cortical neurons (Uvarov et al., 2007; Markkanen et al., 2017), but have different subcellular localization *in vivo* (in neurons of the deep cerebellar nucleus, the pons and the medulla) and *in vitro* (cultured hippocampal neurons; Markkanen et al., 2014, 2017), suggesting a contribution of the NTD to the subcellular targeting of the transporter in given cells, probably *via* the binding to selective partners.

Based on a study of KCC1 (Casula et al., 2001), the KCC2 NTD has been suggested to be mandatory for KCC2 function (Li et al., 2007). Several groups have therefore used KCC2 lacking the NTD (KCC2-ΔNTD) to study ion-transport independent roles of KCC2 (Li et al., 2007; Horn et al., 2010; Fiumelli et al., 2013). The group of Igor Medina recently described altered exocytosis by truncation of the NTD in N2a cells, HEK 293 cells and cultured hippocampal neurons (Friedel et al., 2017).

In addition to five short extracellular loops, KCC2 contains a long extracellular loop (LEL) between TM5 and TM6 of around 100 amino acids (Williams et al., 1999). Based on

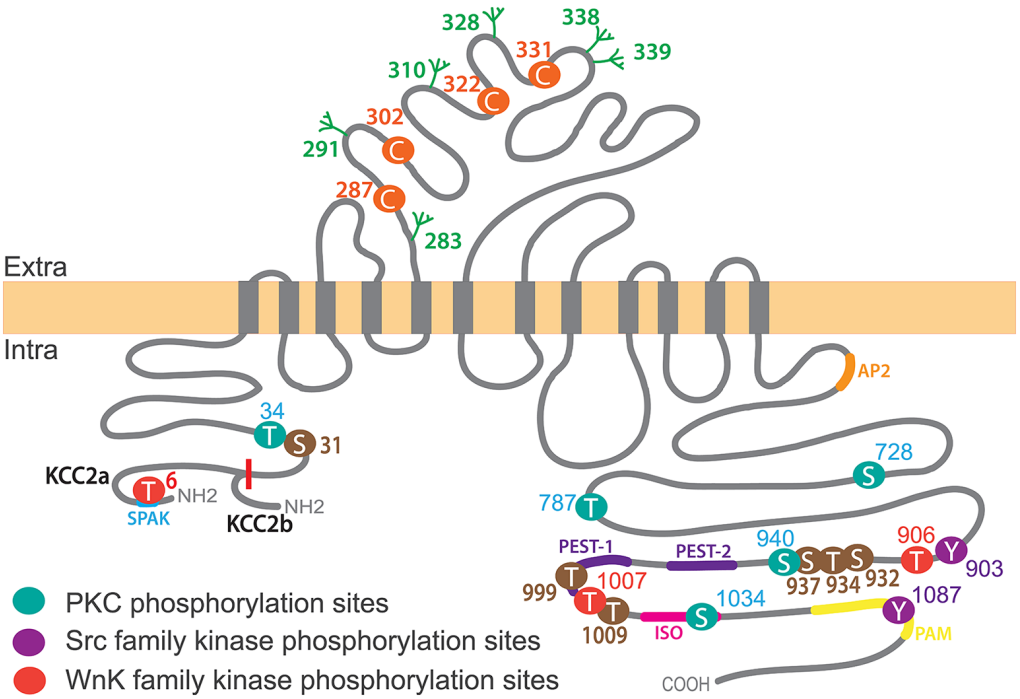

FIGURE 2 | KCC2 structure, key phosphorylation residues and regulatory domains. The KCC2 co-transporter is a ~140 kDa protein with a predicted topology of 12 membrane spanning segments, an extracellular domain between transmembrane domains 5 and 6 containing N-glycosylation sites (green) and key Cysteine residues for ion transport (orange), and is flanked by two cytoplasmic carboxy- and amino- terminal domains. All residues (besides Threonine T6 which is only present in KCC2a) are numbered according to human KCC2b. Note that the mouse sequence lacks amino acid 1,000 and thus beyond this residue the numbering is shifted (e.g., T1007 in human corresponds to T1006 in mice). KCC2a and KCC2b isoforms differ from their intracellular amino-terminal domain with KCC2a having a 23 amino acids longer sequence bearing a SPAK-binding domain (blue). Other regulatory sequences of KCC2 are located within the large intracellular carboxy-terminal domain (CTD), such as protein kinase C (PKC) phosphorylation sites (Serine S728, T787, S940 and S1034), Src family kinase phosphorylation sites (Tyrosine Y903, Y1087) and WNK-SPAK-OSR1 kinases (T906, T1007). Other regulatory sequences are present on this region: the ISO domain (pink) allowing for KCC2 activity under isotonic conditions, and protein associated with Myc (PAM; yellow), Proline/E/S/T (PEST; purple), SPAK (blue) and adaptor protein-2 (AP2; orange) binding sequences. Note that KCC2 activators staurosporine and N-ethylmaleimide (NEM) act by controlling (de)phosphorylation of series of residues (brown).

TABLE 1 | Key regulatory sites and sequences on KCC2.

Site	Localization	References
Protein Associated with Myc (PAM) LVLLNMPGPPRNRNGDENYM	1069-1088	Garbarini and Delpire (2008)
PEST sequence	PEST-1: 949-966 PEST-2: 974-1002	Mercado et al. (2006)
ISO domain PSPVSSEGIKDFFSM	1021-1035	Mercado et al. (2006)
AP-2 interaction domain LLRLEE	657-662	Zhao et al. (2008)
SPAK-OSR1 interaction domain		
RFTV	4–7	Piechotta et al. (2002)
Cysteines	287-302-322-331	Hartmann et al. (2010)
N-Glycosylation sites	283-291-310-328-338-339	Agez et al. (2017)
Tyrosines	34-787-906-1007	Rinehart et al. (2009), de Los Heros et al. (2014), Weber et al. (2014) and Cordshagen et al. (2018)
	34, 999, 1009	
	34, 1009	
Serines	728-940-1,034	Lee et al. (2007), Weber et al. (2014) and Cordshagen et al. (2018)
	31, 913, 932, 988	
	25, 26, 937, 1022, 1025, 1026	

Positions are shown relative to the human KCC2b protein.

KCC4 studies, N-linked glycosylation in the KCC2 LEL was proposed to be crucial for the membrane targeting of KCC2 (Hartmann and Nothwang, 2015). Six glycosylation sites on KCC2 were subsequently identified (Agez et al., 2017). Three human KCC2 mutations associated with severe early-onset epileptic encephalopathy showed reduced protein glycosylation and cell surface KCC2 (Stödberg et al., 2015), implicating KCC2 glycosylation in the control of its membrane expression. In addition, four highly conserved cysteine residues within the LEL were shown to be important for KCC2 activity but not membrane expression in HEK 293 cells probably due to their implication in inter- or intra-molecular di-sulfide bonds and correct protein folding (Hartmann et al., 2010).

The KCC2 CTD contains most of the KCC2 regulatory sequences. Complete truncation of the CTD reduces membrane expression of KCC1, 2 and 3 in *Xenopus laevis* oocytes and HEK 293 cells (Payne, 1997; Casula et al., 2001; Howard et al., 2002). Using live-cell surface labeling, Friedel et al. (2017) recently showed in cultured hippocampal neurons that KCC2 CTD is dispensable for membrane delivery of the transporter but is required for its membrane stabilization. Consistent with these observations, truncation of the KCC2 CTD by the Ca^{2+}-dependent protease calpain at an unknown site leads to the internalization and lysosomal degradation of KCC2 in rat brain slices (Puskarjov et al., 2012). Moreover, the interaction of KCC2 CTD with the clathrin-binding adaptor protein-2 (AP-2) *via* a di-leucine motif induces a constitutive, dynamin-dependent and clathrin-mediated endocytosis of KCC2 in HEK 293 cells (Zhao et al., 2008). The CTD also hosts the majority of KCC2 phosphorylation residues (**Figure 2**) which influence KCC2 membrane stability and thereby function through regulation of the transporter's lateral diffusion, oligomerization, clustering, and endocytosis (see below).

In contrast to other KCCs, KCC2 is constitutively active under isotonic conditions (Payne, 1997). A short sequence called ISO domain (1,022–1,037) located in the CTD has been shown to be responsible for this specific feature in Xenopus oocytes and hippocampal neurons (Mercado et al., 2006; Acton et al., 2012). Thus, replacement of this sequence by the corresponding KCC4 amino acids abolished constitutive KCC2 activity (Acton et al., 2012). Interestingly, KCC2 transporters lacking the ISO domain can still be activated under hypotonic conditions, indicating that two distinct domains are involved in KCC2 activation under isotonic vs. hypotonic conditions.

TEMPORAL AND SPATIAL EXPRESSION PATTERN OF KCC2

KCC2 expression can be observed throughout the central nervous system (CNS) including spinal cord (Hübner et al., 2001), thalamus (Barthó et al., 2004), cerebellum (Williams et al., 1999), hippocampus (Rivera et al., 1999), cortical structures (Gulyás et al., 2001) and the auditory brainstem (Blaesse et al., 2006). Although KCC2 expression is very broad in the CNS, the reversal potential of $GABA_AR$-mediated currents (E_{GABA}) varies among neuronal populations and brain structures (Chavas and Marty, 2003; Watanabe and Fukuda, 2015). These differences are thought to reflect changes in CCC expression and function.

Developmental Expression

Developmental upregulation of KCC2 expression has been described in different systems including human (Dzhala et al., 2005; Sedmak et al., 2016), mouse (Hübner et al., 2001), rat (Gulyás et al., 2001), zebrafish (Zhang et al., 2010), *C. elegans* (Tanis et al., 2009) and other species (for review Blaesse et al., 2009; Kaila et al., 2014). The KCC2 expression profile is well correlated with the sequential maturation of different brain regions (Watanabe and Fukuda, 2015), and follows the rostro-caudal axis of neuronal maturation (Li et al., 2002; Stein et al., 2004). Interestingly only the KCC2b isoform is developmentally upregulated, while KCC2a expression remains constant over brain maturation (Yeo et al., 2009). In the neonatal mouse brainstem KCC2a therefore contributes to about 20%–50% of the total KCC2 mRNA expression, while in the mature cortex its contribution decreases down to 5%–10% (Uvarov et al., 2009). KCC2a is expressed in the basal forebrain, hypothalamus and spinal cord, but is absent from the hippocampus (Markkanen et al., 2014). In contrast to full KCC2 knockout mice, which die at birth due to respiratory failure (Hübner et al., 2001), KCC2b knockout mice are viable until postnatal age 15 (P15; Woo et al., 2002). This suggests that both KCC2a and KCC2b isoforms are essential but contribute differentially to brain development and the establishment of inhibitory neurotransmission. Indeed, Dubois et al. (2018) recently showed a transient role of KCC2a at birth controlling the pontine neuromodulation of the respiratory motor circuits.

Subcellular Expression

At the cellular level, KCC2 expression can be found in the somatodendritic plasma membrane in most brain regions, such as cerebellum (Williams et al., 1999), hippocampus (Rivera et al., 1999; Gulyás et al., 2001) or cortex (Szabadics et al., 2006). KCC2 membrane expression is enriched near inhibitory and excitatory synapses and in spine heads of hippocampal neurons (Gulyás et al., 2001; Hübner et al., 2001; Blaesse et al., 2006; Gauvain et al., 2011; Chamma et al., 2012). At the presynaptic level, only developing photoreceptor cells (Zhang et al., 2006) and retinal bipolar cells (Vardi et al., 2000) exhibit KCC2 expression. Axonal exclusion of KCC2 from CNS axons, including axon initial segment (Williams et al., 1999; Hübner et al., 2001; Chamma et al., 2012), leads to higher $[Cl^-]_i$ in axons than in the somatodendritic compartment (Price and Trussell, 2006). As a consequence, activation of $GABA_AR$ by GABA spillover or axo-axonic GABAergic synapses leads to increased axonal excitability (Stell et al., 2007; Ruiz et al., 2010; Pugh and Jahr, 2011, 2013; Stell, 2011).

Association of KCC2 with the plasma membrane increases during neuronal maturation. Hence, immature neurons show brighter intracellular labeling than mature neurons (Gulyás et al., 2001; Szabadics et al., 2006) and KCC2 forms clusters at the surface of mature neurons (Gulyás et al., 2001; Hübner et al., 2001; Barthó et al., 2004; Watanabe et al., 2009; Chamma et al., 2012, 2013; Heubl et al., 2017). In primary

cultures of hippocampal neurons, KCC2 protein expression can be observed already at 3 days *in vitro* (div) in the soma, while the somatodendritic labeling peaks only at div 15 (Ludwig et al., 2003).

Markkanen and colleagues were the first to compare the subcellular distribution of the two KCC2 isoforms, KCC2a and KCC2b, in the deep cerebellar nucleus, the pons and the medulla, in hippocampal cultured neurons (Markkanen et al., 2014, 2017). The authors showed that in these neurons, KCC2a and KCC2b only partly colocalize and that the two isoforms are not localized in the same subcellular compartments in mature neurons (with stronger labeling of KCC2b on the soma and plasma membrane in general). The functional consequence of this distinct isoform localization however remains unclear.

MOLECULAR AND CELLULAR MECHANISMS OF REGULATION OF KCC2

KCC2 is regulated at the transcriptional and post-transcriptional level (e.g., through phosphorylation/dephosphorylation of key residues) which in turn influence its cellular trafficking (cell surface delivery, membrane diffusion-trapping, clustering, surface removal and intracellular degradation).

Transcriptional Regulation of KCC2

The neuron-specific KCC2 expression pattern is tightly regulated by transcription factors and neuron-restrictive silencing elements (NRSE) in the KCC2 gene *Slc12a5* (Karadsheh and Delpire, 2001; Uvarov et al., 2005, 2006; Yeo et al., 2009). Two NRSE sequences were found in intron 1 of the *Slc12a5* gene (Karadsheh and Delpire, 2001) and in the upstream regulatory region (Yeo et al., 2009). Binding of each of the restrictive elements to a neuron-restrictive silencing factor/repressor-element transcription factor (NRSF/REST) is sufficient to repress gene transcription (Yeo et al., 2009). In addition to these negative regulatory elements, two positive regulatory regions in the *Slc12a5* gene have been reported. Binding of the neuron specific transcription factor Egr4 (early growth response 4) to the Egr (early growth response) binding site activates KCC2 transcription (Uvarov et al., 2006). Similarly, Markkanen et al. (2008) found that binding of upstream stimulation factors, USF1 and 2, to an enhancer box (E-box) activates KCC2 expression.

The brain-derived neurotrophic factor (BDNF) has been shown to modulate KCC2 expression (Poo, 2001; Rivera et al., 2002; Aguado et al., 2003; Gottmann et al., 2009; Watanabe and Fukuda, 2015). While BDNF promotes KCC2 expression in immature neurons, exposure of mature CA1 pyramidal neurons to BDNF leads to decreased chloride extrusion (Rivera et al., 2004). Conversely, GABA increases BDNF expression in immature hippocampal and cerebrocortical neurons but not in mature hippocampal neurons, indicating a synergistic effect of GABAergic maturation and BDNF (Berninger et al., 1995; Kuczewski et al., 2011; Porcher et al., 2011). However, BDNF depletion (as shown in BDNF knockout mice) does not affect the developmental upregulation of KCC2 expression and function (Puskarjov et al., 2015). These results contrast with the reduced hippocampal KCC2 expression observed in TrkB knockout mice (Carmona et al., 2006) and the BDNF-induced increase in KCC2 mRNA expression in immature hippocampal neurons (Aguado et al., 2003; Rivera et al., 2004; Ludwig et al., 2011). Altogether, these results support a role of BDNF and TrkB in the developmental upregulation of KCC2.

Other trophic factors such as insulin-like growth factor 1 (Kelsch et al., 2001) and neurturin (Ludwig et al., 2011) have been implicated in the regulation of KCC2 expression. These data indicate that several signals control KCC2 expression and interact to increase KCC2 expression during neuronal development. The correlation of synaptic maturation with KCC2 upregulation therefore suggests their reciprocal influence.

Posttranslational Regulatory Mechanisms

Ion-transport activity of KCC2 does not only depend on KCC2 expression levels but also on the abundance and activity of numerous other proteins such as scaffolding proteins, cytoskeleton interactors/regulators, kinases and phosphatases that regulate its cellular trafficking.

Exocytosis

Consistent with the developmental switch of GABA/glycine neurotransmission, translocation of KCC2 from the cytoplasm to the plasma membrane indicates that exocytosis contributes to the control of KCC2-mediated chloride extrusion. Dynamic visualization of membrane insertion or internalization using recombinant proteins linked to pH-sensitive fluorophores helped to determine exocytosis-endocytosis trafficking of several neurotransmitter receptors (Petrini et al., 2014; Zhang et al., 2015). Since the NTD and CTD of KCC2 are both cytosolic, a pH-sensitive pHluorin tag was inserted in the second or third extracellular loop of the transporter (Friedel et al., 2015, 2017). Insertion of the tag loop of the transporter did not perturb the function of the protein and therefore this construct constitutes a useful tool to study KCC2 trafficking (Friedel et al., 2017). The expression of pHluorin-tagged KCC2 mutants with deletions of the N terminal (ΔNTD) or C terminal (ΔCTD) domain and the use of live-cell surface immunolabeling of heterologous cells or cultured hippocampal neurons revealed that the NTD is essential for KCC2 plasma membrane delivery whereas the CTD is critical to its membrane stability (Friedel et al., 2017).

Recently, insights into the regulatory mechanisms of KCC2 exocytosis were obtained as transforming growth factor β2 (TGF-β2) was shown to mediate translocation of KCC2 from intracellular pools to the plasma membrane in developing and mature hippocampal neurons (Roussa et al., 2016). The mechanism for TGF-β2-mediated KCC2 membrane translocation involves the Ras-associated binding protein 11b (Rab11b). KCC2-Rab11b interaction was recently confirmed in a native KCC2 interactome study (Mahadevan et al., 2017).

Oligomerization

Multimeric assembly has been demonstrated for a large number of members of the CCC family (Moore-Hoon and Turner, 2000; Casula et al., 2001, 2009; Starremans et al., 2003; Blaesse et al., 2006; Simard et al., 2007; Warmuth et al., 2009). KCC2 was shown to form KCC2a and KCC2b homo-dimers,

as well as KCC2a-KCC2b, KCC2-KCC4 and KCC2-NKCC1 hetero-dimers, in biochemical assays from neuronal and heterologous cell lysates (Blaesse et al., 2006; Simard et al., 2007; Uvarov et al., 2009).

There are discrepancies in the literature regarding the proportions of KCC2 monomers, dimers and higher-order oligomers in neurons. Blaesse et al. (2006) showed that an increase of KCC2 oligomers parallels transporter activation in the developing brainstem (between P2 and P30) whereas Uvarov et al. (2009) found oligomerization already at P2 in various brain regions. Mahadevan et al. (2014, 2017) using native PAGE reported that KCC2 form monomers, dimers as well as higher molecular mass complexes. However, using similar approaches, Agez et al. (2017) detected KCC2 monomers and dimers but not higher-order oligomers. These discrepancies may arise from differences in both experimental assays (native perfluorooctanoate-PAGE vs. 3%–8% Tris-acetate NuPAGE; Blaesse et al., 2006; Uvarov et al., 2009) as well as detergents used for sample preparation (CALX-R3 vs. C12E9; Agez et al., 2017; Mahadevan et al., 2017). These limitations also apply to SDS-PAGE studies, as differences in sample preparation influence the proportion of KCC2 dimer-like complexes (Medina et al., 2014). In conclusion, it is not possible to compare the relative abundance of KCC2 monomers, dimers and higher-order oligomers between studies.

The oligomerization domain has not been identified to date. However, several studies showed self-assembling capability for the CTD of NKCC1 and an Archean CCC (Simard et al., 2004; Warmuth et al., 2009) and decreased oligomerization of KCCs truncated on the C-terminus, or mutated on tyrosine residue 1087 (Simard et al., 2007; Watanabe et al., 2009). This observation suggests that KCC2 CTD might be involved in the assembly of the transporters as observed in Xenopus oocytes and hippocampal cultures (Simard et al., 2007; Watanabe et al., 2009). Whether the monomeric KCC2 is active remains unclear. Several studies reported a correlation between decreased KCC2 oligomerization and reduced transport activity (Watanabe et al., 2009; Mahadevan et al., 2014). For instance, neuropilin and tolloid like-2 (Neto-2) assemble with the oligomeric forms of KCC2 and this interaction increases KCC2-mediated Cl^- extrusion in cultured hippocampal neurons (Ivakine et al., 2013). Similarly, the kainate receptor GluK2 subunit interacts with KCC2 and is critical to KCC2 oligomerization, surface expression and ion-transport function in hippocampal neurons (Mahadevan et al., 2014; Pressey et al., 2017). However, since changes in KCC2 oligomerization and surface expression occur in parallel, these observations do not demonstrate a causal link between KCC2 oligomerization and Cl^- transport.

Clustering

KCC2 forms clusters in the neuronal plasma membrane (Gulyás et al., 2001; Hübner et al., 2001; Barthó et al., 2004; Watanabe et al., 2009; Chamma et al., 2012, 2013; Heubl et al., 2017). Interestingly the majority of KCC2 clusters are found at excitatory and inhibitory synapses in hippocampal cultures, without preferential accumulation at one type of synapses (Chamma et al., 2013). Ultrastructural studies indicate that KCC2 accumulates at the periphery of synapses in dendritic spines as well as on the dendritic shaft (Gulyás et al., 2001; Báldi et al., 2010).

KCC2 clustering could help to localize and/or stabilize transporters in sub-membrane compartments (e.g., near excitatory and inhibitory synapses), and to form a barrier in dendritic spines surrounding glutamatergic postsynaptic densities. Moreover, KCC2 clustering has been proposed to regulate the cotransporter function. Watanabe et al. (2009) showed that inhibition of tyrosine phosphorylation or deletion of a nearby region (Δ1089–1116) both lead to disruption of KCC2 clustering and transport activity without any change in the neuronal membrane pool. This suggests that the KCC2 CTD is involved in cluster formation and that clustering and function of the transporter are tightly correlated. Overexpression of the CTD on the other hand causes a decrease in KCC2 cluster size with no alteration of cluster density or chloride transport in hippocampal neurons (Chamma et al., 2013), suggesting KCC2 clustering does not rely exclusively on its CTD binding to the cytoskeleton. Association of KCC2 with lipid rafts was proposed to influence KCC2 clustering. Watanabe et al. (2009) observed that association with lipid rafts increases KCC2 clustering and function in neuronal cultures, while Hartmann et al. (2009) found larger clusters and enhanced transport activity after disruption of lipid rafts. The later study, however, was performed in HEK 293 cells and showed an overall increase in KCC2 surface expression. It therefore remains unclear how clustering of KCC2 in lipid rafts modifies its transport activity in neurons.

Lateral Diffusion

Lateral diffusion is a key mechanism controlling rapid activity-dependent changes in neurotransmitter receptor number (and therefore clustering) at synapses, a phenomenon underlying the tuning of synaptic transmission and plasticity (Choquet and Triller, 2013). Receptors constantly alternate between periods of free Brownian-type motion outside synapses and constrained diffusion at synapses. They are captured and confined at synapses by transient interactions with postsynaptic scaffolding molecules that anchor them to the underlying cytoskeleton. A reduced density of scaffolding proteins at synapses and/or a weakening of receptor-scaffold interactions increases the escape of receptors from synapses and thereby clustering and synapse efficacy. Since KCC2 is similarly clustered near excitatory and inhibitory synapses, we addressed the role of lateral diffusion on KCC2 subcellular distribution and function. This was analyzed using Quantum-based Single Particle Tracking (QD-SPT) in hippocampal cultures (Chamma et al., 2012, 2013; Heubl et al., 2017).

These experiments showed that KCC2 displays free Brownian-type motion outside clusters while it is slowed down and confined within clusters located near excitatory and inhibitory synapses (Chamma et al., 2012, 2013). However, KCC2 escapes clusters faster near inhibitory synapses than excitatory synapses, reflecting stronger molecular constraints at excitatory synapses. Further investigations suggested specific tethering of KCC2 near excitatory synapses through actin-

binding of the CTD of KCC2 *via* the actin binding protein 4.1 N, whereas KCC2 is confined at inhibitory synapses by a distinct mechanism (Chamma et al., 2013). Therefore, KCC2 undergoes a diffusion-trap mechanism similar to neurotransmitter receptors.

KCC2 lateral diffusion is rapidly tuned by activity. Enhancing glutamatergic excitation or reducing GABAergic inhibition both increased KCC2 membrane diffusion (Chamma et al., 2013; Heubl et al., 2017) through reduced phosphorylation of S940 and increased phosphorylation of T906/1007, respectively. Changes in transporter diffusion were accompanied by cluster dispersion and increased membrane turnover of the transporter. Therefore, we propose that different subpopulations of transporters exist in the plasma membrane: freely moving KCC2 outside clusters and transporters confined in clusters in the vicinity of synapses. These two pools of transporters are in a dynamic equilibrium that can vary in response to changes in synaptic activity. The extra-cluster pool of transporters can be considered as a reserve pool in equilibrium with the perisynaptic pool. Transitions between these compartments by lateral diffusion may then participate in the fine tuning of synapses in response to local fluctuations of synaptic activity (**Figure 3**). Since changes in KCC2 mobility occur within tens of seconds (Heubl et al., 2017), lateral diffusion is probably the first cellular mechanism modulating the transporter membrane stability. This may represent a rapid mechanism for adapting Cl- homeostasis to changes in synaptic activity.

Endocytosis

While activity-dependent KCC2 endocytosis was shown to rapidly decrease its neuronal membrane pool (Lee et al., 2011; Chamma et al., 2013; Heubl et al., 2017), KCC2 turn-over rate under basal conditions is controversial. Two studies showed a high turn-over rate (of about 20 min) of the transporter in neuronal cultures (Lee et al., 2010) and rat hippocampal slices (Rivera et al., 2004). In contrast, Puskarjov et al. (2012) observed no change in KCC2 membrane pool in hippocampal slices after 4 h inhibition of protein synthesis (by cycloheximide) or degradation (by leupeptin). Although the authors concluded that KCC2 has a rather low turnover, what they were testing in this study was the lifetime of the transporter. Once KCC2 is synthesized and inserted in the membrane, it undergoes several cycles of endocytosis and exocytosis until final degradation. The lifetime of the transporter therefore seems to be >4 h whereas KCC2 turn-over rate at the membrane is in the range of 20–30 min.

Lee et al. (2010) reported increased surface expression of endogenous KCC2 in hippocampal cultured neurons after a 45 min exposure to dynasore, a cell-permeable inhibitor of dynamin. Using co-immunoprecipitation experiments, Zhao et al. (2008) showed that endogenous KCC2 interacts with the clathrin-binding AP2, suggesting that KCC2 internalization may be controlled by the clathrin-mediated endocytic pathway. Then, they identified in HEK293 cells a constitutive, non-canonical endocytic 657LLRLEE662 motif in the KCC2 CTD. Both di-leucine residues are required to mediate efficient transporter endocytosis but the L658 residue is the most important. The two glutamic acid residues downstream regulate the function of the di-leucine endocytic motif. This motif is highly conserved among KCC family members but not in NKCC1, NKCC2 or NCC proteins (Zhao et al., 2008). Furthermore, protein kinase C (PKC) and casein kinase substrate in neurons protein 1 (PACSIN1), which are involved in clathrin-mediated endocytosis and vesicle transport in neurons (Schael et al., 2013), were recently identified in a KCC2 interactome study (Mahadevan et al., 2017). Altogether these results suggest that KCC2 membrane retrieval may require AP2 and PACSIN1. PACSIN1 has been shown to regulate the activity-dependent AMPAR surface recycling in cerebellar neurons (Anggono et al., 2013; Widagdo et al., 2016). More work is now needed to test whether PACSIN1 plays a similar role in activity-dependent membrane recycling of KCC2.

However, regarding clathrin-dependence of KCC2 endocytosis, it is important to mention that the motif identified by Zhao et al. (2008) in an artificial overexpression system is non-canonical. Whether this motif plays a role in neurons and whether other regions on KCC2 are critical for internalization remains to be tested. Endocytosis of most transmembrane molecules involves post-translational modifications favoring interaction with the clathrin pathway that have not been clearly demonstrated for KCC2.

Degradation

Constitutively internalized transporters are not targeted for lysosomal degradation in HEK 293 cells (Zhao et al., 2008). Only upon increased glutamatergic activity does KCC2 undergo lysosomal degradation. This has been shown in spinal cord neurons following peripheral nerve injury (Zhou et al., 2012), in cultured hippocampal neurons and hippocampal slices upon application of the glutamate receptor agonist NMDA (Lee et al., 2011; Puskarjov et al., 2012) or interictal-like activity induced by Mg^{2+} depletion (Puskarjov et al., 2012). This process has been shown to require Ca^{2+}-activated calpain cleavage of the KCC2 CTD (Puskarjov et al., 2012; Zhou et al., 2012). The exact location of the proteolytic cleavage site remains unknown. However, since it was proposed that KCC2 S940 dephosphorylation is a pre-requisite for calpain cleavage (Chamma et al., 2013), the calpain cleavage site may be positioned near the S940 residue.

Phospho-Regulation of KCC2

Phosphorylation or dephosphorylation of KCC2 key tyrosine, serine or threonine residues tune KCC2 activity mainly by controlling its membrane stability. KCC2 stability and clustering at the plasma membrane is directly regulated *via* its CTD and notably Y1087 and Y903 residues in HEK 293 cells, GT1-7 cells and hippocampal neurons (Watanabe et al., 2009; Lee et al., 2010). Other pathways have also been identified, and their consequences on KCC2 membrane stability and function characterized (Lee et al., 2007; Rinehart et al., 2009; Heubl et al., 2017). Thus, PKC-dependent phosphorylation of KCC2 S940 was shown to increase the transporter membrane stabilization in HEK 293 cells and in hippocampal neurons (Lee et al., 2007). Interestingly, S940 phosphorylation and calpain-mediated cleavage have been negatively correlated in cultured hippocampal neurons (Chamma et al., 2013). Studies in HEK 293 cells reported that T1007 phosphorylation is mediated by

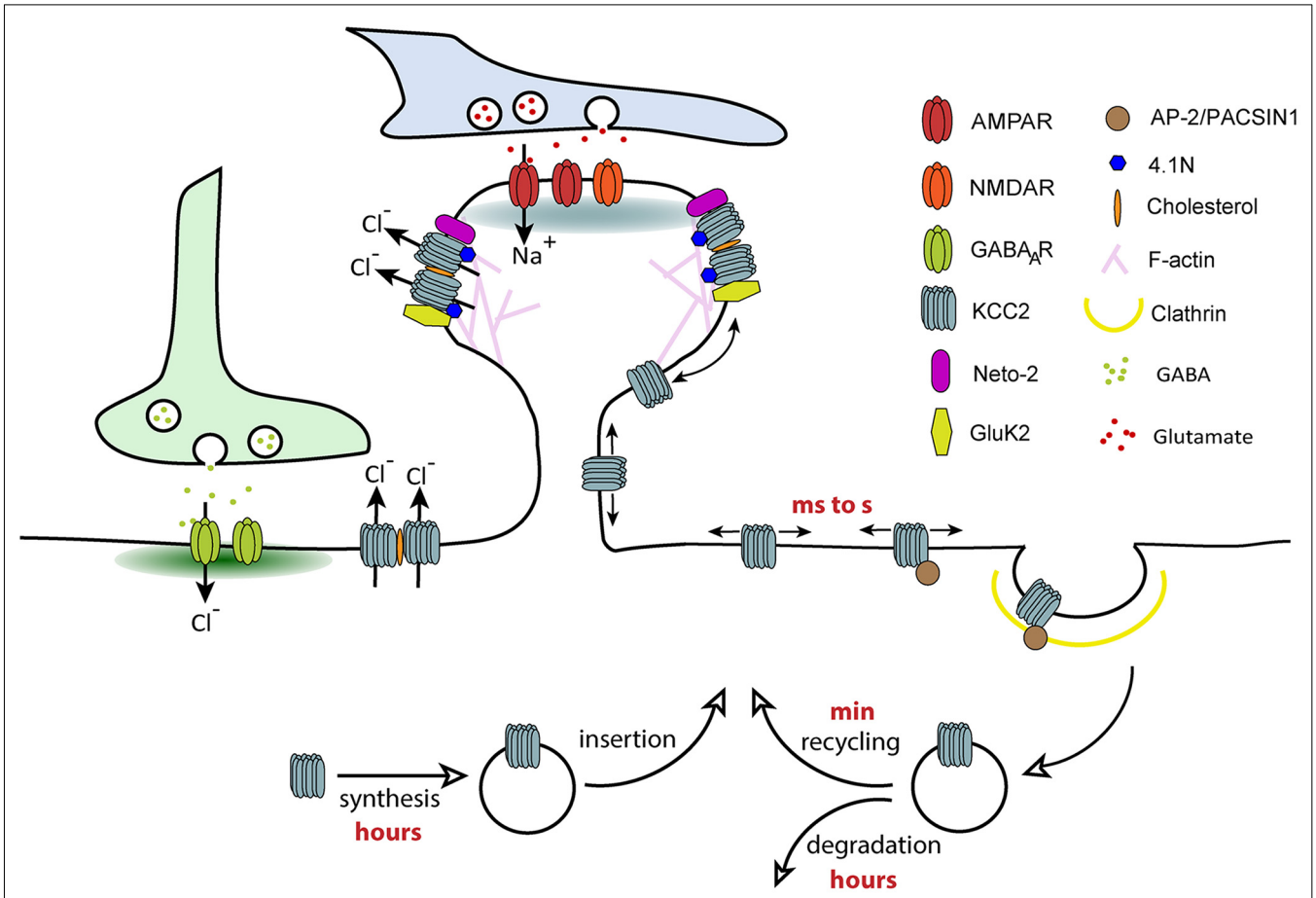

FIGURE 3 | Regulation of KCC2 membrane trafficking by lateral diffusion, clustering and endocytosis. Different subpopulations of KCC2 exist in the plasma membrane: freely moving KCC2 outside of clusters and confined KCC2 in membrane clusters. KCC2 clustering probably results from its accumulation in lipid-rafts, interaction with the cytoskeleton *via* protein 4.1N and oligomerization of the transporter. Freely moving molecules are more susceptible to interact with molecules involved in clathrin-dependent endocytosis such as AP-2. Confinement of KCC2 in membrane clusters may therefore prevent transporter endocytosis, a mechanism favoring chloride extrusion. The balance between "freely moving" and "clustered" pools of KCC2 can be rapidly changed by activity through phosphoregulations, which regulate the overall density of transporters localized at the membrane.

the serine/threonine kinase WNK1 [With No lysine (K) serine-threonine kinase 1] and its downstream effectors SPAK and OSR1 (Rinehart et al., 2009; de Los Heros et al., 2014). In contrast, T906 is not the target of WNK, SPAK or OSR1 in HEK 293 cells (de Los Heros et al., 2014; Zhang et al., 2016). The nature of the kinase phosphorylating T906 is still unknown (Zhang et al., 2016). The phosphorylation of T906 and T1007 keeps E_{GABA} depolarized by decreasing the membrane pool of KCC2 both in immature neurons (Friedel et al., 2015) as well as in mature neurons in response to reduced $GABA_AR$ activation (Heubl et al., 2017).

Other KCC2 phospho-sites have been identified in large-scale phospho-proteomics studies: S31, T34, S913, S932, S988, T999, T1009 (according to human sequence; Cordshagen et al., 2018), and S25, S26, T34, S937, T1009, S1022, S1025 and S1026 (Weber et al., 2014). Several of these sites tune KCC2 transport activity but unlike Y1087, S940, T906 and T1007, this regulation does not involve changes in total or surface expression levels of the transporter. Phosphorylation of S932, T934, S937 and dephosphorylation of T1009 enhance KCC2 transport function in HEK 293 cells (Weber et al., 2014; Cordshagen et al., 2018). Moreover, two potent KCC2 activators, N-Ethylmaleimide (NEM) and staurosporine, differentially impact KCC2 transport activity through a complex mechanism of (de)phosphorylation of several of these phospho-sites (Weber et al., 2014; Conway et al., 2017; Cordshagen et al., 2018). Staurosporine triggers phosphorylation of S932 and dephosphorylation of T1009. The action of staurosporine on T1009 occurs indirectly by inhibiting a kinase while its effect on S932 would be due to an indirect inhibition of a phosphatase (Cordshagen et al., 2018). NEM increases the phosphorylation of S940 while it decreases the phosphorylation of T1007 (Conway et al., 2017). NEM is thought to dephosphorylate T1007 through the control of SPAK phosphorylation/activity (Conway et al., 2017). Furthermore, a complex regulatory mechanism of KCC2 activity by staurosporine and NEM likely involves a change in the transporter conformational state through the (de)phosphorylation of several, partly overlapping phospho-sites

that include S31, T34 and T999 for staurosporine and S31, T34 and S932 for NEM (Cordshagen et al., 2018). The function of other phosphorylation sites (e.g., S25, S26, S1022, S1025 and S1026), however, remains unclear.

REGULATION OF KCC2 CELLULAR TRAFFICKING BY NEURONAL ACTIVITY

KCC2 mRNA, protein, and surface expression are known to be down-regulated under pathological conditions such as epilepsy or in experimental paradigms leading to enhanced excitatory activity, including long term potentiation (LTP; Wang et al., 2006a), rebound burst activity (Wang et al., 2006b), repetitive postsynaptic spiking activity (Fiumelli et al., 2005), coincident pre- and post- synaptic spiking (Woodin et al., 2003), NMDAR activation (Kitamura et al., 2008; Lee et al., 2011), and epileptiform activity (Reid et al., 2001; Rivera et al., 2004; Pathak et al., 2007; Li et al., 2008; Shimizu-Okabe et al., 2011). Most of these paradigms result in a depolarizing shift in E_{GABA} due to a reduced KCC2 function and/or expression. Recently, KCC2 down-regulation was also observed in conditions of reduced GABAergic inhibition in mature neurons (Heubl et al., 2017). This raises questions about the cellular and molecular mechanisms controlling KCC2 activity. A mechanism has emerged that involves phospho-regulation of key KCC2 serine and threonine residues that in turn influence the membrane dynamics, clustering, endocytosis, recycling and/or degradation of the transporter (Lee et al., 2011; Puskarjov et al., 2012; Zhou et al., 2012; Chamma et al., 2013; Heubl et al., 2017).

KCC2 Downregulation by Neuronal Excitation

Under conditions of increased neuronal activity, KCC2 diffusion is rapidly increased leading to the dispersal of KCC2 clusters, transporter internalization, degradation and ultimately deficits in chloride transport (Lee et al., 2011; Puskarjov et al., 2012; Zhou et al., 2012; Chamma et al., 2013). These effects are mediated by NMDAR-dependent Ca^{2+} influx, Ca^{2+}-induced protein phosphatase 1 (PP1) dephosphorylation of KCC2 S940 and Ca^{2+}-activated calpain protease cleavage of KCC2 CTD. These data suggest that deficits in KCC2 activity induced by dephosphorylation of S940 may contribute to the development of status epilepticus *in vivo*. However, the importance of KCC2 S940 phospho-regulation *in vivo* remains unclear. KCC2 dephospho-mimetic S940 (S940A) knock-in mice display normal basal KCC2 expression levels and activity in the hippocampus and do not exhibit any overt behavioral abnormality. Only in conditions of hyperactivity, S940 mice showed increased lethality to kainate-induced seizures (Silayeva et al., 2015). It remains to be determined whether increased lethality reflects enhanced seizure severity due to altered chloride transport or a brainstem-mediated respiratory arrest. On the other hand, phosphorylation of T906/1007 inhibits KCC2 function (Rinehart et al., 2009). Mice in which T906/1007 phospho-dependent inactivation was prevented by mutation into alanine showed increased KCC2 transport function in basal conditions (Moore et al., 2018). This effect was not associated with increased KCC2 surface expression but seems to involve changes in the intrinsic properties of the transporter. Importantly, increased KCC2 function attenuates chemically-induced epileptiform activity in T906A/1007A mice, both in acute hippocampal slices and *in vivo* (Moore et al., 2018), suggesting that enhancing KCC2 activity through T906/1007 dephosphorylation may be an effective approach in epilepsy treatment.

Implication in Glutamatergic Long-Term Potentiation

LTP of glutamatergic synapses in cortical neurons relies mainly on NMDAR activation and Ca^{2+}-dependent activation of intracellular kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Poncer, 2003). Consistent with the Ca^{2+}-dependent regulation of the transporter, persistent (>1 h) KCC2 downregulation has been reported during hippocampal LTP (Wang et al., 2006a). Reduced KCC2 function was then hypothesized to dampen GABAergic transmission and to promote LTP at excitatory synapses (e.g., Ferando et al., 2016), although this hypothesis has not been tested experimentally. Instead, chronic KCC2 knockdown by RNA interference was shown to preclude LTP expression in hippocampal neurons (Chevy et al., 2015). This effect was independent of Ca^{2+} and CaMKII activation but instead involved the direct interaction of KCC2 with the Rac1-specific guanilyl exchange factor betaPIX. Loss of this interaction upon KCC2 suppression led to enhanced activation of Rac1 and its downstream effectors PAK1 and LIM kinase, which inhibit the actin-severing protein cofilin (Chevy et al., 2015; Llano et al., 2015). Thus, KCC2 suppression prevented actin depolymerization required for activity-driven AMPAR exocytosis during LTP (Gu et al., 2010). This effect however was observed upon chronic KCC2 knockdown. How acute downregulation of KCC2 upon physiologically induced LTP influences subsequent plasticity therefore remains to be tested.

Regulation of KCC2 by GABAergic Inhibition

Several studies have shown that KCC2 can be rapidly downregulated by enhanced neuronal activity and glutamatergic neurotransmission (see above). However, little was known until recently about the regulation of KCC2 by synaptic inhibition. A study by Woodin et al. (2003) reported that increased GABAergic transmission also leads to KCC2 downregulation. However, this study was carried out in immature neurons displaying mainly depolarizing excitatory $GABA_AR$-mediating synaptic responses associated with activation of voltage-dependent Ca^{2+} channels (VDCCs) and intracellular Ca^{2+} signaling pathways (Woodin et al., 2003). Therefore, this study did not reveal regulation of KCC2 by synaptic inhibition *per se* but instead by excitatory GABAergic transmission.

A recent study from our group investigated the direct contribution of GABAergic inhibition in the regulation of KCC2 and chloride homeostasis in mature neurons (Heubl et al., 2017). In order to isolate the effect of GABAergic inhibition on KCC2 diffusion and membrane stability, we

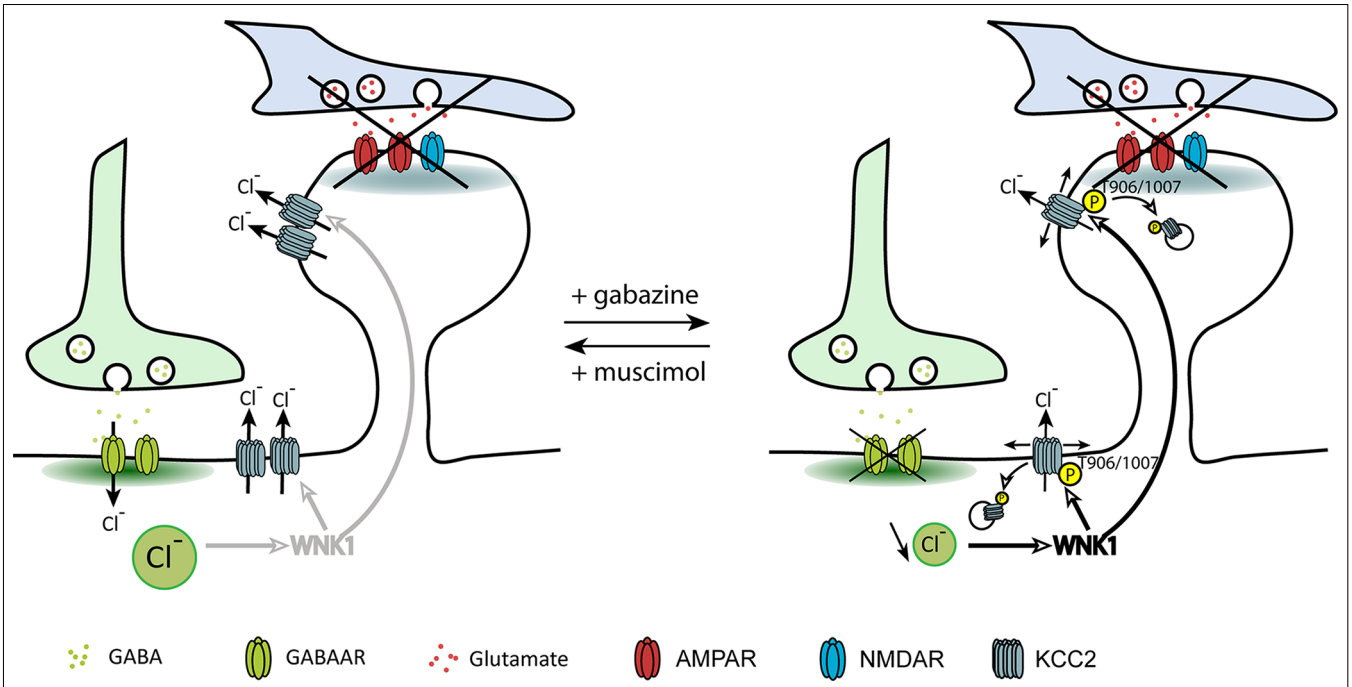

FIGURE 4 | Regulation of KCC2 diffusion and membrane stability by GABAergic inhibition. The impact of inhibitory activity on KCC2 diffusion was explored while blocking neuronal activity and glutamate receptors activity with the Na^+ channel blocker tetrodotoxin, the ionotropic glutamate receptor antagonist kynurenic acid, and the group I/group II mGluR antagonist R, S-MCPG. Chloride influx through $GABA_A$Rs inhibits WNK1, leading to KCC2 dephosphorylation on threonines 906/1007 and membrane stabilization. Upon $GABA_A$R blockade, reduced intracellular chloride activates WNK1 leading to KCC2 phosphorylation on threonines 906/1007 and increased KCC2 diffusion and subsequent internalization. From Heubl et al. (2017).

adjusted neuronal inhibition while blocking action potentials and glutamate receptors. In these conditions, increasing neuronal $GABA_A$R-mediated synaptic inhibition with muscimol enhanced KCC2 diffusion constraints and membrane stability. On the other hand, $GABA_A$R activity blockade with gabazine increased KCC2 diffusion while reducing its membrane clustering and stability (**Figure 4**). Although these observations reflect the influence of bath application of $GABA_A$R agonists and antagonists on KCC2 membrane diffusion and stability, one could imagine that such regulation may also take place locally near GABAergic synapses.

The search for the signaling pathway underlying the $GABA_A$R-dependent regulation of KCC2 demonstrated for the first time that Cl^- acts as a genuine second intracellular messenger to rapidly tune inhibitory synaptic transmission (Heubl et al., 2017). Thus, lowering intracellular Cl^- levels activates the Cl^--sensing WNK1 kinase which in turn phosphorylates and activates the SPAK and OSR1 kinases. Activated SPAK and OSR1 phosphorylate KCC2 T1007, leading to decreased KCC2 activity (Heubl et al., 2017). Conversely, increasing $[Cl^-]_i$ *via* photostimulation of halorhodopsin eNpHR, the light-activated microbial chloride pump, significantly reduced diffusion coefficients and increased the confinement of KCC2 transporters. This mechanism would therefore allow neurons to locally increase or decrease their KCC2 pools to match GABAergic synaptic activity and subsequent need to extrude Cl^- (**Figure 5**). We concluded that GABAergic inhibition in mature neurons tunes itself *via* rapid regulation of KCC2-mediated changes in intracellular Cl^- levels. Since the effect of eNpHR was observed 10 s after light exposure, diffusion-trap of KCC2 appears to be a very rapid mechanism to control Cl^- homeostasis in neurons.

However, the published values of the Cl^- sensitivity of WNK1 measured in an *in vitro* kinase assay (Piala et al.,

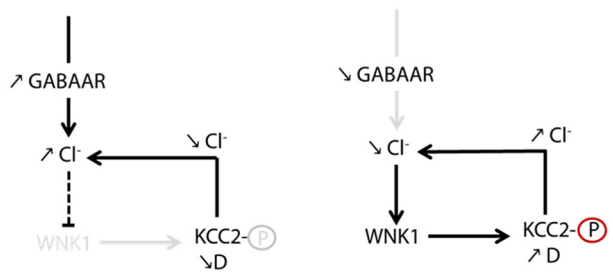

FIGURE 5 | Homeostatic regulation of intracellular chloride *via* WNK1-dependent KCC2 phosphorylation. $GABA_A$R-dependent chloride influx inhibits WNK1 kinase activity and stabilizes KCC2 in the membrane. Increased KCC2-dependent chloride extrusion can therefore counteract efficiently chloride influx due to activation of GABAergic synapses. In conditions of decreased $GABA_A$R activation, a decrease in the intracellular chloride level activates WNK1. Subsequent increase in KCC2 diffusion (D) and loss of KCC2 in the membrane contribute to restore intracellular chloride concentration. From Heubl et al. (2017).

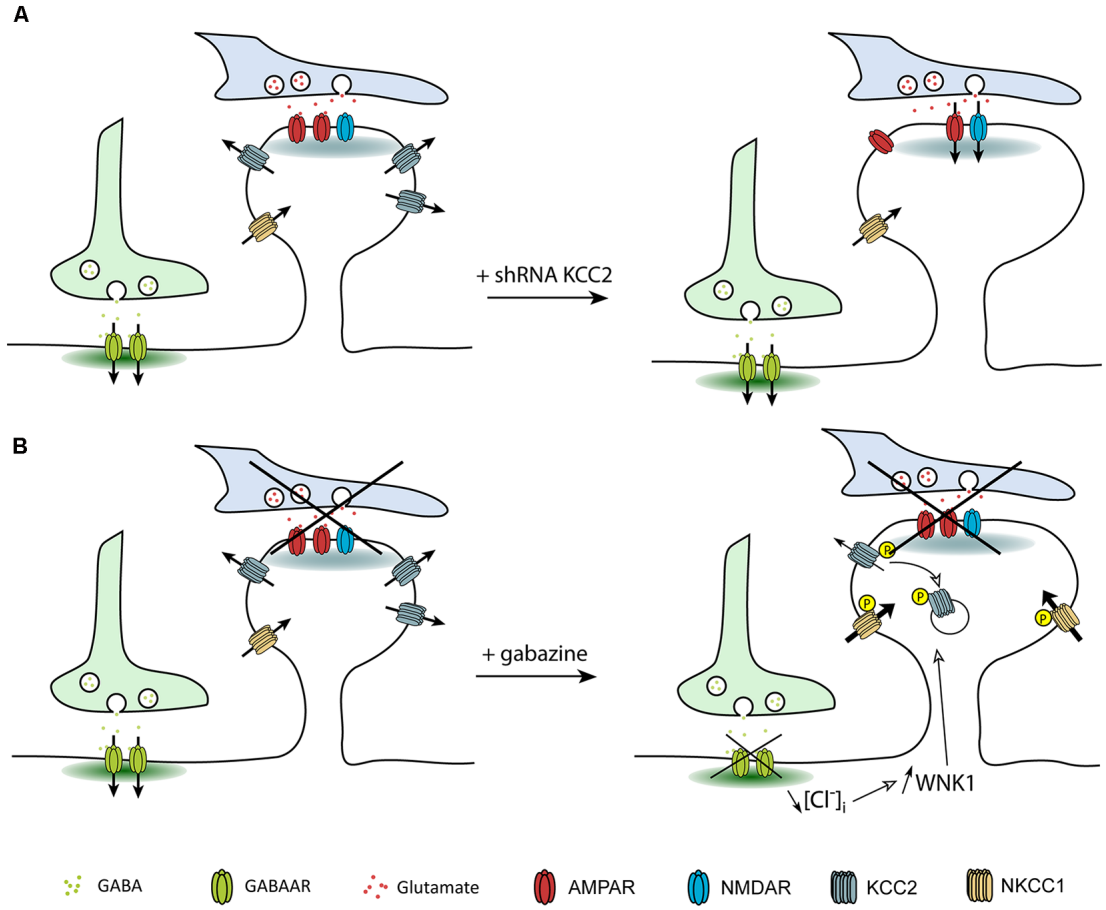

FIGURE 6 | Regulation of dendritic spine head volume upon changes in KCC2 membrane expression. Arrows indicate water fluxes associated with the activity of secondary active transporters and ionotropic receptors. **(A)** Suppression of KCC2 expression leads to increased spine head volume due to the loss of KCC2-associated water extrusion. **(B)** Activation of WNK1 upon $GABA_AR$ blockade leads to KCC2 and NKCC1 phosphorylation and subsequent decrease and increase in the membrane stability of KCC2 and NKCC1, respectively. Increased water influx associated with NKCC1 activity cannot be counteracted by KCC2, leading to spine head swelling.

2014) cannot account for the activation of this signaling pathway upon $GABA_AR$ activity changes in neurons (Heubl et al., 2017). Indeed, intracellular chloride levels in these cells are expected to be in the range of 5–10 mM under control conditions, down to 4–6 mM after $GABA_AR$ blockade and up to 10–15 mM upon $GABA_AR$ activation. The IC50 of chloride was about 20 mM for WNK1 autophosphorylation (Piala et al., 2014) and 100 mM for phosphorylation of its target SPAK in an *in vitro* kinase assay (Terker et al., 2016). If transposable *in situ*, these values would suggest WNK1 would be constantly activated in neurons. Instead, we showed modulation of WNK1 activity even upon subtle intracellular chloride changes (Heubl et al., 2017). The chloride sensitivity of WNK1 in neurons remains to be determined. Additional mechanisms such as membrane translocation (Zagórska et al., 2007) or interaction with other ion-sensitive molecules could modulate the chloride sensitivity of WNK1 upon $GABA_AR$ blockade. Further characterization of WNK1 localization and activity in neurons would therefore provide invaluable insights into how changes in osmolarity and ion concentration can tune its kinase activity.

This signaling pathway may also participate in the onset of pathological conditions. Indeed, a single subcutaneous injection of the $GABA_AR$ antagonist pentylenetetrazole (PTZ) to elicit epileptic seizures *in vivo* increased WNK1, SPAK and OSR1 phosphorylation/activities and promoted KCC2 T906/1007 phosphorylation, which resulted in KCC2 inhibition in neuronal cells (Heubl et al., 2017). Interestingly, PTZ injection also increased NKCC1 T203/T207/T212 phosphorylation. Considering threonine phosphorylation was shown to have opposite effects on KCC2 vs. NKCC1 activity (McCormick and Ellison, 2011), the downregulation of the WNK/SPAK/OSR1 pathway could be a very efficient mechanism to adjust neuronal Cl^- homeostasis in disorders associated with altered inhibition like epilepsy, schizophrenia, autism and neuropathic pain.

Since KCC2 S940 residue is crucial for the regulation of KCC2 transport activity (see above), one may wonder

about the interplay between KCC2 T906/1007 and S940. KCC2 diffusion was increased upon gabazine application even when S940 was mutated to aspartate, indicating that threonine phosphorylation can destabilize KCC2 in the membrane independently of its S940 phospho-status (Heubl et al., 2017). On the other hand, NMDAR-dependent S940 dephosphorylation in neurons in which KCC2 T906/1007 were mutated into alanine also destabilizes KCC2 independently of the threonine phosphorylation status. Hence, the kinase pathways involved in KCC2 regulation by GABAergic inhibition and neuronal excitation appear to be largely independent.

Impact at Glutamatergic Synapses

Several studies showed that, in addition to its role in setting $[Cl^-]_i$ in mature neurons, KCC2 also influences spine head volume as well as the efficacy of glutamatergic neurotransmission (e.g., Gauvain et al., 2011; Chevy et al., 2015). KCC2-mediated spine head volume regulation may rely on water fluxes associated with ion transport, as demonstrated for KCC- (Zeuthen, 1991a,b) and NKCC-mediated transport in epithelial cells (Hamann et al., 2005). Instead, the effect of KCC2 on glutamatergic transmission was shown to depend on its interaction with actin-related proteins (Gauvain et al., 2011). Thirty minutes of $GABA_AR$ blockade with gabazine also induced dendritic spine swelling while $GABA_AR$ activation with muscimol had no effect on dendritic spines (Heubl et al., 2017). The effect of gabazine is reminiscent of what was observed upon chronic KCC2 knockdown or pharmacological blockade. Spine swelling upon KCC2 blockade may thus result from ion and water influx associated with ionotropic receptor activation. Under basal activity, the number of opened receptors is low in hippocampal cultures, with spontaneous EPSP frequency ranging 20–50 Hz. However, gabazine-induced spine swelling occurred on a much faster time scale than with ionotropic glutamate receptors blockade (Gauvain et al., 2011; Heubl et al., 2017). Another mechanism could therefore be at play in the gabazine effect. WNK kinases regulate KCC2 and NKCC1 in opposite directions. Activation of WNK1 in conditions of reduced neuronal inhibition possibly leads to KCC2 removal from the plasma membrane while in the meantime NKCC1 is stabilized at the membrane (Vitari et al., 2006; McCormick and Ellison, 2011). Therefore, spine head swelling observed upon gabazine application may primarily involve increased NKCC1 membrane stabilization and water influx (Zeuthen and Macaulay, 2012). NKCC1 being very efficient in mediating water influx, this might explain the rapidity of the gabazine effect on dendritic spines as compared to that observed upon KCC2 blockade only (**Figure 6**). It would be interesting to directly test NKCC1 involvement in spine swelling upon gabazine application, for instance using the NKCC1 blocker bumetanide.

KCC2 knockdown leads to actin reorganization in spine heads (Chevy et al., 2015). Thus, reduced KCC2 content at the plasma membrane potentially weakens the molecular barrier formed by KCC2 in dendritic spines. This could in turn increase AMPAR escape from spines and reduce the efficacy of glutamatergic synapses as shown upon KCC2 knockdown (Gauvain et al., 2011). Therefore, we predict that KCC2 membrane removal upon reduced $GABA_AR$ activity may act to homeostatically adjust GABAergic and glutamatergic synaptic transmission.

Implication in Energy Loss

The regulation of KCC2 may not only permit a rapid reaction to changes in $[Cl^-]_i$ but also preserve energy consumption. Thus, the loss of KCC2 in conditions of decreased $GABA_AR$-dependent Cl^- influx would maintain membrane KCC2 at minimal levels required to keep E_{GABA} hyperpolarized. Indeed, for every Cl^- ion extruded by KCC2, the transporter uses the energy of the electrochemical gradient of one potassium ion. The Na^+/K^+ ATPase that generates the potassium transmembrane gradient is the main energy consumer in the brain (Buzsáki et al., 2007; Harris et al., 2012; de Lores Arnaiz and Ordieres, 2014). Even though the highest energetic cost of the Na^+/K^+-ATPase is used to restore transmembrane potential upon action potential discharge (Harris et al., 2012; Howarth et al., 2012), maintaining low $[Cl^-]_i$ is associated with high metabolic cost (Kaila et al., 2014). Kaila et al. (2014) raising the hypothesis that "the downregulation of KCC2 following neuronal trauma may be part of a general adaptive cellular response that facilitates neuronal survival by reducing the energetic costs that are needed to preserve low $[Cl^-]_i$". Under physiological conditions, rapid redistribution of KCC2 in the membrane could allow neurons to save energy by keeping surface KCC2 molecules at the minimum required density.

In conclusion, normal and pathological excitatory and inhibitory activities rapidly tune KCC2 function at both inhibitory and excitatory synapses. This regulation occurs through phosphorylation-induced changes in KCC2 membrane diffusion, clustering, endocytosis, recycling or degradation. Although alterations in excitatory and inhibitory signaling might have similar effects on KCC2 cellular trafficking and function, the underlying molecular mechanisms are distinct and involve Ca^{2+} vs. Cl^- signaling cascades and key serine and threonine KCC2 residues. The recent discovery of activity-dependent regulation of KCC2 by the Cl^--dependent WNK/SPAK/OSR1 signaling pathway is of particular interest in the pathology since it controls simultaneously KCC2 and NKCC1 in opposite directions. Further investigation will tell whether targeting this signaling pathway efficiently restores chloride homeostasis and synaptic inhibition in epilepsy, neuropathic pain and various neuropsychiatric disorders.

AUTHOR CONTRIBUTIONS

SL supervised the writing of the review and corrected the manuscript. JP gave advice. EC and MH participated equally in the writing of the review and ES wrote sub-sections of the manuscript.

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frontiers in Cellular Neuroscience

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Postsynaptic Stability and Variability Described by a Stochastic Model of Endosomal Trafficking

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Neurons undergo dynamic processes of constitutive AMPA-type glutamate receptor (AMPAR) trafficking, such as the insertion and internalization of AMPARs by exocytosis and endocytosis, while stably maintaining synaptic efficacy. Studies using advanced imaging techniques have suggested that the frequency of these constitutive trafficking processes, as well as the number of AMPARs that are involved in a particular event highly fluctuate. In addition, mechanisms that trigger some forms of synaptic plasticity have been shown to include not only these processes but also additional fluctuating processes, such as the sorting of AMPARs to late endosomes (LEs). Thus, the regulation of postsynaptic AMPARs by the endosomal trafficking system appears to have superficially conflicting properties between the stability or organized control of plasticity and highly fluctuating or stochastic processes. However, it is not clear how the endosomal trafficking system reconciles and utilizes such conflicting properties. Although deterministic models have been effective to describe the stable maintenance of synaptic AMPAR numbers by constitutive recycling, as well as the involvement of endosomal trafficking in synaptic plasticity, they do not take stochasticity into account. In this study, we introduced the stochasticity into the model of each crucial machinery of the endosomal trafficking system. The specific questions we solved by our improved model are whether stability is accomplished even with a combination of fluctuating processes, and how overall variability occurs while controlling long-term synaptic depression (LTD). Our new stochastic model indeed demonstrated the stable regulation of postsynaptic AMPAR numbers at the basal state and during LTD maintenance, despite fast fluctuations in AMPAR numbers as well as high variability in the time course and amounts of LTD. In addition, our analysis suggested that the high variability arising from this stochasticity is beneficial for reproducing the relatively constant timing of LE sorting for LTD. We therefore propose that the coexistence of stability and stochasticity in the endosomal trafficking system is suitable for stable synaptic transmission and the reliable induction of synaptic plasticity, with variable properties that have been observed experimentally.

Keywords: endosome, stochastic model, postsynapse, long-term plasticity, endosomal sorting

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INTRODUCTION

To stably maintain synaptic transmission, stable regulation of the number of postsynaptic receptors is crucial. However, postsynaptic receptors are not static even under basal conditions, but are rather dynamic (Bredt and Nicoll, 2003; Choquet and Triller, 2003; Lau and Zukin, 2007; Luscher et al., 2011; Lu and Roche, 2012). In particular, the dynamics of AMPA-type glutamate receptors (AMPARs) at excitatory synapses have been well studied. Postsynaptic AMPARs constantly move by lateral diffusion along the plasma membrane (Choquet and Triller, 2003). In addition, postsynaptic AMPARs are internalized by endocytosis and are inserted back into the plasma membrane by exocytosis. Therefore, dynamic degrees of freedom in postsynaptic AMPAR regulation arise from several trafficking processes that AMPARs undergo in the cytosol. In neurons as well as other cells, intracellular trafficking of receptors is mediated by intracellular membrane-bound compartments, namely, endosomes, so that the regulation of the endosomal trafficking pathway at least in part determines receptor trafficking processes, such as recycling, degradation, and the supply of receptors (Bacaj et al., 2015; Bredt and Nicoll, 2003; Brown et al., 2005, 2007; Ehlers, 2000; Fernández-Monreal et al., 2012; Gerges et al., 2004; Lu and Roche, 2012; Matsuda et al., 2013; Petrini et al., 2009). Such regulation occurs constantly to maintain a basal level of postsynaptic AMPARs, and is altered by input stimuli that trigger postsynaptically expressed synaptic plasticity.

Two crucial questions in postsynaptically expressed long-term synaptic plasticity are how cellular components are orchestrated to change the number of postsynaptic AMPARs and how this change in AMPAR number is maintained. Previously reported models of cerebellar long-term depression (LTD), which assume an imbalance between endocytosis and exocytosis by a positive feedback molecular switch (Tanaka and Augustine, 2008; Ogasawara and Kawato, 2009b) can answer the former question. However, these models cannot answer the latter question, because this molecular switch is experimentally suggested to be turned off or lose its effect with time, and hence the imbalance would not last as long as the plasticity is maintained (Kim and Tanaka-Yamamoto, 2013). Thus, to answer this latter question, an extended model is required, which includes another regulatory pathway that comes into the picture after the positive feedback switch loses its effect.

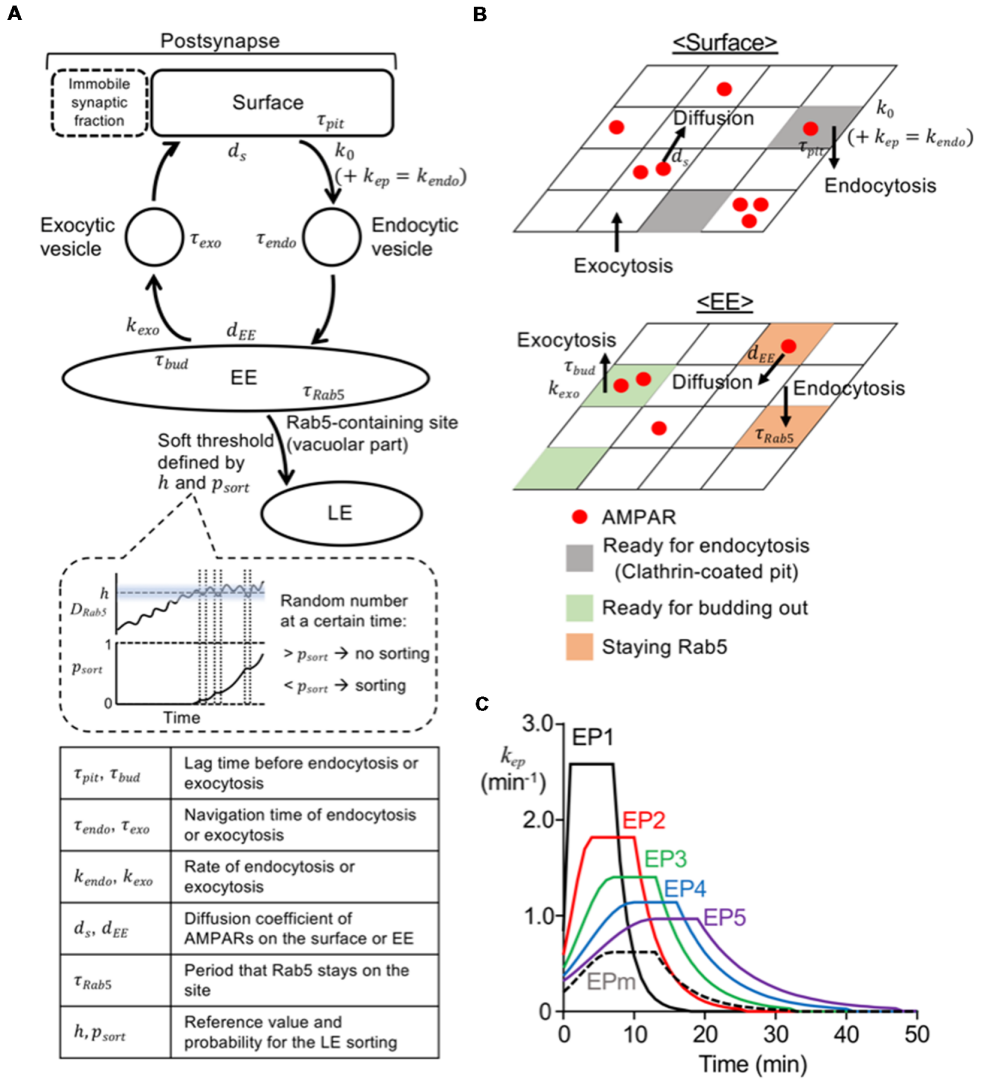
On the other hand, previous studies indicated that endosomal trafficking in the postsynaptic cytosol is necessary for long-term plasticity (Ehlers, 2000; Gerges et al., 2004; Brown et al., 2005, 2007; Petrini et al., 2009; Fernández-Monreal et al., 2012; Matsuda et al., 2013; Bacaj et al., 2015). In particular, our recent work on cerebellar LTD demonstrated that another switch working after the positive feedback molecular switch loses its effect, is likely to be a member of the endosomal trafficking pathway (Kim et al., 2017). The stimulation triggering LTD at synapses between parallel fibers (PFs) and Purkinje cells (PCs) activates a positive feedback loop of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK; Tanaka and Augustine, 2008). This loop enhances the internalization of AMPARs by endocytosis, which results in an imbalance between endocytosis and exocytosis as mentioned above, and in LTD expression. However, the activity of this loop is not required to maintain LTD in the long term. In our previous study, we optogenetically interfered with endosomal trafficking of cargo from early endosome (EE) to late endosome (LE) at different time points, and observed that LTD was impaired when the LE sorting was blocked at 8–23 min after LTD induction. The deterministic model mimicking characteristics of the Rab5-Rab7 conversion switch, which is an essential process for sorting from EE to LE (Rink et al., 2005; Poteryaev et al., 2010), successfully described long-lasting LTD under the short-lasting imbalance between endocytosis and exocytosis due to the diminished effect of the PKC-MAPK positive-feedback loop. In addition, we analyzed individual examples of experimental results and found two distinct responses to the optogenetic interference of LE sorting at the same time points, suggesting different timing of sorting in individual examples. Our results demonstrated that the timing of sorting was partially determined by the speed of LTD expression, and our deterministic model further predicted that another parameter determining the timing is the variable threshold of the Rab5-Rab7 conversion switch. However, experimental observation of endosomal trafficking suggests the existence of other candidates that may be involved in creating the variability in timing of sorting, yet their involvement has not been tested to date.

In this study, we introduced the experimentally known stochasticity of each endosomal trafficking process including the sorting switch from EE to LE, to create a stochastic postsynaptic LTD model. This simplified trafficking model only contains essential dynamic processes but reliably reproduces the time course of LTD with high variability in the timing of sorting AMPARs from EE to LE. Our results from this example system of cerebellar LTD reconfirm the idea that endosomal trafficking is a crucial cellular pathway for long-term plasticity (Ehlers, 2000; Brown et al., 2005, 2007; Fernández-Monreal et al., 2012; Matsuda et al., 2013) and support that the variability in observable parameters arises from the innate stochasticity of each microprocess.

MATERIALS AND METHODS

Model Construction—Compartments

The deterministic model that we previously created (Kim et al., 2017) contained all the essential compartments to describe endosomal trafficking as well as lateral diffusion on the synaptic and extrasynaptic surface. The stochastic model in this present study also utilized the same essential compartments (**Figure 1A**), but the detailed structures of two compartments were modified. First, the extrasynaptic fraction originally considered in the deterministic model was simplified and treated as part of the mobile synaptic fraction (S_m), so that the surface compartment (**Figure 1B**) was basically composed of only the territories of S_m. As was the case in the previous



τ_{pit}, τ_{bud}	Lag time before endocytosis or exocytosis
τ_{endo}, τ_{exo}	Navigation time of endocytosis or exocytosis
k_{endo}, k_{exo}	Rate of endocytosis or exocytosis
d_s, d_{EE}	Diffusion coefficient of AMPARs on the surface or EE
τ_{Rab5}	Period that Rab5 stays on the site
h, p_{sort}	Reference value and probability for the LE sorting

FIGURE 1 | Model construction and various endocytosis profiles. **(A)** Diagram of overall structure of current model. The parameters (τ, k, d, h, p_{sort}) are explained at the bottom, and values of these parameters used in the model are shown in **Table 1**. The panel enclosed with a dashed line in the middle shows an illustration presenting an increase in p_{sort} depending on the duration of D_{Rab5} above the h. The sorting occurred, when a random number was below p_{sort} at a certain time. **(B)** Diagram of surface (top) and early endosome (EE; bottom) compartment. Red filled circles represent AMPA-type glutamate receptors (AMPARs) and they could diffuse laterally on surface or EE. Gray, green, and orange parts are the sites ready for endocytosis from the surface, the site ready for exocytosis from the EE, and Rab5 positive EE sites, respectively. **(C)** Time course of the rate for stimulus-dependent endocytosis applied in the simulation. Long-term synaptic depression (LTD) inducing stimuli were represented by endocytic profiles, EP1–5 (solid lines; EP1—black, EP2—red, EP3—green, EP4—blue, EP5—purple), and mild stimulus was described by EPm (black dashed line).

model, the sum of S_m and the fixed immobile synaptic fraction (S_{im}) represents the number of postsynaptic AMPARs. The surface compartment was assumed to be a square lattice made of 50 × 50 homogeneous sites where AMPARs can freely diffuse (**Figure 1B**). Second, the EE was also simplified as a square lattice consisting of the same number of sites as the surface compartment (**Figure 1B**). Two subcompartments of the EE, i.e., one for recycling and one for being sorted to LE (vacuolar part), were introduced. Each EE site could contain AMPARs and a single Rab5 molecule, and the all Rab5-containing sites were assumed to be the vacuolar portion. Each site on the surface or the EE was able to contain an unlimited number of AMPARs, but highly clustered AMPARs in a site were not observed during any of the simulations in this study.

Model Construction—Trafficking Processes of Endocytosis and Recycling

The movement of each AMPAR starting from the surface compartment can be described first by endocytosis and then by either recycling or sorting to LE (**Figure 1A**). For model construction of endocytosis and recycling processes, the surface

TABLE 1 | Parameter values used in the simulation.

Parameters	Values	Note
S_{im}	40	
k_0	1.2 min^{-1}	
$\tau_{pit} \sim U\ (n, m)$	U (1 s, 2 s)	Drawn from uniform distribution of interval *[n, m]*
$\tau_{endo} \sim N\ (\mu, \sigma)$	N (4 min, 1 min)	Drawn from Gaussian random distribution of mean, μ, and standard deviation, σ.
a	0.2	
b	0.02	
C	1.1	
K	20	
d_{EE}	0.05 $\mu m^2 \cdot s^{-1}$	
k_{exo}	1.0 min^{-1}	
$\tau_{bud} \sim U\ (n, m)$	U (1.5 s, 2.5 s)	Drawn from uniform distribution of interval *[n, m]*
d_s	0.05 $\mu m^2 \cdot s^{-1}$	
$\tau_{exo} \sim N\ (\mu, \sigma)$	N (2 min, 0.5 min)	Drawn from Gaussian random distribution of mean, μ, and standard deviation, σ.
$h \sim N\ (\mu, \sigma)$	N (0.4, 0.02)	Drawn from Gaussian random distribution of mean, μ, and standard deviation, σ.
τ_s	0.38 min	

compartment was constantly exposed to endocytosis and exocytosis, and AMPARs on the surface were able to diffuse with the diffusion coefficient d_s. Each lattice site was assumed to randomly form a clathrin-coated pit with a rate of k_0, under no stimulus (Wang and Linden, 2000). To mimic the application of stimuli, the stimulus-representing endocytosis profile (EP, k_{ep} in **Figure 1C**, see "Simulation" section) was added to k_0, which eventually formed the time-dependent k_{endo} (Tao-Cheng et al., 2011). The clathrin-coated pit was endocytosed after a lag time of τ_{pit}. AMPARs at these sites were internalized upon endocytosis of these sites, and they hence existed on the endocytic vesicles. Each endocytic vesicle with or without AMPARs arrived at the EE after a navigation time of τ_{endo}, and immediately fused to a randomly chosen site on the EE, once it arrived. Upon the fusion of vesicles, Rab5 was assumed to be recruited to the site on the EE, and consequently AMPARs on the vesicles were colocalized with Rab5. Whereas Rab5 remained on the fusion site during a period termed τ_{Rab5}, AMPARs diffused on the EE with the diffusion coefficient d_{EE}. Rab5-free EE sites could bud out with a rate of k_{exo}, and then became exocytic vesicles after a period termed τ_{bud}. Exocytic vesicles traveled toward the surface compartment during a period of τ_{exo} and then immediately fused at a random site on the surface compartment. Values of τ_{pit} and τ_{bud} were drawn from a uniform random distribution, and values of τ_{endo} and τ_{exo} were drawn from a Gaussian random distribution.

Model Construction—Rab5 Accumulation and Sorting From EE to LE

As briefly mentioned above, in our model, Rab5-positive EE sites represent the vacuolar portion of the EE. According to the experimental results (Rink et al., 2005; Poteryaev et al., 2010) and modeling study (Del Conte-Zerial et al., 2008), the kinetics of Rab5 accumulation appear to follow the kinetics of autocatalysis, which was introduced by the simplified equation in the previous deterministic model (Kim et al., 2017). In the current model, Rab5 accumulation was simulated by adjusting the lifetime of Rab5 in an EE site (τ_{Rab5}) to be similar to the kinetics of formation of the vacuolar portion in the previous model, using the equation shown below.

$$\tau_{Rab5}\left(D_{Rab5}\right) = \frac{KD_{Rab5}\left(C - D_{Rab5}\right)}{1 + e^{-\frac{D_{Rab5}-a}{b}}}$$

where D_{Rab5} was the fraction of Rab5-positive sites in the EE, representing Rab5 accumulation. The numerator term represents autocatalysis with a limiting factor whereas the denominator term further shaped the rising kinetics. Thus, the coefficients a and b were the shape adjusting parameters, C was the limiting level of accumulation, and K was the scaling parameter. The newly updated τ_{Rab5} was applied to the newly arrived Rab5 but did not affect the already existing Rab5.

In our present model, a soft threshold was assumed for the threshold of sorting from EE to LE. The soft threshold was defined by two parameters, i.e., a reference value (h) and sorting probability (p_{sort}). The h value was drawn from a Gaussian random distribution. The p_{sort} value exponentially increased depending on the total duration of D_{Rab5} above the h (**Figure 1A**), and was described by the following equation:

$$p_{sort}\left(t\right) = \begin{cases} 0, & t \le t_{exc} \\ 1 - min\{e^{-(t - t_{exc} - \tau_{ud})/\tau_s}, 1\}, & t > t_{exc} \end{cases}$$

where t_{exc} was the first moment of D_{Rab5} reaching h, and τ_{ud} was the total period of D_{Rab5} below the h after t_{exc}. By tuning τ_s, the level of softness of the threshold could be adjusted. Sorting from EE to LE occurred when a random number drawn from the uniform distribution on the interval [0,1] was below p_{sort} at a certain time.

Simulation

All simulation procedures were performed by the built script on MATLAB (Mathworks, Natick, MA, USA). The coefficient values used here are shown in **Table 1**. The time step was 0.1 s, and the entire simulation was repeated 100 times. To set the numbers of AMPARs on different compartments, we first assumed that there were 150 AMPARs on the surface and 100 AMPARs on the EE, and ran the trafficking through endocytosis and

recycling until the numbers in both compartments became stable. We then used averaged numbers from 5 min as the initial number of AMPARs on the surface, the EE, and endocytic and exocytic vesicles for the simulation. The external stimulus, which enhanced endocytosis, was represented by several types of EPs (**Figure 1C**). As in the previous deterministic model (Kim et al., 2017), EPs were described by a piecewise-defined concave function, which consists of a Gaussian rising ($0 \leq t < t_{peak} - 3$), a steady value ($t_{peak} - 3 \leq t < t_{peak} + 3$), and an exponential decay($t_{peak} + 3 \leq t$). The peak timing (t_{peak}) of the LTD-inducing stimuli EP1–5 (**Figure 1C**; solid lines) were varied to describe the different speeds of LTD expression, yet integration along the entire stimulation time was tuned to be the same to conserve the magnitude of the stimulus. To describe mild stimuli, EPm (**Figure 1C**; black dashed line) was assumed to have the same peak timing with EP3, but its integration was set to be significantly lower.

RESULTS

Stable Maintenance of Postsynaptic AMPAR Number

We first determined whether the stochastic model we built reproduces the stable maintenance of postsynaptic AMPAR number. Without any perturbing stimulus, the normalized number of postsynaptic AMPARs (N_{syn}) was mostly conserved over time, although there were fluctuations during short time periods (**Figure 2A**), which were confirmed by comparing the average N_{syn} at earlier time points (−10 to 0 min) with that at later time points (40–50 min; **Figure 2B**). To confirm the stability of the model system under a weakly perturbing stimulus, EPm (**Figure 1C**) was applied at $t = 0$, which altered the time course of N_{syn} and led to a decrease from the baseline (−10 to 0 min) for a finite time period (**Figure 2C**). With time, N_{syn} was recovered to the same level as the baseline (**Figures 2C,D**). These results showed that the newly built stochastic trafficking model was able to describe the stable regulation of the number of postsynaptic AMPARs, despite its rapid fluctuation.

Rab5 Accumulation in the EE

As previous experimental and theoretical studies on intracellular trafficking have indicated (Huotari and Helenius, 2011; Vilar and Saiz, 2011), an essential function of EEs, i.e., the sorting from EE to LE, can be described by the Rab5-Rab7 conversion switch. Regarding the mechanism of this switch, the autocatalytic accumulation of Rab5 in the EE is crucial, which was deterministically modeled in a previous model (Kim et al., 2017). We conserved the autocatalytic accumulation of Rab5 with a competitive degradation term (Del Conte-Zerial et al., 2008); however, before investigating AMPAR trafficking during the switch-on, we tried to confirm reliable Rab5 accumulation in the EE using the current stochastic model. For this purpose, we used other endocytosis profiles (EP1–EP5), which are considered to be triggered by stronger stimuli, and Rab5 accumulation was measured by calculating the fraction of Rab5-positive sites in the EE (D_{Rab5}). As shown in **Figure 1C**, EP1–EP5 had different endocytosis speeds, yet had similar magnitudes of stimulation, as seen in the conserved integration along the entire stimulation time. For the early period (5–15 min) after the start of the stimulus-representing endocytosis profile, concentrated endocytosis within a short period (EP1) showed a higher D_{Rab5} (**Figure 3A**). As time went by, the difference in D_{Rab5} between focused endocytosis (EP1) and dispersed endocytosis (EP5) became smaller, as seen during 15–25 min and 25–35 min (**Figures 3B,C**). These results indicated that Rab5 accumulation proportionally followed the time course of the endocytosis profile until a certain saturation limit of the accumulation, as shown in the past experimental results (Rink et al., 2005; Poteryaev et al., 2010). Thus, we confirmed the ability of the current model to regenerate Rab5 accumulation, as expected previously. Needless to say, individual examples (shown by filled circles in **Figure 3** as well as the following figures) of Rab5 accumulation varied due to the properties of the stochastic model.

Proportional Accumulation of Internalized AMPARs With Higher Variability

The variability of the number of AMPARs in endocytic vesicles was observed by electron microscopy of hippocampal neurons (Tao-Cheng et al., 2011). We included a property to the current model, namely, that the number of AMPARs internalized or leaked out is not uniform across each unit vesicle or unit site of the EE, which is a source of innate stochasticity and a crucial difference of the current model from the previous deterministic model (Kim et al., 2017). Another assumption that we included in the current model is that AMPAR localization in the vacuolar portion (the Rab5-positive portion) of the EE was independent of Rab5, and the AMPARs could spontaneously diffuse out, because to our knowledge, Rab5-dependent regulation of AMPAR localization has not been reported to date. An interesting consequence of these newly introduced variabilities in the current model was detected when AMPAR accumulation in the vacuolar portion of the EE was monitored, as has been done for Rab5 accumulation, shown in **Figure 3**. AMPAR accumulation was presented as the number of AMPARs coexisting with Rab5 on the EE that was normalized by basal levels of postsynaptic AMPAR number ($N_{EE\text{-}Rab5}$). Overall, averaged AMPAR accumulation in the EE appeared similar to Rab5 accumulation (**Figures 4A–C**). However, unlike Rab5 accumulation, AMPAR accumulation had a lower response to EP1 than EP2 during the earlier period (5–15 min) of monitoring (**Figure 4A**) and showed quicker accumulation of EP4 and EP5 during 15–25 min (**Figure 4B**), suggesting that the time course of AMPAR accumulation following application of the endocytosis profile was slightly different from Rab5 accumulation. Additionally, a comparison of the coefficients of variation (CVs) demonstrated that AMPAR accumulation had higher variability than Rab5 accumulation (**Figure 4D**). AMPAR accumulation monitoring in our stochastic model indicated that AMPARs mostly followed endosomal trafficking, but the distinct trafficking between AMPARs and vesicles resulted in different variability.

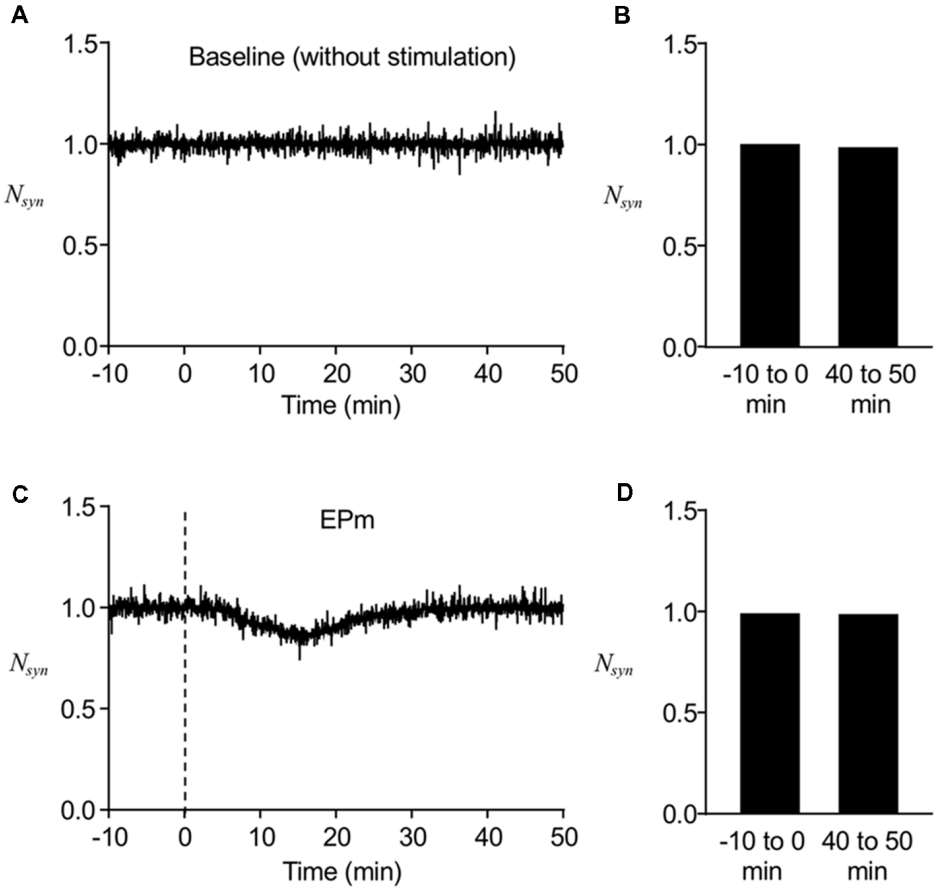


FIGURE 2 | Stable regulation of AMPAR numbers in a postsynapse. **(A)** A representative time course of normalized postsynaptic AMPAR numbers (N_{syn}) without stimulation. **(B)** Averaged N_{syn} comparison between −10 to 0 min and 40–50 min. **(C)** A representative time course of N_{syn} with mild stimulus, EPm. **(D)** Averaged N_{syn} comparison between period before (−10 to 0 min) and 40–50 min after the start of EPm latter of which is the time when EPm is supposed to be phased out. Dashed lines in this and subsequent figures show the time when the stimulation (endocytic profiles) started.

Endocytosis Profile-Independent Occurrence of Sorting to LE With a Soft Threshold

In the deterministic model, the sorting from EE to LE immediately started once the accumulation of Rab5 reached the constant threshold level for sorting (Kim et al., 2017). Although a previous study modeled Rab5-Rab7 conversion as a cut-off switch with a threshold (Del Conte-Zerial et al., 2008), the experimental results implied that the conversion was actually more flexible, which may be a result of other sources of stochasticity. First, molecular interactions or reactions intrinsically contain stochasticity, which probably caused the noisy accumulation of Rab5 in the experiments. Second, the experimental results showed that it was very difficult to predict the timing of conversion, even after Rab5 accumulation appeared to be reaching saturation levels (Rink et al., 2005; Poteryaev et al., 2010). Thus, we introduced a soft threshold in the current model, which increased the probability of the sorting depending on the time of Rab5 accumulation exceeding the reference value that slightly varied around its mean value. Under conditions of this soft threshold, we observed that all endocytosis profiles had a minimum of 64% occurrence of sorting from EE to LE among the repetitions (100 times), as indicated in **Figure 5**. This is not a very high success rate of sorting, but considering that this model was built with high variability for a small scale (a single synapse), it appears to be sufficient to lead to multisynaptic LTD, which can usually be observed experimentally (Wang et al., 2000). An interesting part of the results was similar success rates in sorting occurrence for all endocytosis profiles (**Figure 5**), which might be due to the combined effects of a soft threshold with Rab5 accumulation properties, eventually reaching saturation levels, even by the dispersed endocytosis profile, as indicated in **Figure 3**.

Variable Levels of LTD Maintenance Starting at Various Times

As the current model successfully worked for both stable regulation of the number of postsynaptic AMPARs and sorting from EE to LE, we next tested whether this may also describe LTD maintenance with the involvement of sorting from EE to LE (**Figure 6A**). For this purpose, we only used the successful sorting

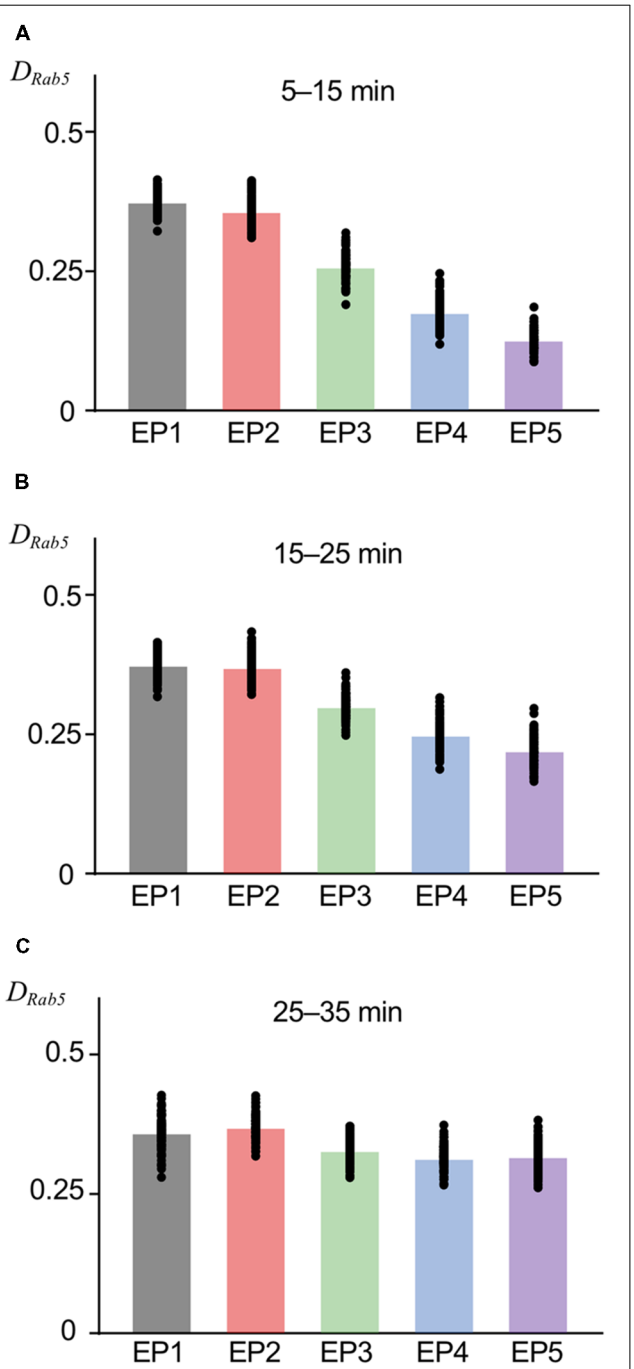


FIGURE 3 | Rab5 accumulation in EE at various timing. The fraction of Rab5 positive sites in EE (D_{Rab5}) at 5–15 min **(A)**, 15–25 min **(B)**, and 25–35 min **(C)**. Black filled circles show the value from the individual simulation, and bar graphs show averaged values, in this figure as well as subsequent figures showing colored bar graph with black filled circles.

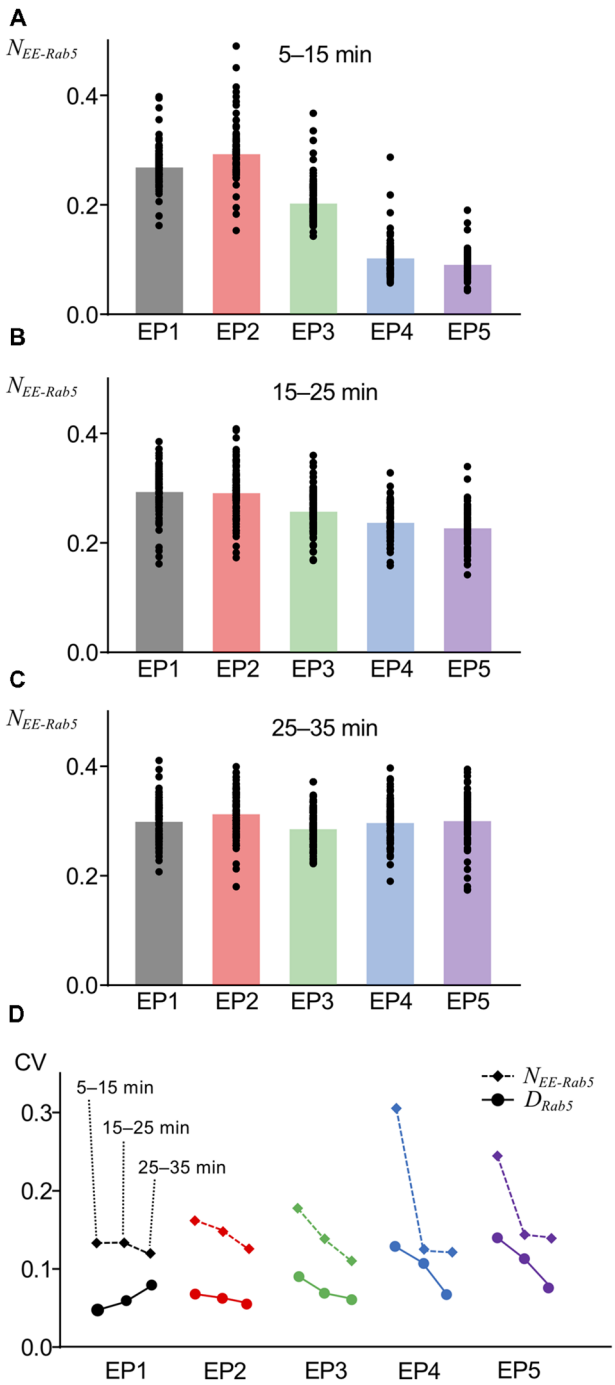


FIGURE 4 | AMPAR accumulation at various timing. **(A–C)** The fraction of AMPARs coexisting with Rab5 in EE ($N_{EE\text{-}Rab5}$) at 5–15 min **(A)**, 15–25 min **(B)**, and 25–35 min **(C)**. **(D)** Comparison of coefficients of variations (CVs) between D_{Rab5} (filled circles, solid lines) and $N_{EE\text{-}Rab5}$ (filled diamonds, dashed lines). Three points from left to right show the results at 5–15 min, 15–25 min, and 25–35 min.

examples mentioned above, because LE sorting is required for LTD maintenance (Kim et al., 2017), and first calculated the averaged N_{syn} of these examples during 40–50 min, which indicates the maintenance level of LTD. Subtraction of the maintenance level of LTD from the basal level of N_{syn} was defined to be the amount of LTD. **Figure 6A** shows that a more focused endocytosis profile resulted in higher depression levels than dispersed profiles. This result appears to be highly relevant to the

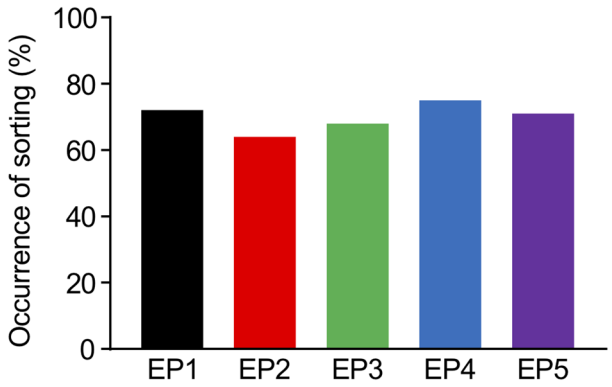


FIGURE 5 | The percentages of successful occurrence of sorting from EE to LE upon the different endocytosis profiles.

results of AMPAR accumulation shown in **Figure 4**. Despite the dependency of the amount of LTD on the types of endocytosis profiles, the amounts of LTD were within the range of 20%–35% on average for all endocytosis profiles, which appears to be a reasonable range, as shown in previous experimental studies (Hansel et al., 2001; Tanaka and Augustine, 2008). This suggested that we can expect LTD to be maintained, as long as EE to LE sorting occurs. In addition, the amount of LTD also varied in individual examples even when the same endocytosis profile was applied (individual data plot in **Figure 6A**). Examples of time courses of N_{syn} by EP1 (**Figure 6B**) and EP3 (**Figure 6C**) demonstrate that different amounts of LTD can be made by similar types of stimulation, whereas similar LTD amounts can be made by different types of stimulation.

In our previous study, we found that the timing of Rab5-Rab7 conversion, namely, the timing of LE sorting for LTD was varied in individual examples, and that such varied timing partially correlated with the speed of LTD expression (Kim et al., 2017). In addition, the deterministic model predicted that varying thresholds of LE sorting may be another factor of the variability in timing of LE sorting. The currently used model includes stochastic properties in endosomal and AMPAR trafficking, which appears to be reasonable based on previous experimental observations, whereas the speed of LTD expression was directly represented as the stimulus-representing endocytosis profiles EP1–EP5. The results of our new stochastic model showed that the timing of LE sorting in individual examples was highly variable regardless of the type of endocytosis profiles, yet the averaged timing of LE sorting correlated with the types of endocytosis profiles (**Figure 7A**). These results indicate that the current model reproduced the two characteristic properties of the timing of LE sorting during LTD, i.e., not only variability, but also partial correlation with the speed of LTD expression. These characteristic properties were observed in the LTD samples (**Figures 7B,C**). As the new stochastic model was able to reproduce our previous experimental results, the origin of the variability can be considered to be stochasticity in the trafficking process, including the number of AMPARs in individual vesicles or in portions of the EE, as well as threshold for LE sorting.

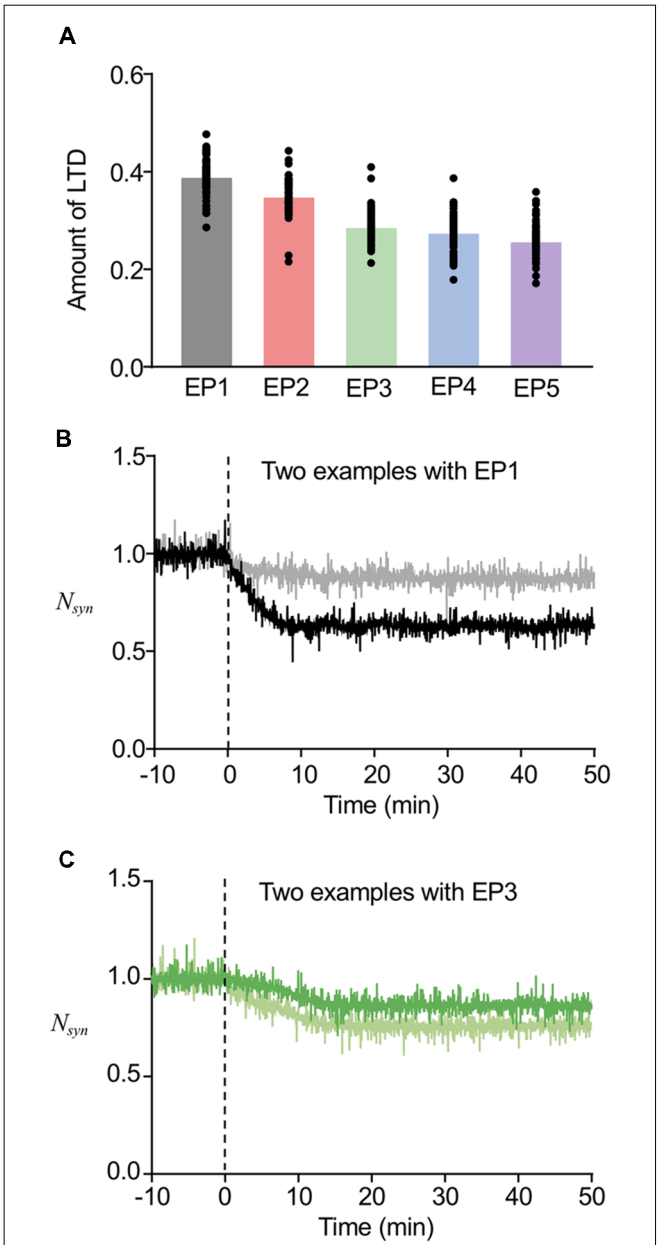


FIGURE 6 | Variability in the amount of LTD. **(A)** The amount of LTD upon the different endocytosis profiles. **(B,C)** Time course of changes in N_{syn} upon the EP1 **(B)** and EP3 **(C)**. Two example traces in the same panel showed different amount of LTD, while they showed similar timing of sorting from EE to late endosome (LE). For **(B)**, the sorting occurred at around 6 min, and for **(C)**, the sorting occurred at around 14 min.

Comparisons of Model Results With Experimental Results

To further confirm the reproducibility of the experimental results by the current stochastic model, we directly compared the results from the model with the experimental results of LTD from our previous study. In the comparison, we also added the results obtained from our previous deterministic model. As expected from the average amount of LTD (**Figure 6A**)

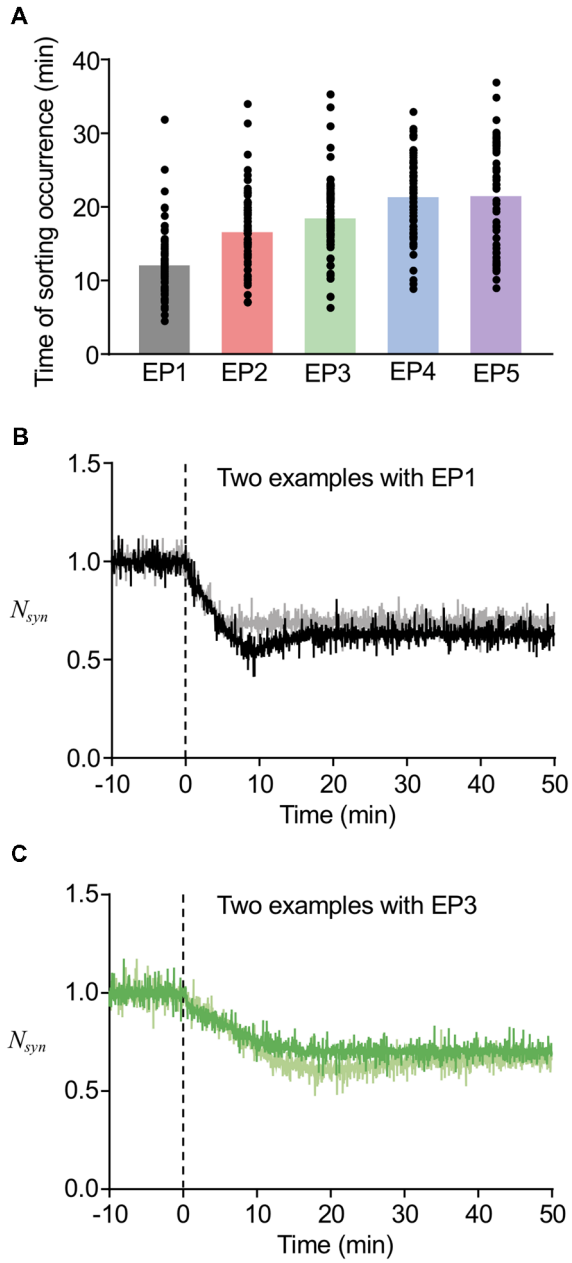


FIGURE 7 | Variability in the timing of sorting occurrence. **(A)** The timing of sorting occurrence upon the different endocytosis profiles. **(B,C)** Time course of changes in N_{syn} upon the EP1 **(B)** and EP3 **(C)**. Two example traces in the same panel showed similar amount of LTD, while they showed different timing of sorting from EE to LE. In **(B)**, the sorting occurred at 18 min for black line and at 5 min for gray line, and in **(C)** the sorting occurred at 16 min for green line and at 29 min for light green line.

and the time of sorting occurrence (**Figure 7A**), the averaged time course of LTD elicited by EP1–EP5 in the present model showed different kinetics of LTD expression and a maximum level of LTD maintenance, yet the overall time course was similar to the experimental results (**Figure 8A**). Our previous deterministic model also produced similar time course of LTD (**Figure 8A**). We also calculated CVs of the amount of LTD at several time points, to quantify the variability. As expected, the CVs in the deterministic model were 0, unless we manually modified the parameters. In contrast, the CVs in the current stochastic model were approximately 0.1 at 5–15 min, similar to the experimental results (0.111, **Figure 8B**). The CVs at other time points were also equivalent to the experimental results (**Figure 8B**). Thus, whereas the deterministic model reproduced the overall time course of LTD, but not individual variability in LTD, the current stochastic model was able to reproduce both the overall time course and variability.

Even though our experimental results demonstrated that the varied timing of LE sorting partially correlated with the speed of LTD expression, the results further led to the conclusion that LE sorting occurred mostly at the intermediate time period (13–18 min), because optogenetic disturbance of LE sorting at this time prevented LTD in 75% of the cells recorded (Kim et al., 2017). To test whether the current stochastic model could reproduce this property, we plotted a histogram of sorting occurrence within the three different time periods, i.e., the early (6–13 min), intermediate (13–20 min), and late (20–27 min) time periods (**Figure 8C**, open bars), using the results showing LE sorting in response to EP1–EP5. Similar to our previous experimental results mentioned above Kim et al. (2017), sorting occurrence was high at the intermediate times. On the other hand, we manually generated a less variable data set with the same average values, and the histogram of the generated data showed an unbiased distribution of occurrence across the three time periods (**Figure 8C**, hatched bars). Thus, high variability resulting from the introduced stochasticity led to an increase in the probability of sorting occurrence at the intermediate time period, when any type of endocytosis profiles triggering LE sorting can be applied. These results imply that the stochasticity in the system helps to produce the experimental results exhibiting a relatively constant time course of LTD maintenance, despite the varied speed of LTD expression. Based on the results of this analysis, we propose that stochasticity may be linked to reliability, even though the high variabilities observed in the stochastic model would superficially give the impression that stochasticity severely harms the reliability of the system.

DISCUSSION

The number of postsynaptic AMPARs is stably regulated by constitutively dynamic trafficking processes. Additionally, when a postsynapse goes through a major change, such as long-term plasticity by strong stimuli, it is still able to reliably control the change of postsynaptic AMPAR numbers even though there is high variability. In this study, we extended the previously constructed cerebellar PF-PC LTD model, which included intracellular endosomal trafficking, particularly sorting from EE to LE, and built a new model including stochasticity in the trafficking process. As a result, we were able to reproduce the stable maintenance of postsynaptic AMPAR numbers both before and after LTD induction, and the variability observed in

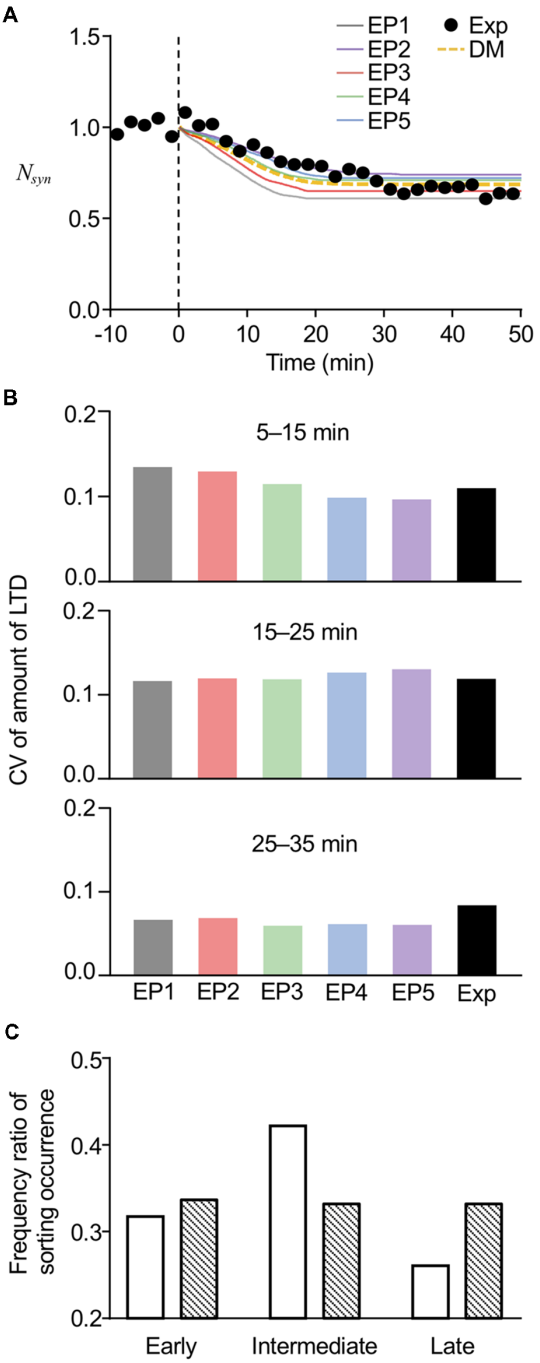


FIGURE 8 | Comparisons of model results with experimental results. **(A)** Averaged time course of LTD produced by the current stochastic model (solid lines) or previous deterministic model (DM, yellow dotted line), superimposed on time course of experimentally recorded LTD (Exp, black circles). **(B)** Comparison of CVs of LTD amounts at different periods between stochastic model results and experimental results (Exp). Results of experiments were modified from data used in the previous study (Kim et al., 2017). **(C)** Comparison of frequency ratio of sorting occurrence between model results (open bars) and less variable data set (hatched bars) at early (6–13 min), intermediate (13–20 min), and late (20–27 min) periods. The less variable data set were manually generated in a way that data set had low variability yet had the same average values.

previous studies, such as the amount of LTD (Tanaka et al., 2007) and timing of sorting from EE to LE (Kim et al., 2017).

Several studies have demonstrated the involvement of endosomal trafficking in the postsynaptic regulation of AMPAR number (Gerges et al., 2004; Brown et al., 2005, 2007; Fernández-Monreal et al., 2012; Matsuda et al., 2013; Bacaj et al., 2015), and endosomal trafficking has been included in qualitative working models (Shepherd and Huganir, 2007; Langemann et al., 2008; Anggono and Huganir, 2012; Lu and Roche, 2012; Colgan and Yasuda, 2014). However, computational modeling approaches have treated endosomes as a passive component that linearly accepts and releases transported AMPARs (Earnshaw and Bressloff, 2008; Bressloff and Earnshaw, 2009; Manninen et al., 2010; Antunes and De Schutter, 2012; Czöndör et al., 2012; Gallimore et al., 2016). This idea is able to explain the relatively short time scale of synaptic plasticity and postsynaptic responses to a mild stimulus that basically enhances the recycling of AMPARs. In principle, the passive component has also been powerful to describe long-term synaptic plasticity under the assumption that the plasticity is maintained by a long-term imbalance between AMPAR internalization and reinsertion (Kuroda et al., 2001; Ogasawara and Kawato, 2009b). In reality, however, it has been shown that in cerebellar LTD, the positive feedback molecular switch leading to an imbalance is no longer required for the maintenance of LTD after a certain time (Ogasawara and Kawato, 2009a; Kim and Tanaka-Yamamoto, 2013). In our previous study, based on the experimental results showing that LE sorting is crucial for the initiation of the maintenance of LTD, we built the first model to our knowledge of postsynaptic LTD composed of AMPAR trafficking, including a nonlinearly responding endosomal component (Kim et al., 2017), namely, the Rab5-Rab7 conversion switch that controls sorting from EE to LE. This deterministic model was able to predict the source of variability, by running the simulation with varied parameter values. In our present study, we simply introduced innate stochasticity into the previous model, and were able to reproduce the high variability without affecting the trends that we observed previously. Considering that these two models are able to explain several features of cerebellar LTD, the involvement of endosomal trafficking in the regulation of postsynaptic AMPAR number should no longer be considered as a passive process, but rather needs to be included as an active controller with a stochastic nature.

As a previous study on the molecular mechanism of the Rab5-Rab7 conversion switch described (Del Conte-Zerial et al., 2008), the intracellular regulation of AMPAR number by the sorting from EE to LE appears to work as a leaky integrator that filters out high frequency noise. Comparing the PKC-MAPK positive-feedback loop switch, which integrates calcium ion flux (Kuroda et al., 2001; Tanaka et al., 2007; Tanaka and Augustine, 2008; Ogasawara and Kawato, 2009b), endosomal sorting has more complexity and integrates endocytosis more slowly. Thus, it is reasonable that the endosomal sorting switch works at a later time than the positive feedback loop switch. The difference in their functioning time scales implies that the endosomal sorting switch may filter out the fluctuation or small changes in AMPAR internalization by endocytosis, while

initiating LTD maintenance. In other words, leaky integrator properties of the endosomal sorting switch enable reliable progression of LTD. In our present model, we introduced experimentally suggested stochasticity, to explain the variabilities of LTD. The important differences of the current stochastic model from the previous deterministic model are summarized as: (i) variable numbers of AMPARs in individual units of vesicles or membrane portions in the EE; (ii) diffusing out of AMPARs from the Rab5-accumulated fraction; and (iii) the soft threshold of the endosomal sorting switch. In our present model, AMPAR displacement was separated from vesicular dynamics, because of (i), and AMPAR accumulation was also separated from Rab5 accumulation because of (ii). These separations of AMPAR dynamics from typical vesicular dynamics generated the high frequency fluctuation even when there was no external stimulus. In general, a leaky integrator system accumulates inputs, yet gradually leaks small amounts of input over time. In the case of the Rab5-Rab7 conversion switch, the input is endocytosis vesicles and the leak is spontaneous diffusion of AMPARs on the EE. Because of the separation of AMPAR dynamics from vesicular dynamics in our present stochastic model, the Rab5-Rab7 conversion switch for AMPAR sorting to LE can be considered as a leaky integrator with a high amount of noise in both input of AMPAR internalization and leak of diffusing out of AMPARs. Combining the noisy leaky integrator with the soft threshold mentioned in (iii) eventually produces variable responses.

The current study demonstrated that including stochasticity in the model could clearly explain the experimentally observed variabilities, suggesting that the stochastic processes are involved in the regulation of postsynaptic AMPARs through the endosomal trafficking system. This raises the question regarding the biological advantages of the stochastic processes in AMPAR regulation. A previous theoretical study showed that synaptic efficacy fluctuations due to the stochastic exchange of AMPARs between the intracellular pool and postsynaptic receptor slots are stronger in small synapses (Triesch et al., 2018). Therefore, investigating the effects of stochastic fluctuations on LTD in synapses of different sizes is an important topic for future research.

In addition, the variability arising from the stochasticity appeared to also be beneficial for producing constant time course of LTD. As shown in **Figure 8C**, highly variable responses to the same stimulus eventually increased the probability of sorting within the intermediate time period, when any type of endocytosis profile triggering LE sorting could be applied. This implies that once the conditions, such as the stimulus profile and the threshold of sorting, fulfilled the requirements for successful initiation of sorting occurrence, stochasticity compensates for the variability of the stimulus profiles and reduces the variation in the timing of sorting. This phenomenon reminds us of the consequences of stochastic focusing (Paulsson et al., 2000), which indicates the beneficial effects of noise in the maintenance of LTD. Less variance in the timing of sorting also suggested the possible synchronized timing of sorting in multiple EEs. In our previous study (Kim et al., 2017), we observed two distinct responses by optogenetic disturbance of LE sorting; recovery or LTD. Considering that PF stimulation for cerebellar LTD induction is usually applied to multiple synapses due to technical difficulties in accurately stimulating a single PF, multiple EEs may be involved and some synapses may even share one EE. The two distinct responses, but not gradual and partial recovery, indicate that the sorting times from all EEs involved fall within a certain range. Even though our present model based on single synapses led to 64% sorting occurrence and consequent LTD maintenance, this synchrony in the timing of sorting of multiple EEs may result in the reliable occurrence of multisynapse LTD. This hypothesis can be evaluated by experimental studies on endosome distribution in postsynaptic areas and on LTD in single synapses, and further by a more realistic endosomal trafficking model (Vagne and Sens, 2018) of multiple synapses based on experimental observations.

DATA AVAILABILITY

The datasets for this manuscript are available from the corresponding authors upon reasonable request. The model script is available at https://sites.google.com/view/closeyes.

AUTHOR CONTRIBUTIONS

TK built the model and performed simulation. Both authors designed the model and wrote the article.

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frontiers in Cellular Neuroscience

REVIEW
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Alterations in $GABA_A$-Receptor Trafficking and Synaptic Dysfunction in Brain Disorders

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$GABA_A$ receptors ($GABA_AR$) are the major players in fast inhibitory neurotransmission in the central nervous system (CNS). Regulation of $GABA_AR$ trafficking and the control of their surface expression play important roles in the modulation of the strength of synaptic inhibition. Different pieces of evidence show that alterations in the surface distribution of $GABA_AR$ and dysregulation of their turnover impair the activity of inhibitory synapses. A diminished efficacy of inhibitory neurotransmission affects the excitatory/inhibitory balance and is a common feature of various disorders of the CNS characterized by an increased excitability of neuronal networks. The synaptic pool of $GABA_AR$ is mainly controlled through regulation of internalization, recycling and lateral diffusion of the receptors. Under physiological condition these mechanisms are finely coordinated to define the strength of GABAergic synapses. In this review article, we focus on the alteration in $GABA_AR$ trafficking with an impact on the function of inhibitory synapses in various disorders of the CNS. In particular we discuss how similar molecular mechanisms affecting the synaptic distribution of $GABA_AR$ and consequently the excitatory/inhibitory balance may be associated with a wide diversity of pathologies of the CNS, from psychiatric disorders to acute alterations leading to neuronal death. A better understanding of the cellular and molecular mechanisms that contribute to the impairment of GABAergic neurotransmission in these disorders, in particular the alterations in $GABA_AR$ trafficking and surface distribution, may lead to the identification of new pharmacological targets and to the development of novel therapeutic strategies.

Keywords: $GABA_A$ receptor trafficking, epilepsy, brain ischemia, Alzheimer's disease, Huntington's disease, Parkinson's disease

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INTRODUCTION

The appropriate equilibrium between excitatory and inhibitory neurotransmission, which is mainly mediated by glutamate and γ-aminobutyric acid (GABA), respectively, is necessary for the correct function of neuronal circuits in the central nervous system (CNS; Smith and Kittler, 2010). Therefore, the control of GABAergic synaptic strength and transmission plays a crucial role in the maintenance of the excitatory/inhibitory synaptic balance (Smith and Kittler, 2010; Mele et al., 2016). An impairment of these mechanisms leading to neuronal hyperexcitability is a common and early event that characterizes several brain disorders (McCormick and Contreras, 2001; Saxena and Caroni, 2011).

The neurotransmitter GABA acts, in part, through activation of GABA$_A$ receptors (GABA$_A$R), which are heteropentameric chloride channels, composed in most cases of 2α-, 2β-, and 1γ2-subunits (Rudolph and Möhler, 2004). GABA$_A$R with different subunit compositions have different physiological and pharmacological properties, are differentially expressed throughout the brain and are targeted to different subcellular regions (Fritschy and Mohler, 1995; Nusser et al., 1998b). Receptors composed of α1, α2 or α3 subunits together with β and γ subunits are benzodiazepine-sensitive and largely synaptically located, mediating most phasic inhibition in the brain (Rudolph and Möhler, 2004). The synaptic localization of these receptors is determined by the direct interaction of the alpha subunits with the scaffold protein gephyrin (Tretter et al., 2008, 2011; Mukherjee et al., 2011). On the other hand, GABA$_A$R composed of α4, α5 or α6 subunits, together with β and δ subunits, are predominantly extrasynaptic, mediate tonic inhibition resulting mainly from synaptic "spillover" and are insensitive to benzodiazepine modulation (Brünig et al., 2002; Glykys and Mody, 2007; Jacob et al., 2008). The tonic inhibition in CA1 and CA3 pyramidal neurons is mediated by α5 and δ subunit-containing GABA$_A$R (Glykys and Mody, 2006) that detect low, ambient concentrations of GABA in the extracellular space and desensitize slowly. Accordingly, deletion of the α5 subunit eliminates about half of the tonic currents mediated by GABA$_A$R in hippocampal CA1 and CA3 pyramidal neurons; the remaining current was found to be mediated by GABA$_A$R containing δ subunits (Glykys and Mody, 2006). Moreover, studies using mice bearing a point mutation in position 105 of the GABA$_A$R α5 subunit, which downregulates the expression of the receptors exclusively in hippocampal pyramidal neurons, showed an important role for these subunits in cognitive processes (Crestani et al., 2002).

Under normal physiological conditions GABA$_A$R respond to the binding of GABA by opening an integral chloride channel and allowing chloride to enter the neuron. The result is a membrane hyperpolarization and neuronal inhibition. This mechanism of inhibition by GABA$_A$R depends on the electrochemical potential for chloride. Therefore changes of the intracellular Cl^- concentration ($[Cl^-]_i$) may regulate the response to the activation of GABA$_A$R (Jedlicka et al., 2011). For example, in immature neurons GABA$_A$R are mostly excitatory due to the fact that the intracellular chloride concentration is above the equilibrium. Maturation of the CNS is accompanied by a decrease of neuronal $[Cl^-]_i$, which accounts for the hyperpolarizing effect of the receptor (Watanabe and Fukuda, 2015).

Neuronal $[Cl^-]_i$ is mostly regulated by two chloride cotransporters, KCC2 (K^+-Cl^- cotransporter; KCC type 2) and NKCC1 (the Na^+-K^+-$2Cl^-$ cotransporter type 1; Russell, 2000; Blaesse et al., 2009). KCC2 expression is neuronal specific and under normal physiological conditions the transporter extrudes Cl^- out of the cell. NKCC1 is present in a variety of cells and generally loads cells with Cl^-. Furthermore, the relative expression pattern of the two transporters differs across development (Russell, 2000; Ben-Ari, 2002). The NKCC1 transporter is more expressed earlier in development than KCC2, and this accounts for the high $[Cl^-]_i$ observed in immature neurons. In the mature brain, the increased abundance of KCC2 contributes to a lower $[Cl^-]_i$ when compared with the extracellular concentration, favoring the influx of Cl^- through the GABA$_A$R channel and consequent membrane hyperpolarization upon activation of the receptors (Kaila et al., 2014).

The activity of GABA$_A$R is also regulated by "cross-talk" with other receptors (Shrivastava et al., 2011a). Since GABA$_A$R can be found in heterologous synapses (Nusser et al., 1996; Renner et al., 2012; de Luca et al., 2017), such receptor cross-talk may be mediated by a direct interaction with other receptors or through activation of intracellular signaling pathways. For example, GABA$_A$Rs have been demonstrated to heteromerize with GABA$_B$R (Balasubramanian et al., 2004), dopamine D5 receptors (Liu et al., 2000), purinergic P2X receptors (Jo et al., 2011; Shrivastava et al., 2011b), nicotinic acetylcholine receptors (Lee et al., 2010) and adenosine A_1 receptors (Hu and Li, 1997). In particular, the cross-talk between GABA$_B$R/GABA$_A$R may contribute to their regulation at pre- and postsynaptic levels. For instance, a direct interaction of GABA$_{B1}$ subunits with γ2S subunits of GABA$_A$R was observed in the rat brain, and co-expression of GABA$_{B1}$ subunits with GABA$_A$R increases the inhibitory responses mediated by the latter receptors (Balasubramanian et al., 2004). Of particular interest is the NMDA receptor (NMDAR) mediated modulation of GABA$_A$R. It has been demonstrated that activation of NMDAR downregulates GABA$_A$R function due to calcium dependent activation of phosphatase 2B/calcineurin followed by dephosphorylation of GABA$_A$R (Stelzer and Shi, 1994; Chen and Wong, 1995; Marsden et al., 2007; Bannai et al., 2009). A recent study showed that GABA$_A$Rs are trapped at glutamatergic synapses in response to glutamatergic stimulation, thereby limiting GABA$_A$R inter-synaptic diffusion (de Luca et al., 2017). The evidence that a hetero-synaptic interaction is modulated by neuronal activity suggests that cross-talk between GABA$_A$R and other receptors may be considered a mechanism for tuning inhibition in the CNS.

Deficits in the functional expression of GABA$_A$R have been implicated in the pathogenesis of several neurological and psychiatric diseases (Schwartz-Bloom and Sah, 2001; Rudolph and Knoflach, 2011; Kaila et al., 2014). GABA$_A$R are assembled within the endoplasmic (ER) and are then transported to the Golgi. In the ER, unassembled receptor subunits are subjected to poly-ubiquitination that targets them for proteasomal degradation (Kittler et al., 2002), a phenomenon that is dependent on the level of neuronal activity (Saliba et al., 2007). This process is negatively regulated by Plic-1 (the protein that links integrin-associated protein with the cytoskeleton-1; Bedford et al., 2001), which binds directly to the α- and β-subunits of the receptor, prolonging their residence times in the ER (**Figure 1**). Inside the Golgi, GABA$_A$R receptors bind to GABA$_A$R associated protein (GABARAP)/N-ethylmaleimide-sensitive factor (NSF) complexes, facilitating their transport to the plasma membrane (Leil et al., 2004). This mechanism mediates the increase

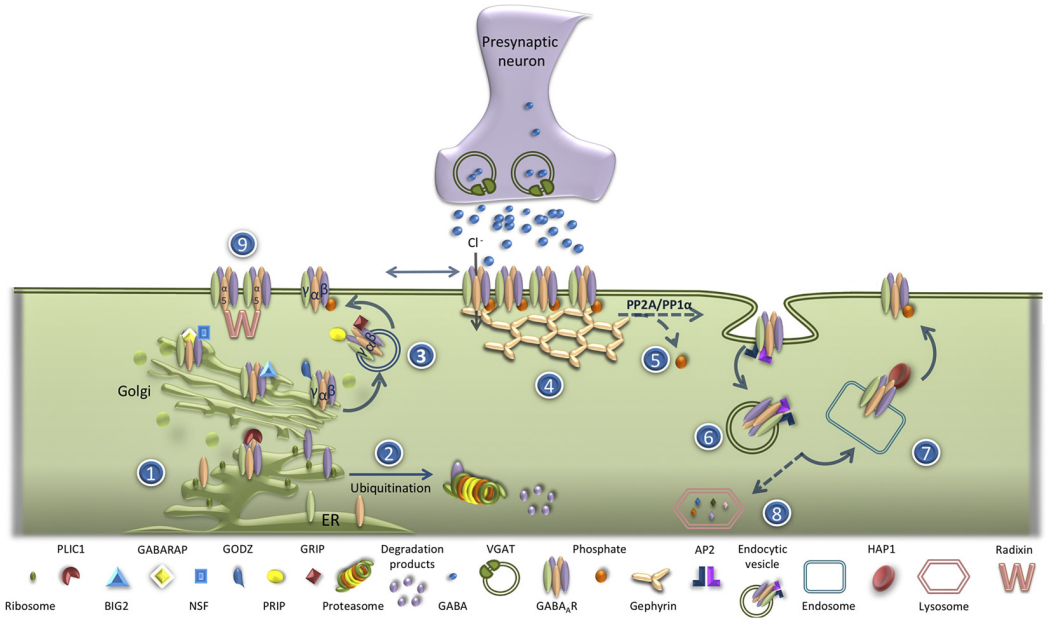

FIGURE 1 | GABA$_A$ receptor (GABA$_A$R) trafficking under physiologic condition. (1) GABA$_A$R are assembled in the ER. (2) In the ER, unassembled receptor subunits are subjected to poly-ubiquitination and targeted for proteasomal degradation. (3) GABA$_A$R transport to the Golgi is a process negatively regulated by Plic-1. Inside the Golgi, GABA$_A$R bind to GABA$_A$R associated protein (GABARAP)/N-ethylmaleimide-sensitive factor (NSF) complex that facilitates their transport to the plasma membrane. The delivery of GABA$_A$R to the plasma membrane is also regulated by GODZ, Big2, glutamate receptor-interacting protein (GRIP) and PRIP. (4) At the plasma membrane, GABA$_A$R quickly exchange between synaptic and extrasynaptic locations, and the accumulation of the receptor at the inhibitory synapses is regulated by its scaffold protein gephyrin. (5) The phosphorylation of β3 or γ2 GABA$_A$R subunits on their intracellular loop negatively regulates GABA$_A$R internalization. (6) The process of GABA$_A$R endocytosis is AP2/clathrin/dynamin-mediated. (7) Most internalized GABA$_A$R are rapidly recycled back to the plasma membrane by a mechanism dependent of the interaction with huntingtin-associated protein 1 (HAP1). (8) The non-recycled GABA$_A$R are targeted for lysosomal degradation.

in the exocytosis of GABA$_A$R observed upon stimulation of cultured hippocampal neurons with N-Methyl-D-aspartate (NMDA; Marsden et al., 2007). The delivery of GABA$_A$R to the plasma membrane is regulated by Golgi-specific DHHC (Asp-His-His-Cys) zinc finger protein (GODZ), a Golgi resident palmitoyltransferase responsible for the palmitoylation of γ subunits. GODZ interacts with the GABA$_A$R γ2 subunit recognizing a 14-amino acid cysteine-rich domain conserved in the intracellular domain of γ1–3 subunits, NH_2-terminal to the GABARAP binding site (Rathenberg et al., 2004). The γ2 subunit is palmitoylated at all four cysteines within the GODZ binding domain (Rathenberg et al., 2004; Vithlani et al., 2011). The ADP ribosylation factor (Arf) guanine nucleotide exchange factor (GEF) Big2 (brefeldin A-inhibited GDP/GTP exchange factor 2) also plays a role in the delivery of GABA$_A$R from the Golgi to the plasma membrane by promoting the budding and trafficking of vesicles from this compartment (Charych et al., 2004b). This protein interacts with the intracellular loop of all GABA$_A$R β2 subunits (Charych et al., 2004b). Additional proteins important in the trafficking of GABA$_A$R from the Golgi to the plasma membrane are the glutamate receptor-interacting protein (GRIP; Charych et al., 2004b; Kittler et al., 2004a), the phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2; Kanematsu et al., 2002; Uji et al., 2002), the GABA$_A$R-interacting factor (GRIF-1; Beck et al., 2002) and Maf1 interacting coiled-coil protein (Macoco; Smith et al., 2010). The insertion into the membrane of the vesicles containing GABA$_A$R also depends on SNAP23-syntaxin1A/B-VAMP2 complexes (Gu et al., 2016).

Once in the membrane, GABA$_A$R are very dynamic, exchanging between synaptic and extrasynaptic locations (Jacob et al., 2005; Thomas et al., 2005; Bogdanov et al., 2006), being the accumulation of the receptors at the inhibitory synapses regulated by the scaffold protein gephyrin (Fritschy et al., 2008; Tyagarajan and Fritschy, 2014). Gephyrin recruitment to inhibitory synapses is a fundamental phenomenon for their long-term potentiation (iLTP). Studies using a chemical protocol to induce iLTP in cultured hippocampal neurons, consisting in a moderate activation of NMDARs, showed an increased synaptic clustering of GABA$_A$R by a mechanism involving a CaMKII-dependent phosphorylation of GABA$_A$R β3 subunits on S383 (Petrini et al., 2014). Potentiation of inhibitory synapses in the same model was found to be mediated by recruitment of gephyrin from extrasynaptic regions, downstream of GABA$_A$R phosphorylation, as shown by single-particle tracking (SPT) analysis (Petrini et al., 2014). Recent studies using single-molecule super-resolution imaging with a novel clustering analysis, showed a rearrangement of synaptic gephyrin molecules during iLTP, with the formation of gephyrin nanodomains within the synaptic area (Pennacchietti et al., 2017).

GABA$_A$R are in a continuous cycle between the plasma membrane and the intracellular compartments (Jacob et al., 2008; Mele et al., 2016). Regulation of the total GABA$_A$R surface expression plays a key role in the control of the postsynaptic receptor pool size and the strength of synaptic inhibition (Mele et al., 2016). The process of GABA$_A$R endocytosis occurs mainly *via* clathrin- and dynamin-dependent mechanisms upon interaction of GABA$_A$R β and γ subunits with the adaptor protein 2 (AP2) clathrin adaptor protein complex (Kittler et al., 2000, 2005, 2008). In the brain, GABA$_A$R interact with AP2 through a direct binding of the β1–3 and γ2 GABA$_A$R subunits (Kittler et al., 2000). The first sequence motif important for AP2/clathrin/dynamin-mediated endocytosis of GABA$_A$R was identified in an heterologous system and corresponds to a di-leucine motif present in β subunits (Herring et al., 2003, 2005). Additional studies performed in neurons, identified an amino acid sequence motif (KTHLRRRSSQLK in the β3 subunit), which includes a major phosphorylation site conserved in the cytoplasmic loop region of β1–3 subunits (Ser^{408}, Ser^{409} in β3), as an important motif for AP2/clathrin/dynamin-mediated GABA$_A$R internalization (Kittler et al., 2005, 2008). This motif also contains the major sites of phosphorylation by cAMP-dependent protein kinase A (PKA) and calcium/phospholipid-dependent PKC within this class of receptor subunits: Ser^{409} in β1, Ser^{410} in β2, and Ser$^{408/9}$ in β3 (McDonald et al., 1998; Brandon et al., 2002, 2003; Kittler et al., 2005; Smith et al., 2008). Furthermore, a sequence of three arginine residues (^{405}RRR^{407}) was identified within the β3 subunit that is responsible for the interaction of GABA$_A$R with AP2 and in the stabilization of the receptors at dendritic endocytic zones where they are internalized (Smith et al., 2012). The GABA$_A$R internalization rate is negatively regulated by phosphorylation of β3 or γ2 GABA$_A$R subunits on their intracellular loop. Thus, NMDAR signaling is known to control the stability of synaptic GABA$_A$R *via* calcineurin-mediated dephosphorylation of the receptors (Muir et al., 2010). Moreover, a tyrosine-based AP2-μ2 adaptin-binding motif (Y^{365}GY^{367}ECL) was identified in the GABA$_A$R γ2 subunit, which is also conserved in the γ1 and γ3 subunits (Moss et al., 1995; Kittler et al., 2008). These tyrosine residues are the major sites for phosphorylation by Fyn and Src kinases (Nishikawa et al., 2002; Jacob et al., 2005; Bogdanov et al., 2006), and their phosphorylation reduces AP2 binding (Kittler et al., 2008).

The internalized GABA$_A$R may be rapidly recycled back to the neuronal plasma membrane or targeted for lysosomal degradation. The destiny of receptors following endocytosis is determinant for the regulation of surface/synaptic receptor abundance. The interaction of GABA$_A$R β1–3 subunits with huntingtin-associated protein 1 (HAP1) determines whether endocytosed GABA$_A$R are recycled (Kittler et al., 2004b). HAP1 is a GABA$_A$R associated protein that binds the intracellular loop of β subunits *in vitro* and *in vivo* (Kittler et al., 2004b). Overexpression of HAP1 in neurons inhibits GABA$_A$R degradation and consequently increases receptor recycling (Kittler et al., 2004b). Furthermore, HAP1 overexpression was shown to increase surface levels of GABA$_A$R and miniature inhibitory postsynaptic current (mIPSC) amplitude in cultured hippocampal neurons (Kittler et al., 2004b).

The balance between the insertion, lateral diffusion, internalization and recycling of GABA$_A$R in the neuronal plasma membrane determines the strength of GABAergic synapses. Defects in GABA$_A$R trafficking have been reported as triggers of GABAergic dysfunction in a number of brain pathological conditions (Hines et al., 2012). The following sections will address the alterations in GABA$_A$R trafficking, in acute brain disorders, as well as in neuropsychiatric and neurodegenerative diseases (**Figure 2**).

ALTERATIONS IN THE RATE OF CONSTITUTIVE DEGRADATION AND ON THE TRAFFICKING OF GABA$_A$R IN EPILEPSY

Epilepsy is a chronic disorder of the brain characterized by the presence of recurrent spontaneous seizures. The disease affects approximately 65 million people worldwide, from all ages and both genders (Jacobs et al., 2009; Hesdorffer et al., 2013). In temporal lobe epilepsy, the most common form of partial epilepsy in humans, an initial insult is followed by a seizure-free period before the development of spontaneous seizures. The process by which the brain become hyperexcitable and prone to generate seizures is defined as epileptogenesis (Sharma et al., 2007; Curia et al., 2008). During the latent (seizure-free) period there is a complex reorganization of neuronal networks, which has been characterized in more detail in the hippocampus (Goldberg and Coulter, 2013). An increase in neuronal excitability may contribute to the genesis and/or propagation of epileptic seizures, and several cellular and molecular changes are thought to be involved in the development of spontaneous seizures following a brain insult (Loscher and Brandt, 2010; Goldberg and Coulter, 2013; Staley, 2015).

Studies in animal models have shown that the pathophysiology related with the appearance of seizures is associated with a dysfunction of GABAergic neurotransmission (El-Hassar et al., 2007). Accordingly, several antiepileptic drugs act as agonists of GABA$_A$R (Czuczwar and Patsalos, 2001) and a dysfunction of GABA$_A$R has been proposed to be involved in the etiology of epilepsy. In fact, mutations or genetic variants of the genes encoding the α1, α6, β2, β3, γ2, or δ subunits have been associated with human epilepsy (reviewed by Hirose, 2014). Also, mutations in GABA$_A$R that enhance the constitutive ER-associated degradation (ERAD) of the receptors have been associated with genetically determined epilepsies, as well as, with idiopathic generalized epilepsies (Cossette et al., 2002; Huang et al., 2014). Furthermore, multiple GABA$_A$R mutations associated with epilepsy result in the abnormal trafficking of the receptors (Kang et al., 2015), perturbing their expression on the plasma membrane and synaptic clustering (Han et al., 2015; Huang et al., 2017; Ishii et al., 2017).

Among genetic epilepsies displaying abnormal GABAergic neurotransmission, a group of pediatric monogenic epilepsies

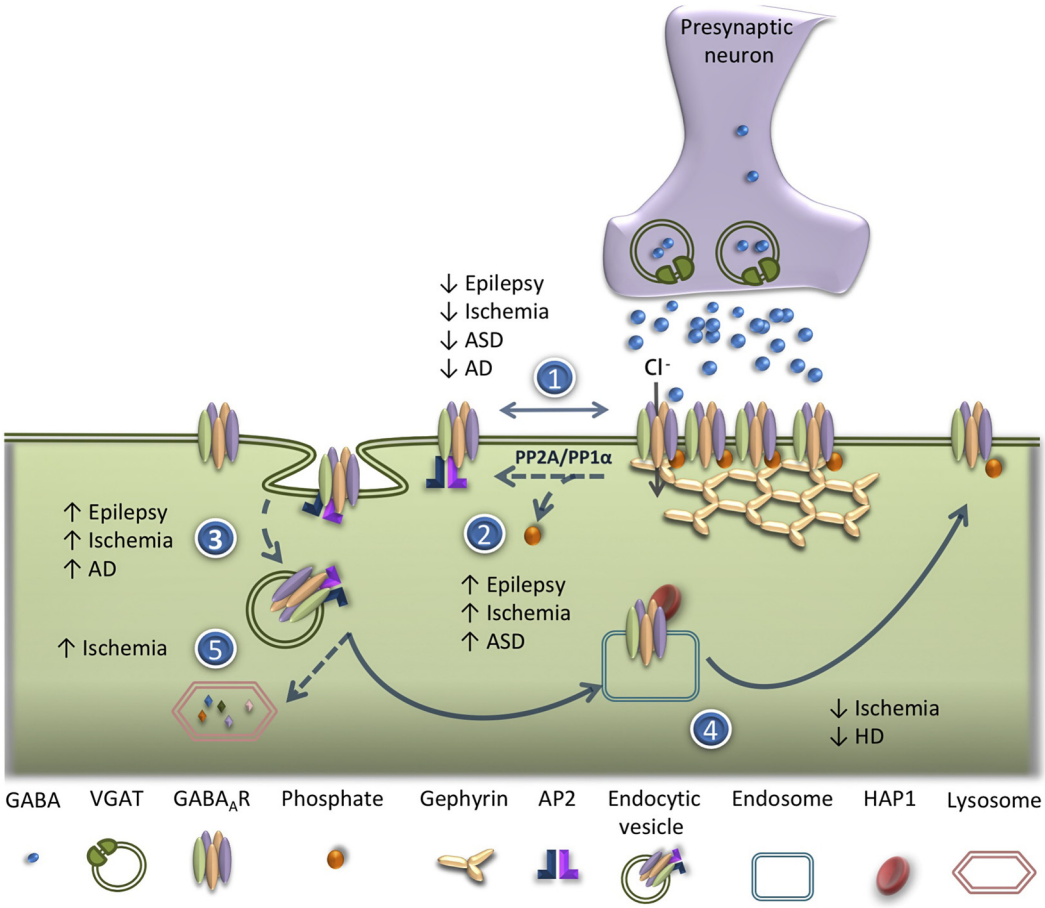

FIGURE 2 | Alterations of GABA$_A$R trafficking in brain disorders. Deficits in GABA$_A$R trafficking have been reported in different pathological conditions in the central nervous system (CNS). (1) Reduced synaptic clustering of GABA$_A$R has been observed in epilepsy, ischemia, autism spectrum disorders (ASDs) and Alzheimer's disease (AD). (2) Increased dephosphorylation of GABA$_A$R β3 subunit on serine residues 408/9 (Ser$^{408/409}$) has been reported in epilepsy, ischemic condition and ASD. (3) An increase in AP2/clathrin/dynamin-mediated endocytosis of GABA$_A$R occurs in epileptic conditions, ischemia, ASD and AD. (4) Impairment in GABA$_A$R recycling has been shown in ischemic conditions and in Huntington's disease (HD). (5) Enhanced lysosomal degradation of GABA$_A$R due to ubiquitination was detected after an ischemic insult.

was characterized in patients with the Dravet and Rett syndromes (Ali Rodriguez et al., 2018; Gataullina et al., 2019). These disorders are associated with neurodevelopmental complications, and autism spectrum disorders (ASD)-like features are common in patients with both syndromes, suggesting a link between epilepsy and ASD (Ali Rodriguez et al., 2018). In fact, epilepsy is quite common in patients with ASD and therefore the association between epilepsy and autism is receiving growing interest (Deykin and MacMahon, 1979; Olsson et al., 1988; Galanopoulou et al., 2000; Giovanardi Rossi et al., 2000; Besag, 2004; Hughes and Melyn, 2005; Kosinovsky et al., 2005). In addition to the most common mutation in the SCN1A gene affecting the α1 subunit of voltage-gated sodium channels (Wu et al., 2015), Dravet syndrome may also result from mutations in genes that alter GABAergic transmission, such as GABRA1, GABRB2, GABRB3, and GABRG2, encoding the corresponding subunits of GABA$_A$R (α1, β1, β2 and γ2 subunits, respectively). Moreover, a recent study identified a de novo heterozygous missense mutation in GPHN, which encodes for gephyrin, in a patient with Dravet-like syndrome (Dejanovic et al., 2017). Human mutations in the protocadherin-19 (PCDH19) gene, which encodes for the PCDH19 protein, also cause early infantile epileptic encephalopathy, associated with intellectual disability and autistic features (Kolc et al., 2019), similar to Dravet syndrome. PCDH19 cytoplasmic region binds to the α subunits of GABA$_A$R thereby regulating the receptor surface expression, suggesting that PCDH19 might be involved in the regulation of GABA$_A$R intracellular trafficking (Bassani et al., 2018). Furthermore, PCDH19 downregulation in hippocampal neurons causes a reduced frequency of mIPSCs (Bassani et al., 2018).

The primary cause of Rett syndrome is a mutation of the gene encoding the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2; Kozinetz et al., 1993). Between 60 and 80% of females with Rett syndrome suffer from epilepsy (Vignoli et al., 2017). Studies performed in the Mecp2 KO

animal model of Rett syndrome, showed a dramatic loss of GABAergic neurons (Chao et al., 2010). Moreover, recent evidence demonstrated that Mecp2 targets KCC2, and neurons differentiated from induced pluripotent stem cells from patients with Rett syndrome showed a reduced expression of KCC2 and a delayed switch in the excitatory to inhibitory responses to GABA during development (Tang et al., 2016).

The major problem in the therapy of status epilepticus (SE), and recurrent epileptiform discharges, is the time-dependent pharmacoresistance; about 30% of the patients become resistant to the treatment (Regesta and Tanganelli, 1999; French, 2007). A potential mechanism accounting for the impairment of inhibitory neurotransmission, characteristic of SE, and for the development of pharmacoresistance to benzodiazepines (De Koninck, 2007), is a reduction in the availability of functional GABA$_A$Rs associated with the plasma membrane, which may arise from an altered pattern of receptor trafficking (**Figure 2**). Accordingly, *in vitro* studies performed in hippocampal neurons exposed to a medium lacking Mg^{2+}, to induce epileptiform discharges, showed a reduction of about 50% in the surface expression of GABA$_A$R after 1 h of SE, as demonstrated by a biotinylation assay (Cho et al., 2017). Furthermore, experiments using cultured hippocampal neurons incubated in a medium lacking Mg^{2+}, an *in vitro* model of SE, showed a reduction in the surface stability of GABA$_A$R as determined by live-cell imaging of SE pHluorin (SEP)-tagged α_2 subunits. The observed decrease in the surface expression of GABA$_A$R was mediated by activation of NMDARs for glutamate and was sensitive to inhibition of the phosphatase calcineurin (Eckel et al., 2015). Additional studies using the same *in vitro* model of SE combined with electrophysiological and cellular imaging techniques, showed that prolonged epileptiform bursting leads to a reduction of GABA-mediated synaptic inhibition; the constitutive internalization of GABA$_A$R accelerated by the increased neuronal activity was associated with seizure activity. Moreover, inhibition of neuronal activity reduced the effect of SE on the rate of GABA$_A$R internalization as well as the downstream reduction in the surface expression of the receptors that may contribute to the downregulation of inhibitory neurotransmission observed during seizures (Goodkin et al., 2005). This model is supported by evidence obtained in *in vivo* studies using the lithium-pilocarpine model of TLE, which showed a reduction in the amplitude of mIPSCs mediated by postsynaptic GABA$_A$R when tested in dentate gyrus granule cells (Naylor et al., 2005). In contrast, the amplitude of extrasynaptic GABA$_A$R tonic currents was increased during SE (Naylor et al., 2005). These results also suggests a possible increase in extracellular GABA concentration during SE, which may be coupled to an upregulation of extrasynaptic tonic currents, while synaptic currents may be decreased under the same conditions due to desensitization and internalization of GABA$_A$R (Naylor et al., 2005). In fact, inhibition of GABA$_A$R endocytosis in epileptic cultures resulted in both a recovery of the levels of membrane associated GABA$_A$R and a total blockade of spontaneous recurrent epileptiform discharges (Blair et al., 2004).

In accordance with the role of GABA$_A$R phosphorylation in the regulation of their surface expression (see above), SE reduces PKC-dependent phosphorylation of GABA$_A$R β3 subunit on the serine residues 408/9 ($Ser^{408/409}$; Terunuma et al., 2008). These residues contain a binding motif for the clathrin AP AP2, being a critical regulator of GABA$_A$R endocytosis (Nakamura et al., 2015). Pharmacological activation of PKC or the specific blockade of GABA$_A$R binding to AP2, during SE, restores the surface expression of the receptors, re-establishing the efficacy of synaptic inhibition (Terunuma et al., 2008).

The proper trafficking of GABA$_A$R required to maintain the number and localization of the receptors at the neuronal surface is also dependent on the function of different proteins that interact with GABA$_A$R directly or through adaptor proteins linked with microtubules (Mele et al., 2016). The expression of key scaffolding proteins associated with GABA$_A$R is altered during epileptogenesis. For example, SE downregulates the expression of gephyrin and GRIP in the hippocampal CA1 region 4–8 days after the insult (pilocarpine injection; González et al., 2013). These alterations are correlated with changes in the plasma membrane expression and assembly of GABA$_A$R (González et al., 2013). To what extent the downregulation of GRIP contributes to the observed reduction in the surface expression of GABA$_A$R remains to be investigated. In fact, GRIP interacts with GABARAP (Kittler et al., 2004b) and is expressed at inhibitory postsynapses (Dong et al., 1999; Charych et al., 2004a; Li et al., 2005). Therefore, the SE-induced decrease in GRIP protein levels may impair the GABARAP-mediated delivery of GABA$_A$R to the plasma membrane (Marsden et al., 2007).

Alterations in gephyrin clustering and expression during epileptogenesis were also detected in the hippocampus and in the cerebral cortex (Thind et al., 2010; Fang et al., 2011). The epileptogenic period is characterized by a reduction in the number of gephyrin puncta and GABAergic synapses in dentate gyrus, while an increased number of gephyrin clusters was detected during the chronic period (Thind et al., 2010). Moreover, studies performed in the neocortex showed that gephyrin expression gradually decreases during the epileptogenic period and returns to basal levels during the chronic phase (Fang et al., 2011). Thus, gephyrin downregulation may contribute to the instability of GABA$_A$R clustering, amplifying the deficit in GABAergic neurotransmission observed in epileptic condition.

The ezrin/radixin/moesin (ERM) family protein radixin acts a scaffold to anchor α5βγ2 GABA$_A$R to the actin cytoskeleton at extrasynaptic sites (Loebrich et al., 2006). This interaction is regulated by an activity-dependent manner through the RhoA-ROCK pathway (Hausrat et al., 2015). The dissociation of the receptors from the radixin anchor allows the lateral diffusion of GABA$_A$R to increase their synaptic expression (Hausrat et al., 2015). However, whether this type of mechanism regulates the surface expression of GABA$_A$R containing α5 subunits remains to be investigated.

Recent evidence indicates that alterations in chloride homeostasis may also contribute to the impairment of the GABA inhibitory activity (Rivera et al., 2004). These alterations have been attributed to a downregulation of the K^+-Cl^- cotransporter KCC2. The resulting increase in the intracellular

Cl^- concentration may account for the positive shift of the $GABA_AR$ reversal potential, and the consequent depolarizing effects of GABA, observed in hippocampal slices exposed to conditions mimicking status epilepticus (Coull et al., 2003). Interestingly, two independent studies reported that rare variants of KCC2 confer an increased risk of epilepsy in humans (Kahle et al., 2014; Puskarjov et al., 2014). However, whether the SE-induced alteration in $GABA_AR$ trafficking depends on the alteration in Cl^- gradient was not yet confirmed.

Taken together, the studies mentioned above indicate that during seizures, the persistent cell firing and GABA release may lead to the extracellular accumulation of GABA, causing desensitization and internalization of postsynaptic $GABA_AR$. Moreover, alterations of scaffolding proteins associated with $GABA_AR$, mainly gephyrin, contribute to the ultimate failure of inhibition observed in epilepsy. These mechanisms could account for the maintenance of recurrent seizure activity and benzodiazepine pharmacoresistance.

A DECREASE IN GABA$_A$R ANCHORING AT THE SYNAPSE AND IN RECEPTOR RECYCLING IMPAIR INHIBITORY SYNAPSES IN BRAIN ISCHEMIA

Cerebral ischemia is a pathological condition caused by insufficient blood supply to the brain, which leads to an increase in glutamatergic neurotransmission coupled to excitotoxic neuronal death. The down-regulation of GABAergic synapses in brain ischemia resulting from $GABA_AR$ desensitization (Gyenes et al., 1994) and a reduction of cell surface density of $GABA_AR$ (Nusser et al., 1997, 1998a), is one of the major factors contributing to excitotoxicity (Mele et al., 2014).

One of the first direct evidence suggesting that ischemic insults decrease the cell surface expression of $GABA_AR$ through an increase in receptor internalization (**Figure 2**) came from *in vitro* studies using ELISA, as a cell surface receptor assay (Mielke and Wang, 2005). These studies showed that transient incubation of cultured cortical neurons in the absence of oxygen and glucose to mimic global ischemia decreases cell surface $GABA_AR$ without altering the total expression of receptors. In fact, inhibition of receptor endocytosis with hypertonic sucrose treatment prevented receptor internalization. In the same study, the authors suggested that $GABA_AR$ internalization could contribute to neuronal death (Mielke and Wang, 2005). Similarly, studies using quantitative membrane protein biotinylation assays and immunocytochemistry confirmed that the abundance of plasma membrane-associated $GABA_AR$ was significantly decreased in cortical and hippocampal neurons exposed to oxygen and glucose deprivation (OGD). In this set of experiments the activation of phosphatidylinositol 3-kinase/Akt-dependent signaling pathway, through PTEN downregulation, was shown to protect neurons from the toxic effects of OGD by preventing the reduction in the surface expression of $GABA_AR$ (Smith et al., 2012). Results obtained with antibody feeding assay also showed that OGD-induces the internalization of $GABA_AR$-α1 and β3 subunits in cultured hippocampal neurons by a dynamin-dependent mechanism (Mele et al., 2014). Additionally, it was reported that the down-modulation of $GABA_AR$ from dendritic clusters during OGD is dependent on the AP2 pathway for cell surface removal of the receptors. Moreover, blockade of this pathway reduced the neuronal death induced by OGD (Kittler et al., 2008).

The interaction between β3-subunit and AP2 seems to be critical for $GABA_AR$ reduction in synapses during ischemic insult (Smith et al., 2012). The identification of the intracellular domains (ICD) region of the β3-subunit that mediates the interaction with the clathrin adaptor AP2 also revealed the presence of three arginine residues (^{405}RRR^{407}) within this binding motif that are essential for the interaction with μ2–AP2; mutation of these residues impairs receptor recruitment to clathrin-coated pits, significantly reducing receptor endocytosis (Smith et al., 2012). Studies performed with a β3-subunit RRR motif mutant with a deficient AP2 binding site showed that the acute loss of synaptic $GABA_AR$ during OGD is mediated by an AP2/β3 interaction. Furthermore, blocking the internalization of $GABA_AR$ using a peptide competing with β3 for the binding to AP2 reduces OGD-induced cell death (Smith et al., 2012).

Interestingly, the β3-subunit RRR motif is located adjacent to a phosphorylation site, Ser^{408}/Ser^{409}, which is known to negatively regulate the internalization of the receptor when phosphorylated (Kittler et al., 2005, 2008). These phosphorylation sites are also regulated during an ischemic insult, both *in vivo* (using the transient middle cerebral artery occlusion—MCAO, a model of focal ischemia) and *in vitro* (OGD). In particular, it was found that brain ischemia induces the dephosphorylation of $GABA_AR$ β3-subunit (Ser^{408}/Ser^{409}) *in vitro* and *in vivo* (Mele et al., 2014). Studies with cultured hippocampal neurons subjected to OGD confirmed that the dephosphorylation of this domain is responsible for the observed increase in receptor internalization (Mele et al., 2014). Again, the consequent reduction in the surface expression of $GABA_AR$ was correlated with ischemia-induced cell death, since the transfection of hippocampal neuron with a phospho-mimetic mutant of $GABA_AR$ β3 subunit (SS$^{408/409}$AA), which does not undergo internalization, reduced significantly the OGD-induced apoptotic neuronal death (Mele et al., 2014).

The destiny of $GABA_AR$ after endocytosis depends on their interaction with HAP1 (Kittler et al., 2004b). Under physiologic conditions most internalized $GABA_AR$ are rapidly recycled back to the plasma membrane, by a mechanism dependent of HAP1, while the remaining pool of receptors undergoes lysosomal degradation (Kittler et al., 2004b). Cultured hippocampal neurons subjected to OGD showed an impairment in receptor recycling that was correlated with a decrease in the interaction of the receptor with HAP1. This protein is indeed downregulated during OGD condition by a calpain mediated mechanism. When overexpressed, HAP1 protected hippocampal neurons from OGD-induced cell death (Mele et al., 2017).

The reported reduction in the number of synaptic $GABA_AR$ observed in brain ischemia may also be directly related with the ubiquitination-dependent degradation of the receptors. In particular the ubiquitination of lysine residues between amino acids 317–328 within the intracellular domain of the $GABA_AR$

γ2 subunit modulates the lysosomal targeting of the receptor. This process controls the efficacy of neuronal inhibition under basal conditions by regulating the accumulation of GABA$_A$R at inhibitory synapses (Arancibia-Cárcamo et al., 2009). The deficit in neuronal inhibition under conditions of OGD also involves an enhanced degradation of GABA$_A$R due to ubiquitination of a motif located within the intracellular domain of the γ2 subunit, with a consequent deficit in the cell surface stability of the receptors (Arancibia-Cárcamo et al., 2009).

Together, these studies point to postsynaptic alterations of GABAergic synapses as central players in synaptic dysfunction induced by brain ischemia. The internalization of GABA$_A$R that accounts for the impairment in inhibitory neurotransmission may also be related with the synaptic instability of the receptor. Indeed, the gephyrin scaffold protein was found to be cleaved in cultured hippocampal neurons subjected to OGD, by a calpain-dependent mechanism. The resulting disassembly of the gephyrin lattice underneath the plasma membrane is likely to cause an inefficient synaptic anchoring of GABA$_A$R (Costa et al., 2016). OGD also decreases GABA$_A$R/gephyrin interaction, as shown in experiments of surface co-immunoprecipitation of GABA$_A$R α1 subunits and gephyrin (Mele et al., 2014). The decrease in the interaction between GABA$_A$R and its scaffold protein gephyrin suggests a possible alteration in the membrane dynamics of the receptor. An increased mobility of the receptors at the synapse may make them less confined within this compartment, and these receptors would become more prone to be internalized. However, further experiments are needed to better understand the alteration induced by ischemic insults on the lateral diffusion of GABA$_A$R, and the signaling mechanisms involved, contributing to the impairment of GABAergic synapse strength. The internalization of GABA$_A$R after an ischemic injury may explain, at least in part, the failure of receptor agonists or modulators in clinical trials for stroke (Amantea and Bagetta, 2017).

Alteration of the electrochemical gradient may also contribute to the impairment of GABAergic neurotransmission in brain ischemia. Several studies reported a decrease in KCC2 expression in brain ischemia (Galeffi et al., 2004; Papp et al., 2008; Jaenisch et al., 2010). Transient MCAO was found to decrease KCC2 mRNA levels, 1 day after reperfusion, and a consequent downregulation in the protein levels of the transporter was detected 7 days after reperfusion (Jaenisch et al., 2010). An attenuated expression of KCC2 in neurons subjected to an ischemic insult may trigger GABA-evoked depolarizing responses, thereby influencing plasticity and damage induced by stroke.

ANIMAL MODELS OF AUTISM SPECTRUM DISORDERS (ASD) ARE CHARACTERIZED BY A DOWNREGULATION OF GABA$_A$R AND ALTERATION IN THEIR SYNAPTIC DISTRIBUTION

ASD is a group of early-onset developmental disorders characterized by a variety of behavioral deficits and intellectual disability (Mattina et al., 2009). More than 80% of ASD cases are caused by genetic alterations (Rosenberg et al., 2009; Frazier et al., 2014; Baio et al., 2018). However, a huge number of genes have been identified associated to ASD, making difficult the study of the physiological pathways affected by these conditions.

The imbalance between neuronal excitation and inhibition within cortical circuits has been suggested as a cellular mechanism accounting for the behavioral and cognitive symptoms of ASD (Jenks and Volkers, 1992; Ramamoorthi and Lin, 2011; Yizhar et al., 2011). Although the neurobiological bases of ASD have not been clearly established, several genes related to autism were shown to encode synaptic proteins. Accordingly, an aberrant synaptic activity is characteristic of ASD patients (Howell and Smith, 2019). In particular, a dysfunction in the GABAergic system has been suggested to play an important role in the pathogenesis of ASD (Nielsen, 1990; Dhossche et al., 2002; Pizzarelli and Cherubini, 2011; **Figure 2**).

A recent study reported a decreased expression of membrane associated GABA$_A$R-β3 subunits, as well as a downregulation of the phosphorylated form of the receptor subunit, in the sodium valproate (VPA)-induced rat model of ASD. The reduced phosphorylation levels of GABA$_A$R-β3 subunit suggests alterations in the trafficking of the receptor, namely an increase in receptor internalization. The changes in GABAergic neurotransmission induced by prenatal exposure to VPA were also associated to impaired spatial memory, limited exploration, increased anxiety, and reduced sociability (Li et al., 2017b).

Alterations in the phosphorylation of GABA$_A$R γ2 subunits may also be relevant for the ASD phenotype as shown in studies using the Ser$^{408/409}$Ala homozygous mice, in which the receptor subunit shows a low interaction with the AP2 complex which decreases internalization, similarly to the behavior of phosphorylated receptors. These animals are characterized by an increase in the activity of synaptic GABA$_A$R, together with a reduction in the extrasynaptic inhibitory currents, and exhibit the core phenotypes of ASD (Vien et al., 2015). The *fmr1* KO mice which are commonly used as a model to study the fragile X syndrome and ASD also display an increased phosphorylation of GABA$_A$R γ2 on Ser$^{408/409}$ (Vien et al., 2015), further pointing to a role for alterations in the phosphorylation state of this subunit in neuropsychiatric disorders.

Deficits in GABA$_A$R surface expression were also detected in mice with a loss-of-function of *PX-RICS* that results in ASD-like behaviors (Nakamura et al., 2016). These mice recapitulate the pathogenic process of ASD-like behavior characteristic of Jacobsen syndrome (JBS) patients (Mattina et al., 2009). *PX-RICS*$^{-/-}$ mice exhibit a dysfunction of the postsynaptic mechanism for GABA$_A$R trafficking. Cell surface labeling and biotinylation assays revealed that GABA$_A$R γ2 surface expression is significantly reduced in *PX-RICS*$^{-/-}$ hippocampal neurons and in cerebellar granule neurons (CGNs). Moreover, whole-cell patch–clamp experiments detected a reduction in the amplitude of mIPSCs with no significant differences in their frequency, suggesting that the postsynaptic responsiveness to inhibitory input is impaired without alteration in the presynaptic release of the neurotransmitter. Interestingly, stimulation with a GABA$_A$R

agonist improved some autistic-like phenotypes of *PX-RICS*$^{-/-}$ mice (Nakamura et al., 2016). This suggests that a potentiation of postsynaptic GABAergic signaling could be a possible therapeutic strategy for ASD-like behavior.

The impairment of GABAergic neurotransmission in patients with ASD is further supported by evidence showing increased levels of Hrd1 in the middle frontal cortex of patients with ASD (Crider et al., 2014). This E3 ligase ubiquitinates misfolded GABA$_A$R α1 subunits before ERAD in HEK293 cells (Di et al., 2016). Interestingly, a downregulation of GABA$_A$R α1 subunits was also detected in the middle frontal cortex of ASD patients (Crider et al., 2014).

Mutations in several proteins associated with the postsynaptic density (PSD) of excitatory synapses have been associated with neuropsychiatric disorders (Volk et al., 2015; Li et al., 2017a; Gandal et al., 2018). The growing interest in the characterization of the inhibitory PSD (Tyagarajan and Fritschy, 2014) may shed light into the complexity of the mechanisms involved in the regulation of GABAergic neurotransmission and may show novel molecular players involved in the regulation of the surface dynamics of GABA$_A$R with a role in neuropsychiatric disorders, including ASD.

GABA$_A$-RECEPTOR TRAFFICKING INVOLVEMENT IN NEURODEGENERATIVE DISORDERS

Alterations in GABA$_A$R trafficking coupled to the dysregulation of the synaptic excitatory/inhibitory balance are also a common feature of several neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD; **Figure 2**). These alterations might induce changes in synaptic strength and ultimately lead to excitotoxicity and consequent neuronal cell death.

AD is a chronic and progressive neurodegenerative disease characterized by memory deficits and cognitive decline owing to synaptic and neuronal loss in the hippocampus and cerebral cortex. The abnormal deposition of amyloid-β (Aβ) in these brain regions suggests that this peptide plays an essential role in AD pathogenesis (Karran and De Strooper, 2016). In fact, the observed deleterious effects of Aβ were shown to arise, in part, from the interaction of the peptide with NMDAR, causing excitotoxity and neuronal dysfunction (Costa et al., 2012).

GABAergic signaling was also demonstrated to be profoundly altered in the AD brain (Limon et al., 2012). Indeed, GABA currents were shown to desensitize faster and the GABA$_A$R were found to be less sensitive to GABA after micro-transplantation of membranes from the temporal cortex of AD patients into *Xenopus oocytes* (Limon et al., 2012). Aβ was also shown to weaken synaptic inhibition through downregulation of GABA$_A$R *via* receptor endocytosis (Ulrich, 2015). Accordingly, Aβ induced a decline in mIPSCs in layer V pyramidal neurons, an effect that was prevented using an inhibitor of the dynamin-mediated internalization of GABA$_A$R (Ulrich, 2015). This result indicates that the observed hyperexcitability characteristic of AD could be partly related with the loss of functional GABA$_A$R observed in the AD brain (Limon et al., 2012) and with the loss of synaptic inhibitory strength induced by Aβ (Ulrich, 2015).

In the context of AD, GABA$_A$R were also show to suffer several consistent alterations in their subunit composition (e.g., α1, α2, α5, β2, β3 and γ2), in different brain regions, namely in the hippocampus (Kwakowsky et al., 2018). The complexity of these alterations is not compatible with simple compensatory mechanisms, but may reflect instead the reorganization of defined neuronal circuits (Kwakowsky et al., 2018). Despite these results, the effects of Aβ on inhibitory synapses are still poorly understood as most studies have focused on the impairment of excitatory synaptic transmission. In particular, the signaling pathways by which Aβ induce GABA$_A$R endocytosis remain to be investigated. Since Aβ enhances neuronal excitability though NMDA activation and synaptic plasticity (Parihar and Brewer, 2010; Costa et al., 2012; Varga et al., 2014), this may constitute the signal to induce the internalization of GABA$_A$R. Future studies should also address a possible direct interaction of Aβ with GABA$_A$R or with proteins associated with the inhibitory PSD. Whether the tau pathology in AD is also somehow related with alterations in GABA$_A$R traffic also remains to be investigated. Furthermore, the implications of the alteration in GABA$_A$R trafficking in AD progression are still unclear. Several studies suggested that part of the symptoms associated to this disorder might be caused by the loss of the synaptic excitatory/inhibitory balance (Michels and Moss, 2007; McDade et al., 2009; Ulrich, 2015).

Alterations in GABA$_A$R trafficking have also been associated with PD. This long-term neurodegenerative disorder mainly affects the motor system and causes a characteristic combination of motor symptoms (e.g., hypertonia) due to progressive neurodegeneration of dopaminergic neurons (Gilbert et al., 2006; Meder et al., 2018). The symptomatic treatment of hypertonia can be achieved by enhancing GABAergic transmission. Indeed, the regulation of GABA$_A$R homeostasis was reported to be disrupted in a hypertonic mouse model bearing a mutation in the *hyrt* gene, which codes for the trafficking protein kinesin binding 1 (Trak1). This study showed a marked reduction in the levels GABA$_A$R in the CNS, particularly in the lower motor neurons, and, interestingly, Trak1 was found to interact with GABA$_A$R (Gilbert et al., 2006). Trak1 (and Trak2) shares some homology with HAP1 (Li et al., 1995), which has been implicated in intracellular trafficking and transport of GABA$_A$R (Kittler et al., 2004b; Gilbert et al., 2006). In contrast with the effect on the expression of GABA$_A$R, the distribution of the GABA$_A$R anchoring protein gephyrin was not altered in *hyrt* mice. Therefore, the reduction in GABA$_A$R in *hyrt* mice may be due to the dysregulation of GABA$_A$R endocytic trafficking rather than to the destabilization of the plasma membrane complex that stabilizes the receptors at the synapse (Gilbert et al., 2006). Thus, it can be hypothesized that Trak1 may facilitate the targeting of endocytosed receptors back to the membrane or it may block their degradation. Interestingly, no significant degeneration of GABAergic neurons was observed in *hyrt* mice despite the

reduction in the levels of GABA$_A$R subunits in this hypertonic mouse model (Gilbert et al., 2006), as described for AD (Ulrich, 2015).

Other proteins have been associated with the reduction of GABA$_A$R surface expression in PD. GABARAPs are a family of proteins that play a role in vesicle and receptor trafficking (Kittler et al., 2001), and in particular they were shown to interact and regulate the intracellular trafficking of GABA$_A$R (Wang et al., 1999; Chen et al., 2001; Chen and Olsen, 2007). Furthermore, members of this protein family have been implicated in autophagy (Rowland et al., 2006; Schwarten et al., 2009), a mechanism involved in GABA$_A$R clearance (Rowland et al., 2006). A recent study showed that GABARAPs also bind the parkin-associated endothelin-like receptor (PAELR; Dutta et al., 2018), which is localized in the core of Lewy bodies, a PD hallmark (Murakami et al., 2004). Furthermore, PAELR interacts with the GABA$_A$R binding site of GABARAPL2, and this protein together with Parkin and PICK1 are most likely involved in the regulation of PAELR protein levels. This occurs *via* autophagy, ubiquitination and proteasomal degradation (Dutta et al., 2018), which ultimately might lead to the regulation of GABA$_A$R trafficking. However, additional studies are required to establish a role for GABARAPs in PD.

HD is an autosomal dominant progressive neurodegenerative disorder caused by the mutant huntingtin (Htt), with an expanded polyglutamine (polyQ) repeat (McClory et al., 2014). This disorder is characterized by progressive involuntary choreiform movements, emotional disturbances and cognitive decline (Pinborg et al., 2001), associated with degeneration of GABAergic neurons (Fritschy and Brünig, 2003).

An early study using emission tomography methods (PET and SPECT) showed a reduction in the abundance of benzodiazepine receptors in the striatum (but not in the cortex) of HD patients (Pinborg et al., 2001). These binding sites are present in GABA$_A$R containing, for example, α1, α2, α3, or α5 subunits, together with β and γ subunits, and are mainly located at the synapse where they mediate most phasic inhibition in the brain (Jacob et al., 2008). This contrasts with the extrasynaptic GABA$_A$R that mediate tonic inhibition, which are insensitive to benzodiazepines (Jacob et al., 2008). The putative alterations in the expression of GABA$_A$R in HD requires further investigation since immunohistochemistry experiments showed an increase in the abundance of the α1 and γ2 receptor subunits in the globus pallidus of patients with the disease, while the levels of gephyrin were not changed (Thompson-Vest et al., 2003). The discrepancy between the results obtained in the analysis of benzodiazepine receptors and expression of GABA$_A$R subunits may be due to differences in the brain regions analyzed, which was more restricted in the latter case.

In contrast with the evidence showing changes in the abundance of GABA$_A$R in certain brain regions of HD patients, the alterations in receptor trafficking in the disease have been poorly investigated. As mentioned before, HAP1 interacts directly with GABA$_A$R and regulates inhibitory synaptic transmission by modulating GABA$_A$R recycling (Kittler et al., 2004b). GABA$_A$R are trafficked to synapses by the kinesin family motor protein 5 (KIF5), which mediates the insertion of GABA$_A$R into the plasma membrane, and HAP1, the adaptor that links the motor protein to the receptors. Accordingly, HAP1-KIF5 dependent GABA$_A$R trafficking was reported as a fundamental mechanism controlling the strength of synaptic inhibition in the brain (Twelvetrees et al., 2010). Mutant huntingtin containing a polyQ expansion disrupts the HAP1-KIF5 GABA$_A$R trafficking and synaptic delivery (Twelvetrees et al., 2010). Thus, the disruption of this complex by mutant huntingtin may lead to altered synaptic inhibition and increased neuronal excitability in HD (Twelvetrees et al., 2010).

The disruption of GABA$_A$R trafficking and synaptic inhibition was also observed in a mouse model of HD (Yuen et al., 2012). In the latter study, GABA$_A$R-mediated inhibitory transmission was found to be disrupted in the HD at the symptomatic stage, a consequence of a diminished surface GABA$_A$R expression, which may underlie the impaired GABAergic transmission (Yuen et al., 2012). Furthermore, the KIF5-mediated microtubule-based transport of GABA$_A$R was confirmed to be impaired in HD, which may underlie the disruption of GABA$_A$R trafficking to the synaptic membrane. Therefore, the interference in the effect of polyQ-Htt on the HAP1/KIF5-mediated trafficking of GABA$_A$R to synapses may constitute a therapeutic approach for HD, by restoring synaptic function (Yuen et al., 2012).

The chronic neuroinflammation observed in these neurodegenerative disorders induces the upregulation of tumor necrosis factor-α (TNF-α), which might play a role in the observed synaptic excitatory/inhibitory unbalance (Frankola et al., 2011). TNF-α was already described as an important mediator of homeostatic synaptic plasticity (Stellwagen and Malenka, 2006), and, interestingly, it was shown to modulate GABA$_A$R trafficking, thereby downregulating the inhibitory neurotransmission. Indeed, TNF-α enhances the association of protein phosphatase 1 (PP1) with GABA$_A$R β3 subunits and dephosphorylates the amino acid residue of the β3 subunit responsible for the regulation of the phospho-dependent interactions with the endocytic machinery (Pribiag and Stellwagen, 2013).

FINAL REMARKS

Aberrant excitability is a common feature of numerous disorders of the CNS. Dysfunction of GABAergic synapses and in particular alterations of postsynaptic GABA$_A$R trafficking have been reported as a key mechanism that contributes to the unbalance between excitation an inhibition, which ultimately will lead to neuronal hyperexcitability. Interestingly, similar alterations in the mechanism coupled to an increased internalization of GABA$_A$R result in distinct outcomes/symptoms associated to different pathologies of the CNS. Depending on the circuits, the brain region and the developmental stage in which the postsynaptic alteration of GABAergic system is initiated, different structural and molecular modifications of the involved neurons may occur, triggering distinct pathologic responses. However, the disruption of the

GABAergic neurotransmission characteristic of various illnesses may partly account for some common symptoms. For example, patients with cerebral ischemia, as well as certain cases of ASD or HD (Gambardella et al., 2001), may present seizures that are a hallmark of epilepsy. The reviewed studies indicate that the mechanisms involved in the control of plasma membrane and synaptic expression of GABA$_A$R are key players in the modulation of neuronal excitability. However, considering the recent findings showing that the nanoscale redistribution of the scaffold protein gephyrin is a key event in the potentiation of inhibitory synapses, additional studies are required to evaluate the alterations in GABA$_A$R and gephyrin nanoscale redistribution induced by hyperexcitability in pathological conditions. The outcome of this type of studies may contribute to the identification of novel therapeutic targets for various brain disorders characterized by an impaired regulation of the excitation/inhibition balance.

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