IONOTROPIC GLUTAMATE RECEPTORS TRAFFICKING IN HEALTH AND DISEASE

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IONOTROPIC GLUTAMATE RECEPTORS TRAFFICKING IN HEALTH AND DISEASE

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The knowledge about the properties and importance of ionotropic glutamate receptor trafficking is ever increasing. Importantly, the pace of the progress has been accelerated in recent years.

Here, our contributors provide a) reviews on specific topics that present an up-to-date overview of the field, as well as b) original articles with the relevant new findings.

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EDITORIAL
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Editorial: Ionotropic Glutamate Receptors Trafficking in Health and Disease

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Keywords: ionotropic glutamate receptors, trafficking, NMDA, AMPA, kainate

The Editorial on the Research Topic

Ionotropic Glutamate Receptors Trafficking in Health and Disease

Because of their fundamental role in excitatory synaptic function in health and disease, ionotropic glutamate receptors (iGluRs) continue to be the focus of wide-spread research efforts within the neuroscience community.

A core aspect of on-going research is the elucidation of the complex sequence of events that coordinate iGluR processing, delivery to, retention at, recycling, and removal from synapses (collectively known as receptor trafficking). Understanding of the activity dependent regulation of these events in healthy and diseased neurons will likely provide new targets for therapeutic intervention and, we believe, holds tremendous promise for new and improved treatments for neurological and neurodegenerative diseases.

The contributors to this special issue "Ionotropic glutamate receptors trafficking in health and disease" each provide new insights into different aspects of this complex problem, covering a wide range of issues, starting with early stages of trafficking taking place in the ER, through the distribution of receptors along actin tracks to the final stages of insertion into the surface membrane. Taken together with broader overviews, these papers provide a broad picture of current understanding of how postsynaptic iGluRs are integral to the initiation and expression of synaptic plasticity and how this impacts on disease.

With respect to NMDAR subunits, specific sections have been shown to affect the transition between individual steps in receptor trafficking, including their processing and eventual release from the ER. Based on their structure, different rules may apply to individual subunits and it is now shown that specific structural features of GluN2C can also regulate this process (Lichnerova et al.). On a wider scale, the processes surrounding the ER-related events in NMDAR trafficking are also covered by a dedicated article in our special issue (Horak et al.).

Beyond their regulation at the level of transcription and translation, iGluRs are subject to stringent regulation by post-translational modifications. Among these, the phosphorylation of GluN2A at Ser1048 by the Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) can interfere with the internalization of GluN1/GluN2A NMDARs, while also potentiating their activation and increasing the NMDAR-current density (Grau et al.).

The complexity of excitatory synapses is enhanced by AMPAR- and NMDAR-interacting proteins. These interactions can be prolonged or transient and have profound effects on trafficking. For example, carnitine palmitoyltransferase 1C (CPT1C) affects early steps of AMPAR trafficking to control AMPAR availability at synapses (Gratacòs-Batlle et al.).

Another intriguing phase of AMPAR trafficking is their forward transport along tracks provided by rapidly changing actin cytoskeleton. This occurs with the participation of various interacting

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proteins including PICK1 and the ARP2/3 Complex, ADF/Cofilin, as well as molecular motors, such as myosin. All of these interactors have the ability to modify trafficking of AMPARs, thus determining both the basal synaptic transmission, as well as activity-dependent regulation of synaptic strength (Hanley). Once that the receptors reach their targeted surface membrane, they need to be inserted in it and this regulated process, mediated by SNARE proteins, is critical for the postsynaptic expression of various forms of plasticity, as reviewed by Jurado and Chater and Goda.

An emerging concept is that iGluRs interact with other neurotransmitter systems and their receptors, including GABAB (Kantamneni), nicotinic receptors (Zappettini et al.), as well as dopaminergic system. This crosstalk has far-reaching implications, especially for diseases such as Parkinson's and Huntington's, as well as in addiction (Gardoni and Bellone).

Needless to say, with so many roles in synaptic function, it is clear that disturbances in iGluR trafficking give rise to serious neurological and psychiatric diseases. Among proteins affecting iGluR trafficking, SynGAP (and the genetic changes affecting its expression) is now recognized as the pathophysiological substrate for autism spectrum disorder and this fascinating topic is reviewed within our issue (Jeyabalan and Clement).

In conclusion, we believe that the articles presented in this special issue represent a valuable resource that provides a clear overview of the current state of the art of this important and rapidly progressing field of neuroscience.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW ARTICLE
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ER to synapse trafficking of NMDA receptors

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Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. There are three distinct subtypes of ionotropic glutamate receptors (GluRs) that have been identified including 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptors (AMPARs), *N*-methyl-D-aspartate receptors (NMDARs) and kainate receptors. The most common GluRs in mature synapses are AMPARs that mediate the fast excitatory neurotransmission and NMDARs that mediate the slow excitatory neurotransmission. There have been large numbers of recent reports studying how a single neuron regulates synaptic numbers and types of AMPARs and NMDARs. Our current research is centered primarily on NMDARs and, therefore, we will focus in this review on recent knowledge of molecular mechanisms occurring (1) early in the biosynthetic pathway of NMDARs, (2) in the transport of NMDARs after their release from the endoplasmic reticulum (ER); and (3) at the plasma membrane including excitatory synapses. Because a growing body of evidence also indicates that abnormalities in NMDAR functioning are associated with a number of human psychiatric and neurological diseases, this review together with other chapters in this issue may help to enhance research and to gain further knowledge of normal synaptic physiology as well as of the etiology of many human brain diseases.

Keywords: glutamate receptor, excitatory neurotransmission, ion channel, internalization, intracellular trafficking, subcellular compartment

INTRODUCTION

The most common glutamate receptors (GluRs) in mature synapses are 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptors (AMPARs) that mediate fast excitatory neurotransmission; *N*-methyl-D-aspartate receptors (NMDARs) serve mainly to modulate this neurotransmission by controlling the strength and number of AMPARs. However, during early postnatal development of many kinds of synapses, NMDARs predominate prior to the accumulation of AMPARs. The GluRs and their many associated proteins are "embedded" in an elaborate complex of interlinked proteins and cytoskeleton made by the postsynaptic density (PSD). The initial picture of the PSD was that of a static structure where GluRs were present, and were activated upon glutamate release. Over the last 10 years, a new picture of the PSD has emerged, that of a highly dynamic structure that increases or decreases in size and content during the entire life span of the individual, directly impacting spine shape. Such variations influence the physiological response of the postsynaptic side, the level of information storage and ultimately memory. For example, it is now believed that synapse function involves initial activation of AMPARs with glutamate binding, leading to a membrane depolarization that will release the magnesium block of the NMDAR channel, allowing calcium to enter the postsynaptic process via the glutamate-activated NMDAR. This calcium then initiates various metabolic pathways that ultimately can affect the strength of the synapse. Most commonly, these pathways involve various phosphorylation/dephosphorylation events that can activate or deactivate other pathways leading to changes in the strength or number of AMPARs at the synapse. Thus, it is clear that the trafficking and function of both types of GluRs, AMPARs and NMDARs, are tightly regulated by multiple unrelated mechanisms, ensuring that proper numbers and types of synaptic receptors are available in a given excitatory synapse. The interest in all these mechanisms has been strengthened by the recent discovery that pathologies such as Alzheimer's disease and schizophrenia, but also mental retardation, fragile X syndrome, Rett syndrome, or Autism Syndrome Disorder, are due to disruption of synapse shape and function and not to structural permanent brain damage as initially thought. This has fostered new perspectives suggesting that by understanding how synapses form and are regulated, we could develop therapies to treat pathologies that were thought to be out of the reach of any curative intervention.

This review will focus on NMDARs. A functional NMDAR is a heterotetramer composed mainly of two GluN1 subunits and two GluN2 subunits; in some cases the GluN3 subunits are also incorporated into the heterotetramer (Petralia et al., 2009; Traynelis et al., 2010; Paoletti et al., 2013). Based on the crystallography structure of the recombinant NMDARs,

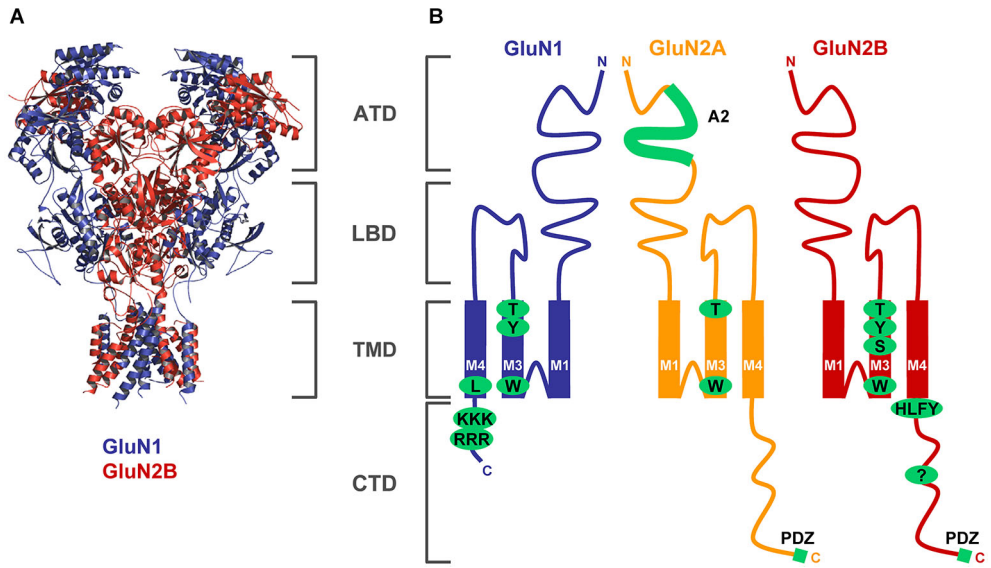


FIGURE 1 | Structural determinants regulating ER processing of functional NMDARs. **(A)** The structure of the NMDAR heterotetramer, based on a recent paper by Gouaux's laboratory, is indicated by blue (GluN1) and red (GluN2) colors (Lee et al., 2014). The membrane topology of the GluN subunits is described in detail in the Introduction section (ATD—amino-terminal domain, LBD—ligand-binding domain, TMD—transmembrane domain, CTD—C-terminal domain). **(B)** Schematic topology of the GluN subunits with indicated trafficking determinants (GluN1—several amino acid residues within the M3 domain and two ER retention signals, RRR and KKK, within the CTD; GluN2A—A2 segment within the ATD, several amino acid residues within the M3 domain; GluN2B—several amino acid residues within the M3 domain, HLFY export signal and unknown ER retention signal within the CTD). Both GluN2 subunits and some GluN1 splice variants (not shown) also contain PDZ-binding motifs in their far C-termini (see text for more details).

recent studies showed that the functional GluN1/GluN2 heterotetramer is formed in a GluN1-2-1-2 subunit arrangement (i.e., 2 GluN1/GluN2 heterodimers combine to form the heterotetramer; **Figure 1**) (Karakas and Furukawa, 2014; Lee et al., 2014), although several other studies suggested that there is a GluN1-1-2-2 subunit arrangement in the NMDAR (Schorge and Colquhoun, 2003; Balasuriya et al., 2013). There are eight different GluN1 splice variants, four GluN2 subunits (GluN2A-D) and two GluN3 subunits (GluN3A-B) expressed in the mammalian CNS. All GluN subunits share similar membrane topology—four membrane domains (M1-M4), an extracellular N-terminus and a loop between M3 and M4 domains, and an intracellular C-terminus (Petralia et al., 2009). It is expected that the presence of relatively long N- and C-termini of the GluN subunits enables an NMDAR to dynamically interact with different proteins during its journey to the synapse, its retention at the synapse, and its removal from the synapse. The first step that shortly follows protein synthesis is the receptor subunit assembly that occurs in the endoplasmic reticulum (ER). Next, the receptors are processed in the Golgi apparatus and packaged in the Golgi complex by means of vesicles, which carry the GluRs to the membrane. They are subsequently internalized and reinserted at extrasynaptic sites before being anchored at the PSD. At each step of the trafficking process GluRs need to be associated with specific partners that allow the maturation and transport of the receptors. While significant progress has been made in identifying the proteins involved in anchoring GluRs at the PSD, little is known about the partners involved in the trafficking processes of these receptors. A deep knowledge of the trafficking from the ER to the membrane is paramount since these processes specify the final destination of the receptor complexes, and their deregulation can profoundly disrupt synaptic function. This review will highlight recent advancements in our understanding of early events in the trafficking of NMDARs as well as mechanisms regulating synaptic NMDARs. We will discuss the structural determinants and protein-protein interactions, both involved in the regulation of NMDARs, in three sections, summarizing; (1) events that happen early in the biosynthetic pathway (largely the ER); (2) events that happen in the transport of receptors after release from the ER; and (3) events that happen at the plasma membrane.

PROCESSING OF NMDARs IN THE ER

What molecular mechanisms control the formation of the functional NMDAR heterotetramers in the ER? The GluN1 subunit is produced in the ER in large excess relative to GluN2 subunits, ensuring that sufficient amounts of GluN1 subunits are available for newly synthesized GluN2 and GluN3 subunits (Chazot and Stephenson, 1997; Huh and Wenthold, 1999). Several working models of the assembly of functional NMDARs in the ER have been proposed. First, several studies suggest that the GluN1-GluN1 and GluN2-GluN2 homodimers, which are initially formed, are required for the formation of the functional heterotetramers (Meddows et al., 2001; Schorge and Colquhoun, 2003; Papadakis et al., 2004; Qiu et al., 2005; Hansen et al., 2010). Second, another study proposed

that the GluN1-GluN2 heterodimers are required for formation of heterotetrameric receptors (Schüler et al., 2008), or that GluN1-GluN1 homomers are the substrate for the oligomeric assembly of the heterotetramer (Atlason et al., 2007). The last model has been extended by a recent study that suggested that the N-terminal domains of the GluN1 subunits initially form homodimers and that the subsequent dimer dissociation is essential for the forming of the functional GluN1/GluN2 heteromers (Farina et al., 2011). Indeed, the reported promiscuity between the GluN1 and GluN2 N-terminal domains could explain the development of different working models of the assembly of NMDARs.

How does the cell ensure that only properly folded NMDAR heterotetramers are transported from the ER to the cell surface? In general, it is expected that the ER employs a quality control mechanism(s) for proteins exported from the ER to prevent the accumulation of unassembled and misfolded protein complexes via the trafficking pathways to the cell surface. In the case of NMDARs, it has been shown that all GluN2 subunits and some GluN1 splice variants are retained in the ER unless assembled (McIlhinney et al., 1998). The basic principle that the unassembled GluN subunits are retained in the ER has been demonstrated also in mice lacking the GluN1 subunit in the hippocampus, resulting in an accumulation of GluN2 subunits in the ER (Fukaya et al., 2003). Similarly, it has been reported that the GluN3A subunit requires the association with GluN1 subunits for its export from the ER (Perez-Otano et al., 2001).

What signals control ER retention of unassembled GluN subunits? Different regions of the GluN subunits have been proposed to regulate the assembly and/or ER retention of NMDA receptors (**Figure 1**). First, the C-termini of some GluN1 splice variants have been shown to contain two specific ER retention motifs, KKK and RRR, in the C1 cassette (Standley et al., 2000; Scott et al., 2001; Horak and Wenthold, 2009). Interestingly, the GluN1 variant, GluN1-3, which contains both the ER retention motifs in the C1 cassette and the PSD-95, Dlg, and Zo-1 (PDZ)-binding motif (-STVV) in the far C-terminus exhibits enhanced surface delivery even when expressed alone, suggesting that specific protein-protein interactions with other proteins such as the PSD-MAGUKs and COPII (which recognizes a divaline motif of the C-terminus of the GluN1-3) can regulate the ER retention of NMDAR subunits (Standley et al., 2000; Scott et al., 2001; Mu et al., 2003). The ER retention of the GluN1 subunit also can be modulated by phosphorylation by PKA and PKC of serine residues that are nearby the RRR motif, as shown using chimeric proteins of the C-terminus and single transmembrane proteins (tac = interleukin-2 receptor α subunit; CD8) (Scott et al., 2001); but our previous report did not confirm this observation using the full-length GluN1 constructs (Horak and Wenthold, 2009). The C-terminus of the GluN2B subunit attached to tac is retained in the ER, suggesting the presence of an ER retention signal (Hawkins et al., 2004). However, efforts to identify this specific signal have been unsuccessful, although truncation of the C-terminus appended to tac leads to increased surface expression in constructs containing the region up to residue 1070 of the GluN2B (Hawkins et al., 2004). This study also identified a short motif, HLFY, localized immediately after the M4 domain of the GluN2B subunits. This motif is likely required as an export signal from the ER for the properly folded NMDAR heterotetramers (Hawkins et al., 2004). However, a later study proposed that the HLFY motif is not necessary for the formation of the surface functional NMDAR, as it can be replaced by alanines if the C-terminus is absent (Yang et al., 2007). Similarly, the deletion of the GluN2B C-terminus including the HLFY motif did not affect the formation of functional receptors when two pieces of the GluN2B subunit, GluN2B truncated before M4 domain and GluN2B M4 domain, were co-expressed together with the GluN1 subunit (Horak et al., 2008a). Together, these data indicate that the HLFY motif may provide a structural role to ensure the proper orientation of the membrane domains and/or the C-termini in the ER processing of the GluN1/GluN2 receptors. Similarly, the GluN3B subunit may also use the RXR motifs for ER retention, which must be negated by the association with the GluN1 subunit (Matsuda et al., 2003).

The structures of the extracellular regions of the GluN subunits were also implicated in the regulation of ER processing of NMDARs. Specifically, Prof. Stephenson's group reported that the structure of the glycine binding site in the GluN1 subunit is critical for the release of the functional NMDAR from the ER (Kenny et al., 2009). Similarly, another study revealed that the structure of the glutamate binding site within the GluN2B subunit controls the early processing of functional NMDARs (She et al., 2012). Given the fact that glutamate is likely present in the ER in the millimolar range (Berger et al., 1977; Meeker et al., 1989), it is plausible to speculate that a newly formed NMDAR heterotetramer is activated by agonists and then assessed for its proper functioning by a specific ER quality control machinery, as has been shown for the AMPARs (Penn et al., 2008). Future studies must resolve whether different affinities for glutamate and glycine among GluN1/GluN2A-D receptors, reported by many studies, regulate the ER processing of NMDARs (Traynelis et al., 2010). Furthermore, an ER retention signal has been identified in the A2 segment of the amino-terminal domain (ATD) of the GluN2A subunit; this must be masked by an interaction with the GluN1 subunit so that the functional NMDAR leaves the ER (Qiu et al., 2009). Interestingly, the appropriate region within the GluN2B subunit does not contain any ER retention signal, although there is relatively high sequence homology between the A2 segments of the GluN2A and GluN2B subunits. Because the identified A2 segment within the GluN2A subunit does not likely control the ER retention of unassembled GluN2A subunits, an additional ER retention signal(s) must exist in the remaining part of the GluN2A subunit (Qiu et al., 2009).

The structures of the membrane domains also likely regulate the ER processing of the functional NMDAR. Our previous reports identified critical structural determinants within the M3 domains of GluN1 and GluN2A-B subunits that cause the unassembled subunits to be retained in the ER (Horak et al., 2008b; Kaniakova et al., 2012a). However, we also showed that the structures of the M3 domains of the GluN1 and GluN2 subunits are critical for the release of the functional NMDARs from the ER, likely because the M3 domains mutually negate

their ER retention signals (Horak et al., 2008b; Kaniakova et al., 2012a). Interestingly, these structural determinants within the M3 domains are present in the other glutamate receptor subtypes including their human variants as well, and thus it is likely that most ionotropic GluRs employ a common mechanism that includes specific inter-membrane domain interactions. This view is supported by recent studies showing that a specific amino acid residue within the GluN1 M4 domain regulates the early processing of NMDARs (Kaniakova et al., 2012b) and specific inter-membrane domain interactions of the M4 domain with the M1/M3 domains are required for surface expression of AMPARs (Salussolia et al., 2011). Moreover, recent data revealed that the M4 domain also controls the tetramerization of AMPARs (Salussolia et al., 2013). Whether the M4 domain also regulates tetramerization of NMDARs needs to be elucidated in future studies. Indeed, lack of precise structural information about the membrane regions of the NMDARs limits our current understanding of the processes that are involved in described phenomena.

Are the functional properties of NMDARs monitored during ER processing, as has been shown for AMPARs and kainate receptors (Priel et al., 2006; Penn et al., 2008)? As mentioned above, there are sufficient concentrations of glutamate present in the ER so that an NMDAR can be activated and monitored by the ER quality control machinery. The NMDARs are thought to have specific conformations associated with closed, open or desensitized states (Traynelis et al., 2010). Interestingly, the presence of the GluN2 subunit determines the functional and pharmacological properties of NMDARs, including their macroscopic desensitization, Mg^{2+} affinities and single-channel conductances (Traynelis et al., 2010; Paoletti, 2011; Siegler Retchless et al., 2012). But desensitization is not likely to be the major trafficking determinant of GluN1/GluN2B receptor subtype (Kaniakova et al., 2012a). Clearly, additional studies are necessary to elucidate molecular mechanisms that are behind the release of functional NMDARs from the ER. One of these mechanisms may include the ER resident chaperone protein, Sigma-1 receptor (σ-1R), which mediates trafficking of NMDARs to the cell surface (Pabba et al., 2014).

Most neurons express at least two of the most common GluN2 subunits, GluN2A and GluN2B, and thus three types of receptors can be formed, GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2A/GluN2B (Al-Hallaq et al., 2007; Tovar et al., 2013). The functional properties of these three receptor types are quite different (Hatton and Paoletti, 2005) and one may speculate that their formation is not due to the random association of the subunits, but is regulated by other factors such as developmental stage and synaptic activity. Interestingly, when GluN2A subunit increases its abundance at P7, the di-heteromeric GluN1/GluN2A and GluN1/GluN2B complexes are present in similar amounts to those seen in the later developmental stages (Al-Hallaq et al., 2007). This indicates that the formation of NMDAR complexes is not dependent only on the relative expression of the GluN2 subunits. A previous study also reported that there is a preference for association of the GluN2 subunits with different GluN1 splice variants (Sheng et al., 1994). Because the GluN1 variants containing the C2' cassette exhibit an accelerated trafficking from the ER (Okabe et al., 1999; Mu et al., 2003; Horak and Wenthold, 2009) and neuronal activity leads to increased expression of C2'-containing GluN1 variants (Mu et al., 2003), it is obvious that the formation of individual NMDAR types and their exit from the ER are highly regulated processes that we are just learning to understand.

FROM THE EXIT OF THE ER TO THE SYNAPSE

TRAFFICKING OF NMDA RECEPTORS FROM THE ER TO THE PLASMA MEMBRANE

After being released from the ER, as for many other membrane proteins, NMDARs are further processed in the somatic Golgi apparatus and then distributed to the *trans* Golgi network (TGN) and endosomes, to finally reach the membrane and spines. While most NMDARs are likely processed in the cell body and then transported to the synapse, some use a non-conventional secretory pathway that bypasses the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) pathway in the cell body and utilizes dendritic ER or Golgi outposts (Wenthold et al., 2003; Jeyifous et al., 2009). Indeed, neurons possess ramified dendrites that contain functional ER and Golgi outposts; even spines may contain such structures. NMDAR complexes seem to use different routes to reach the synapses, specifically bypassing or not the somatic Golgi apparatus.

MAGUKS

PDZ (PSD-95/Discs-large/ZO-1) domain-containing proteins, such as the MAGUK proteins, PSD-95, SAP102, and SAP97, were first identified as the major synaptic scaffolding proteins anchoring NMDARs at glutamatergic synapses (Lue et al., 1994; Kornau et al., 1995; Müller et al., 1995, 1996; Brenman et al., 1996; Kim et al., 1996; Lau et al., 1996; Niethammer et al., 1996; for review, see Sheng, 1996; Sheng and Kim, 1996; Kornau et al., 1997) but many studies have also implicated them in the trafficking of receptors to and/or from synapses (Wenthold et al., 2003; Elias and Nicoll, 2007). SAP102, a multiple PDZ domain with the same organization as PSD-95, got our attention very early because, according to early data, it is enriched both at synapses and in the general neuronal cytoplasm (Müller et al., 1996; Sans et al., 2000). Indeed, from the microsome fraction solubilized with Triton X-100, it was shown that GluN1 subunits could be immunoprecipated with SAP102 but not with PSD-95, showing that at least SAP102 could interact with NMDARs way before they reach the synapse (Standley et al., 2000). Therefore, we and others hypothesized that PDZ proteins in a more global way could be involved in the early events of assembly and delivery of receptors, and that these different events were regulated through interaction with other proteins (**Figure 2**). We performed yeast-two hybrid screens using the MAGUK, SAP102, as bait to identify novel regulators of GluR trafficking. A first screen with the three PDZ domains of SAP102 identified Sec8 as a potential partner of SAP102 (Sans et al., 2003). Sec8 is a member of the exocyst complex, with a previously uncharacterized PDZ-binding domain implicated in the secretory process (Hsu et al., 1996). The exocyst is a multiprotein complex containing eight proteins (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) associated with

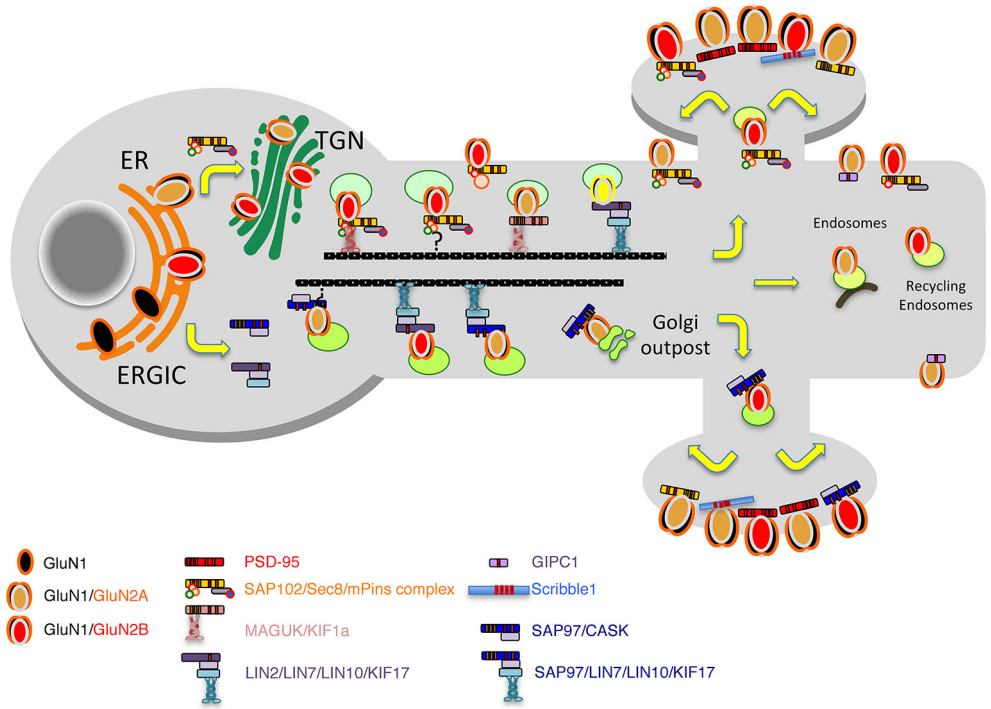
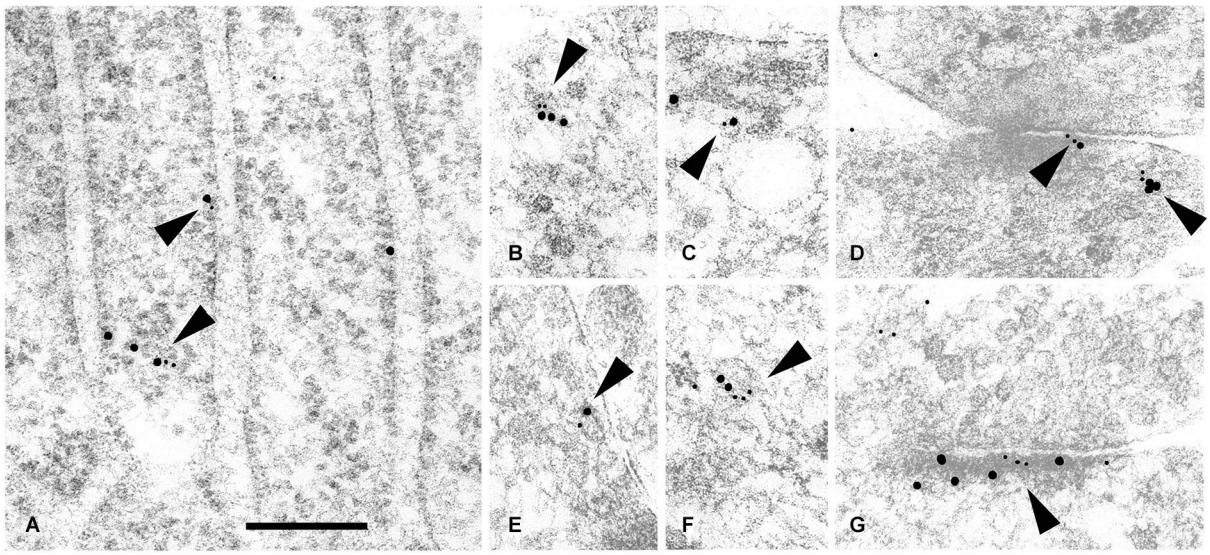


FIGURE 2 | Model of the secretory pathways used by NMDARs. First, after their synthesis and export from the endoplasmic reticulum (ER), receptors travel through the Golgi before being inserted into vesicles and transported directly to the plasma membrane or transported into dendrites by vesicular transport by means of different complexes including the MAGUKs, exocyst and mPINS and kinesins. Alternatively, through an interaction with SAP97 and CASK, NMDAR can exit the ER, bypass the ERGIC pathway and travel in vesicles to dendritic Golgi outposts before reaching the membrane or the synapse.

intracellular compartments in yeast, and implicated in directing intracellular membrane vesicles through the secretory pathway to their sites of fusion with the plasma membrane (Hsu et al., 1999). However, its role in mammalian cells and the mechanisms by which this complex could move cargos are unclear. Using immunogold labeling in the CA1 *stratum pyramidale/stratum radiatum* region of the hippocampus at P10, we showed that Sec8 or Sec6 colocalized with SAP102 and NMDARs in the ER or Golgi region including the adjacent intermediate compartment and TGN (**Figure 3**). In the ER, a complex made up of NMDAR/SAP102 binds to Sec8 and some of the additional subunits of the complex such as Sec6 or Exo70. We showed that these interactions were important for surface delivery of the receptors in heterologous cells and for synaptic delivery in neurons (Sans et al., 2003). mPins was later shown to be involved in the proper folding of SAP102 complexes that participate in receptor trafficking (Sans et al., 2005). mPins interacts with G protein alpha-subunits (Gαi) and these interactions also play a role in the traffic of the receptors. The GDI activity of mPins can be overcome by the guanine nucleotide exchange factor, Resistance to inhibitors of cholinesterase (Ric-8A), which activates Gαi protein stimulating the release of Gαi-GTP (Tall and Gilman, 2005). It is possible that through this balanced action of mPins and Ric-8, Gαi proteins influence the traffic of NMDA receptors. Furthermore, intracellular NMDARs have been shown to colocalize with SAP102 by immunocytochemistry or immunogold labeling (Washbourne et al., 2004; Petralia et al., 2010; Standley et al., 2012). In addition, it has been shown that phosphorylation of Ser1480 on GluN2B can disrupt the interaction with SAP102 and PSD-95, thus leading to decreased targeting and anchoring of GluN2B in neurons (Chung et al., 2004). More recently, the hypothesis that SAP102 mediates trafficking of NMDARs has been strengthened by several studies. Indeed, neurons transfected with a ligand-binding deficient form of SAP102 show decreased synaptic clustering of NMDARs, although the SAP102 mutant forms were efficiently targeted to synapses (Minatohara et al., 2013). Interestingly, we showed in neurons transfected with full-length GluN2B and SAP102, using a switch in temperature from 37°C to 20°C to slow down the trafficking process, that SAP102 was colocalized with NMDARs at the level of the ER (Sans et al., 2005). Recently, Standley et al. confirmed these data, demonstrating that SAP102 interaction with NMDAR occurs very early in the secretory pathway; and using live imaging with Tac-GluN2A or Tac GluN2B chimeras, they showed that SAP102 first interacts with the tail of the receptors and that PSD-95 could also be involved in the traffic of some NMDARs in the post trans-Golgi network (Standley et al., 2012). It should be noted that the PSD-95 antibody used in this study (T60) also recognizes SAP97 (Sans et al., 2000) leaving open the possibility that both PSD-95 and SAP97 can interact with the chimeras. PSD-95 was also shown to interact with the exocyst (Riefler et al., 2003). It is interesting to note that the exocyst has also



H

Soma cytoplasm RER region	2.0±0.1 gold/μm²
Soma cytoplasm Golgi region	4.0±0.6 gold/μm²
Soma cytoplasm Total gold	2.6±0.2 gold/μm²

Apical dendrites Microtubule-associated	56±3% of gold
Apical dendrites T-V organelle-associated	37±10% of gold
Apical dendrites Total gold	3.3±0.3 gold/μm²

Neuropil Postsynaptic processes	6.8±0.6 gold/μm²
Neuropil Presynaptic processes	4.4±0.3 gold/μm²

FIGURE 3 | Immunogold labeling of NMDARs, SAP102 and the exocyst complex. **(A–G)** Double-immunogold labeling (arrowheads), in sections of the CA1 region of the hippocampus **(A)** stratum pyramidale; **(B–G)** stratum radiatum of juvenile animals, with antibodies to Sec6 or Sec8 (5 nm gold) and SAP102 or GluN2A/B (10 nm gold). **(A)** SAP102 and Sec8 in RER in a neuron soma. **(B)** SAP102 and Sec8 associated with a cytoplasmic tubulovesicular structure in a growth cone. **(C)** SAP102 and Sec8 associated with a small vesicle adjacent to a large one, in a large dendrite. **(D)** SAP102 and Sec8 at an early contact between neurites. **(E)** GluN2A/B and Sec8 associated with a cytoplasmic vesicle in a small neurite. **(F)** SAP102 and Sec6 associated with a cytoplasmic vesicle in a large dendrite. **(G)** SAP102 and Sec6 at a synapse on a dendrite shaft. Line scale is 200 nm. **(H)** Distribution of immunogold labeling (5 nm) for Sec8 in the CA1 stratum pyramidale/stratum radiatum region of the hippocampus at P10. Golgi region includes Golgi and the adjacent intermediate compartment and TGN. Microtubule-associated and tubulovesicular (T-V) organelle-associated categories are not mutually exclusive. Figure is a reprint of Figure 3 from Sans et al., 2003.

been involved in the trafficking of NMDAR–dependent AMPAR trafficking through a previously unidentified interaction between the Sec8 N-terminal sequence and GRIP1 (Mao et al., 2010). Exo70, another exocyst component, controls receptor synaptic accumulation (Gerges et al., 2006). All these results raised several interesting questions about the exact composition of the exocyst complex in the trafficking process, but they clearly show that the exocyst is implicated in delivery of receptors. SAP97 is another MAGUK with a high level of expression in the intracellular compartments (Sans et al., 2001), and that could interact with NMDARs (Niethammer et al., 1996; Songyang et al., 1997; Bassand et al., 1999). SAP97 is a GluA1 interactor involved in the precise targeting and clustering of AMPARs (Leonard et al., 1998; Sans et al., 2001; Nakagawa et al., 2004; Schlüter et al., 2006). In 2003, Di Luca et al. showed that SAP97 could indeed bind directly to GluN2A through its PDZ1 domain, and that this interaction is regulated by CaMKII (Gardoni et al., 2003; Mauceri et al., 2007). This interaction may be quite important in immature neurons since SAP97 seems to be able to drive the switch between GluN2B and GluN2A (Howard et al., 2010). It could also be involved in the trafficking of a subpopulation of receptors that do not use the conventional secretory pathway. Actually, Green et al. described a new path taken by some NMDARs associated with SAP97 and CASK and possibly KIF17 (Jeyifous et al., 2009). They showed that some NMDARs are directed from the somatic ER into a specialized dendrite ER subcompartment that targets NMDARs to dendritic Golgi outposts in dendrites. Later work showed that CASK regulates the conformation of SAP97, and thus is responsible for the specificity of SAP97 for AMPARs or NMDARs. In its compact conformation, SAP97 is preferentially associated with GluA1-containing AMPARs, while in the extended conformation due to CASK binding, SAP97 is associated with GluN2B-containing NMDARs (Lin et al., 2013). However, this is in contradiction to the initial finding showing that only GluN2A associates with non-phosphorylated SAP97 (Gardoni et al., 2003) and that GluN1 cannot interact with SAP97 (Leonard et al., 1998). These discrepancies could be due to SAP97 isoforms, which have distinctive roles in the trafficking of AMPARs and NMDARs. It was shown that the synaptic pool of AMPARs is regulated by αSAP97 while βSAP97 is important for the extrasynaptic pools of both AMPARs and NMDARs (Li et al., 2011).

KINESIN MOLECULAR MOTORS

The long distance transport along dendrites or axons depends on microtubules and motor proteins such as kinesins. KIF17 was the first kinesin involved in the trafficking of approximately 50 nm vesicles containing the GluN2B subunit of the NMDARs (Setou et al., 2000). KIF17 interacts with the PDZ domain of mLin10/Mint1/X11, which binds to GluN2B though an additional interaction with the adaptor proteins mLin2/CASK and mLin7/MALS/Velis. Additional work showed that these GluN2B containing vesicles move with a speed of 0.76 μm/sec (Guillaud et al., 2003). This is quite different from the 0.07 mm/sec found for GluN1 subunits (Washbourne et al., 2002) but could represent a different population of receptors. More recently, it has been shown that the interaction between KIF17 and its cargo is regulated by CaMKII (Guillaud et al., 2008) and that synaptic activity can control cargo itinerary (Hanus et al., 2014). Using loss of function experiments, Hirokawa et al. showed that the loss of GluN2B is compensated by an increase in GluN2A subunits at synapses suggesting that KIF17 is somehow specific for GluN2B subunits (Guillaud et al., 2003). However, in a *kif17* KO, both GluN2A and GluN2B subunits are reduced at synapses by 22% and 43% respectively, but by two different mechanisms. GluN2B transport is inhibited and GluN2A subunits are also lost due to an accelerated degradation by the ubiquitin-proteasome system (Yin et al., 2011). Interestingly, mLin2/CASK associates with SAP97 to regulate the traffic of Kir2 or NMDARs early in the secretory pathway (Leonoudakis et al., 2004; Jeyifous et al., 2009; Lin et al., 2013), suggesting that KIF17 may direct post-ER transport of GluN2B through different adaptor complexes including KIF17/Mint1/CASK/MALS and KIF17/Mint1/CASK/SAP97. Another possibility is that the KIF17/Mint1/CASK complex binds to SAP102 as well as SAP97. However, only 43% of the synaptic GluN2B subunits are lost in the *kif17* KO suggesting that other kinesins and complexes are also involved in GluN2B trafficking. Indeed, KIF1bα has been shown to interact directly with PSD-95 and SAP97 (Mok et al., 2002). Even though SAP102 was not tested in this paper, the C terminus sequence of KIF1bα (RETTV) contains a potential class I PDZ domain-binding motif, S/T-X-V (S/T, Ser or Thr; X, any amino acids; V, hydrophobic amino acids) that may interact with PSD-95 relatives such as PSD-93 and SAP102. In summary, SAP102, PSD-95 or SAP97 and Mint1/CASK have been implicated in the early trafficking of NMDARs (**Figure 2**). The data are so far insufficient to attach a specific MAGUK to a specific kinesin or subunit. It is clear that GluN2B has received more attention than GluN2A and may be more often associated with trafficking complexes.

MYOSINS

KIF1b or KIF17 do not seem to enter directly into postsynaptic regions (Mok et al., 2002; Guillaud et al., 2003). Therefore, other means are needed to bring NMDAR to the PSD (Guillaud et al., 2008). It has been shown that the short distance transport inside the spine depends on actin and motor proteins such as myosins (Kapitein and Hoogenraad, 2011). Myosin Va or Vb and Myosin VI have been implicated in the trafficking of AMPARs (Wu et al., 2002; Osterweil et al., 2005; Lisé et al., 2006; Correia et al., 2008) but none of these have been involved in the regulation of NMDARs. While these are clearly involved in spine trafficking, other types of myosins (Lei et al., 2001; Amparan et al., 2005) may be involved in the delivery of NMDAR to the PSD. Other possible mechanisms for reaching the PSD include lateral diffusion along the extrasynaptic membrane (Choquet and Triller, 2013).

MECHANISMS REGULATING SYNAPTIC NMDARs

NMDAR DISTRIBUTION AND FUNCTION AT SYNAPSES AND EXTRASYNAPTIC REGIONS OF NEURONS

Literature on the function of NMDARs at synapses is extensive and we only can summarize it briefly in this review (see reviews: Petralia and Wenthold, 2008; Petralia et al., 2009; Traynelis et al., 2010; Paoletti et al., 2013; Sanz-Clemente et al., 2013b; Horak et al., 2014). The basic components of a synapse include: (1) the presynaptic terminal, which has a presynaptic membrane region called an active zone where synaptic vesicles dock to release glutamate into a synaptic cleft between the pre- and post-synaptic processes; and (2) the postsynaptic membrane, which contains the synaptic receptors that are bound to and/or associate with a complex of proteins that make up the postsynaptic density. Usually the postsynaptic process is either a dendrite shaft or a spine extending from a dendrite shaft. NMDARs are found on all parts of the synapse, including the pre- and postsynaptic membranes as well as extrasynaptic membrane areas that surround the synapse proper.

Typically, NMDARs of mature postsynaptic membranes are made of GluN1 combined with GluN2A or GluN2B, or both, as noted in the first section of this review. Other GluN2 subunits have more restricted distributions, such as GluN2C in the cerebellum and olfactory bulb and GluN2D in central parts of the forebrain and in the midbrain in mature animals. GluN2B and GluN2D are widespread in the embryonic and early postnatal brain. GluN3A is also a common NMDAR subunit in early postnatal development, while GluN3B appears mainly later in development. GluN2B and GluN2D may be the GluN2 subunits of the most common extrasynaptic NMDARs, and in fact, GluN2D may be exclusively extrasynaptic (Brickley et al., 2003; Harney et al., 2008). Mature neurons of the forebrain may show a prevalence for extrasynaptic NMDARs with GluN2B and synaptic ones with GluN2A (Tovar and Westbrook, 1999; reviewed in Hardingham and Bading, 2010; Gladding and Raymond, 2011; Parsons and Raymond, 2014), but other studies have not seen a clear delineation between preferential localization of these 2 subunits (Groc et al., 2006; Thomas et al., 2006; Harris and Pettit, 2007; Petralia et al., 2010). In addition, differences in GluN2A/GluN2B receptor composition in synapses have been found between left and right CA3 inputs onto CA1 pyramidal cells of the adult hippocampus (Shinohara et al., 2008). Synaptic spines that receive presynaptic terminals from the left CA3 (on both sides of the brain) are smaller and have a high density of GluN2B receptors, while spines receiving input from the right CA3 are larger and richer in GluN2A and GluA1 receptors.

Presynaptic NMDARs may be more widespread during neuronal differentiation and may be involved in guidance of the

axonal growth cone (e.g., Wang et al., 2011; they also function at some developing and mature synapses (e.g., Jourdain et al., 2007; Larsen et al., 2011; Duguid, 2013)).

At the synapse, NMDARs are associated with scaffolding proteins of the postsynaptic density, especially the MAGUKs (PSD-95, PSD-93, SAP102, SAP97), as noted in the previous sections. The PDZ binding domain at the C-terminus of GluN2A and GluN2B binds to the first and the second PDZ domains of MAGUKs (Kornau et al., 1995; Niethammer et al., 1996). But NMDARs also may bind to MAGUKs via other domains (Cousins et al., 2009; Bard et al., 2010; Chen et al., 2011). PSD-95 and SAP102 are the main MAGUKs present in most mature forebrain synapses and both of them interact with GluN2A and GluN2B (Sans et al., 2000). PSD-95 is almost immobile in the PSD and forms an organized structure (Blanpied et al., 2008) maybe because of the presence of palmitoylation sites of the protein (El-Husseini et al., 2000); while SAP102 appears to be more widespread in the cytoplasm and extrasynaptic sites, in addition to its presence in the postsynaptic density (Müller et al., 1996; Sans et al., 2000, 2003; Standley et al., 2000). Furthermore, the majority of SAP102 in spines turns over within 5 min and its mobility is dependent on actin and glutamate receptor activation (Müller et al., 1996; Zheng et al., 2010, 2011). Indeed, during development, there may be a more prevalent association of SAP102 with GluN2B-containing NMDARs while PSD-95 may associate more with GluN2A (Sans et al., 2000; Petralia et al., 2005). In the superior colliculus and the visual cortex, after eye opening, synaptoneurosomal PSD-95 is bound to more GluN2A-rich NMDARs and less GluN2B-rich NMDARs, but the amount of the auxiliary protein, stargazin, bound to PSD-95, remains constant (Yoshii et al., 2003). In retinal ganglion cell synapses, GluN2A, the GluN1-c2' variant, PSD-93, and PSD-95 are associated with the PSD, while GluN2B, the GluN1-c2 variant, and SAP102 tend to be perisynaptic (Zhang and Diamond, 2009). However, such a preferential association is not always clear, and mainly at mature synapses (Al-Hallaq et al., 2007; Petralia et al., 2010). In 2008, Nicoll et al. showed that SAP102 can traffic either GluN2A or GluN2B to the synapse, but PSD-95 selectively traffics GluN2A (Elias et al., 2008). Later, Groc et al. showed that this could be due to a specific divalent interaction (Bard et al., 2010). Other proteins may hold NMDARs in the synapse such as EphB receptors that associate with the extracellular N-terminus of NMDARs (Dalva et al., 2000); extracellular matrix proteins such as reelin may also affect NMDAR composition at synapses (Groc et al., 2007). NMDARs also can have an auxiliary subunit, Neto1 (complement C1r/C1s/Uegf/Bmp1 domain-containing neuropilin tolloid-like 1 protein), which may be important for synaptic plasticity (Ng et al., 2009). Electrophysiology and immunogold electron microscopy studies with Neto1 and Neto1/Neto2 knockout mice, respectively, found an increase in GluN2B-containing NMDARs at hippocampal CA3 mossy fiber synapses (Wyeth et al., 2014). Neto1 may form part of a trafficking complex that also includes NMDARs, MAGUKs and amyloid precursor protein (APP; Cousins et al., 2013).

Extrasynaptic NMDARs may associate with scaffolding proteins also, including MAGUKs and GIPC (**Figure 4**; Yi et al., 2007; Petralia et al., 2010; reviewed in Gladding and Raymond, 2011; Petralia, 2012). However, most extrasynaptic NMDAR sites show little ultrastructural specialization, probably because there are relatively few proteins accumulated at these sites (Petralia et al., 2002, 2010; review: Petralia, 2012). Extrasynaptic NMDARs may be associated with a different set of proteins, involved in a different cell pathway, compared to NMDARs of the synapse. At least in some cases, activation of synaptic NMDAR pathways may upregulate neuronal cell functional plasticity and survival, while activation of extrasynaptic ones may turn on pathways leading to neurodegeneration (Liu et al., 2007; Hardingham and Bading, 2010; Gladding and Raymond, 2011; Bartlett and Wang, 2013; Karpova et al., 2013); and synaptic vs. extrasynaptic NMDARs also may be tied to pathways leading to LTP vs. long-term depression (LTD), respectively (Bartlett and Wang, 2013). In addition, NMDAR function is gated by different co-agonists, D-serine and glycine, in synaptic and extrasynaptic NMDARs, respectively (Papouin et al., 2012).

NMDAR function at synapses can be modified either by modulating the function of individual NMDAR complexes or by changing the composition or number of NMDARs in the synapse (see reviews: Rebola et al., 2010; Paoletti et al., 2013). The function of individual complexes can be modulated in many ways, including co-agonist activation, inhibition by extracellular zinc, as well as effects of polyamines and redox modulators. The C-terminus is subject to modulation via phosphorylation at several sites, and these can affect the strength of NMDAR-mediated currents and calcium permeability. Also, many G protein-coupled receptors, such as M1 muscarinic, LPA, metabotropic glutamate, and PACAP1 receptors enhance NMDAR function via phosphorylation events. Unfortunately, there is no room here to discuss these in any detail. Thus we will concentrate in the following sections only on changes in trafficking of NMDARs at synapses.

MECHANISMS OF NMDAR MOVEMENTS: ENDOCYTOSIS AND RECYCLING

While there may be distinct, relatively stable, functional populations of NMDARs in extrasynaptic locations as well as in the synapse, other extrasynaptic NMDARs may be more mobile—trafficking en route to or from a synapse (see previous section of this review, and reviews by Gladding and Raymond, 2011; Petralia, 2012; Paoletti et al., 2013). NMDARs destined for the synapse may exocytose at sites away from the synapse, either along the dendrite or on the sides of spines (Petralia et al., 2003; Washbourne et al., 2004), while endocytosis also may occur in these areas (**Figure 5**; Blanpied et al., 2002; Petralia et al., 2003; Rácz et al., 2004; Pérez-Otaño et al., 2006). Prior to synapse formation in early postnatal development, NMDARs appear to migrate to and from the cell surface in cycles of exo- and endocytosis (Washbourne et al., 2004). The dynamic movements of excitatory and inhibitory receptors involve constant switching between mobile and immobile states, depending on thermal agitation and the reversible binding to stable elements including scaffolding and cytoskeletal anchoring proteins, both in the postsynaptic density and in extrasynaptic sites (reviewed in Choquet and Triller, 2013). Most of the mobility studies of

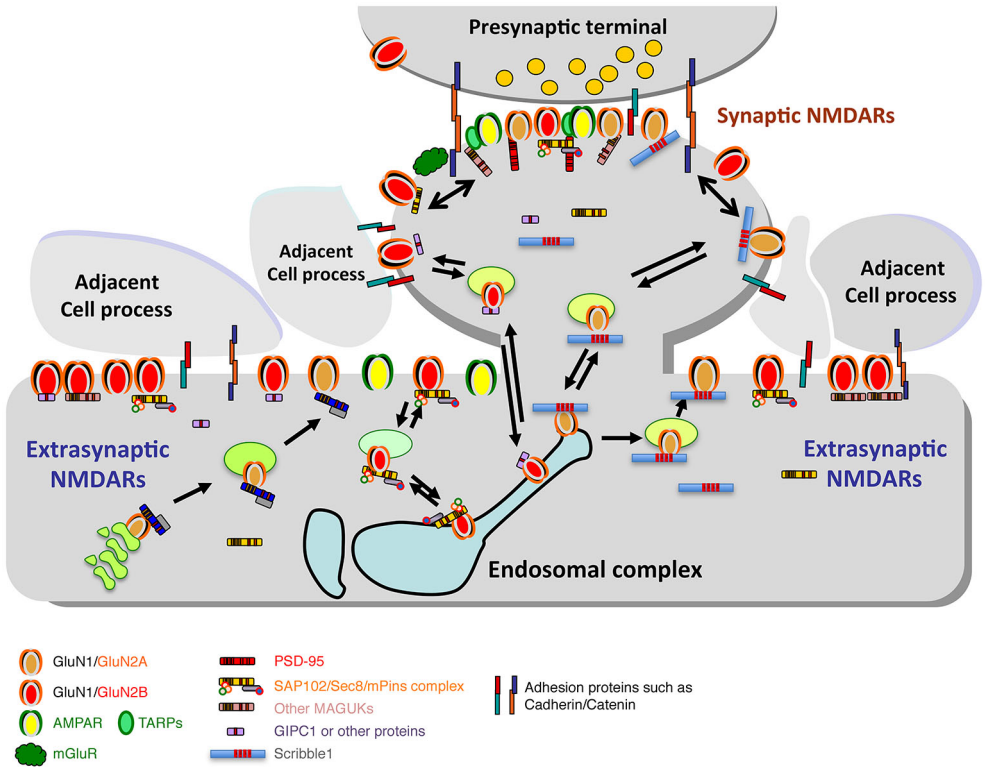


FIGURE 4 | Diagram illustrating the synaptic and extrasynaptic distributions of NMDARs and associated scaffolding and adhesion proteins, and especially the associations of extrasynaptic NMDARs with adjacent cell processes. Note that other GluRs (AMPARs, kainate and delta iGluRs, and metabotropic GluRs (mGluRs)) are found at synapses and in extrasynaptic locations. AMPARs are typically the most abundant GluRs at synapses and may also be more common than NMDARs in extrasynaptic locations in some neurons. mGluRs are also widespread; some forms are particularly abundant in the perisynaptic zone. Like NMDARs, these GluRs also show close associations with other proteins that affect their trafficking and localization (not illustrated here). Trafficking of NMDARs through the Golgi pathway and/or endosomes is mediated by a number of associated proteins such as MAGUKs and Scribble1 (see text for details). Diagram and legend text is modified from Figure 4 of Petralia, 2012.

GluRs have involved AMPARs, but Groc et al. (2006), looking at NMDARs in dissociated hippocampal neuron cultures, found that the latter are more stable overall compared to AMPARs, with GluN2B-containing receptors having more surface mobility than those with GluN2A. Unlike AMPAR mobility, NMDAR mobility does not seem to be affected by TTX or KCl; thus, NMDARs may be more tightly attached to their surface positions (Groc et al., 2004). This is consistent with ideas of stable NMDAR populations both at synapses and in some extrasynaptic areas. Harris and Pettit (2007), using acute hippocampal slices, found little evidence of exchange of NMDARs between synaptic and extrasynaptic pools, which contained about 35% of the dendritic NMDARs. In contrast, Tovar and Westbrook (2002), using dissociated hippocampal neuron cultures, found an exchange of about 65% of synaptic NMDARs with extrasynaptic NMDARs in 7 min. Bard et al. (2010) also found evidence for rapid exchange of NMDARs in cultured hippocampal neurons.

Endocytosis of NMDARs utilizes clathrin-coated vesicles via association of the receptors with the AP-2 adaptor complex (Roche et al., 2001; Petralia et al., 2003; Lavezzari et al., 2004; Prybylowski et al., 2005); however, internalization may occur by an alternative, non-clathrin mediated endocytosis mechanism (Swanwick et al., 2009). Interaction of GluN2A with AP-2 is via a C-terminus dileucine motif (Lavezzari et al., 2004) although an additional AP-2 binding motif may be involved (Vissel et al., 2001). The AP-2 binding motif of GluN2B is YEKL, close to the C-terminus (Roche et al., 2001); MAGUK-dependent, fyn kinase-mediated phosphorylation of Tyr1472 in this motif prevents internalization and increases synaptic NMDAR currents (Prybylowski et al., 2005). NMDARs can take different pathways following their internalization and incorporation into early/sorting endosomes; thus, GluN2A-containing NMDARs preferentially traffic to late endosomes for degradation, while GluN2B-containing NMDARs tend to move to recycling endosomes from where they can return to the surface and to synapses (Lavezzari et al., 2004). Recently, we showed that Scribble1 could prevent GluN2A subunits from undergoing lysosomal trafficking and degradation by increasing their recycling to the plasma membrane following NMDAR activation (Piguel et al., 2014). We also showed that Arf6 and the Arf6-specific guanine nucleotide exchange factor (EFA6) are involved in that process. Interestingly, EFA6 is a partner of sorting nexin-1 (SNX1), a retromer component that is implicated in endosomal sorting and trafficking (Fukaya et al.,

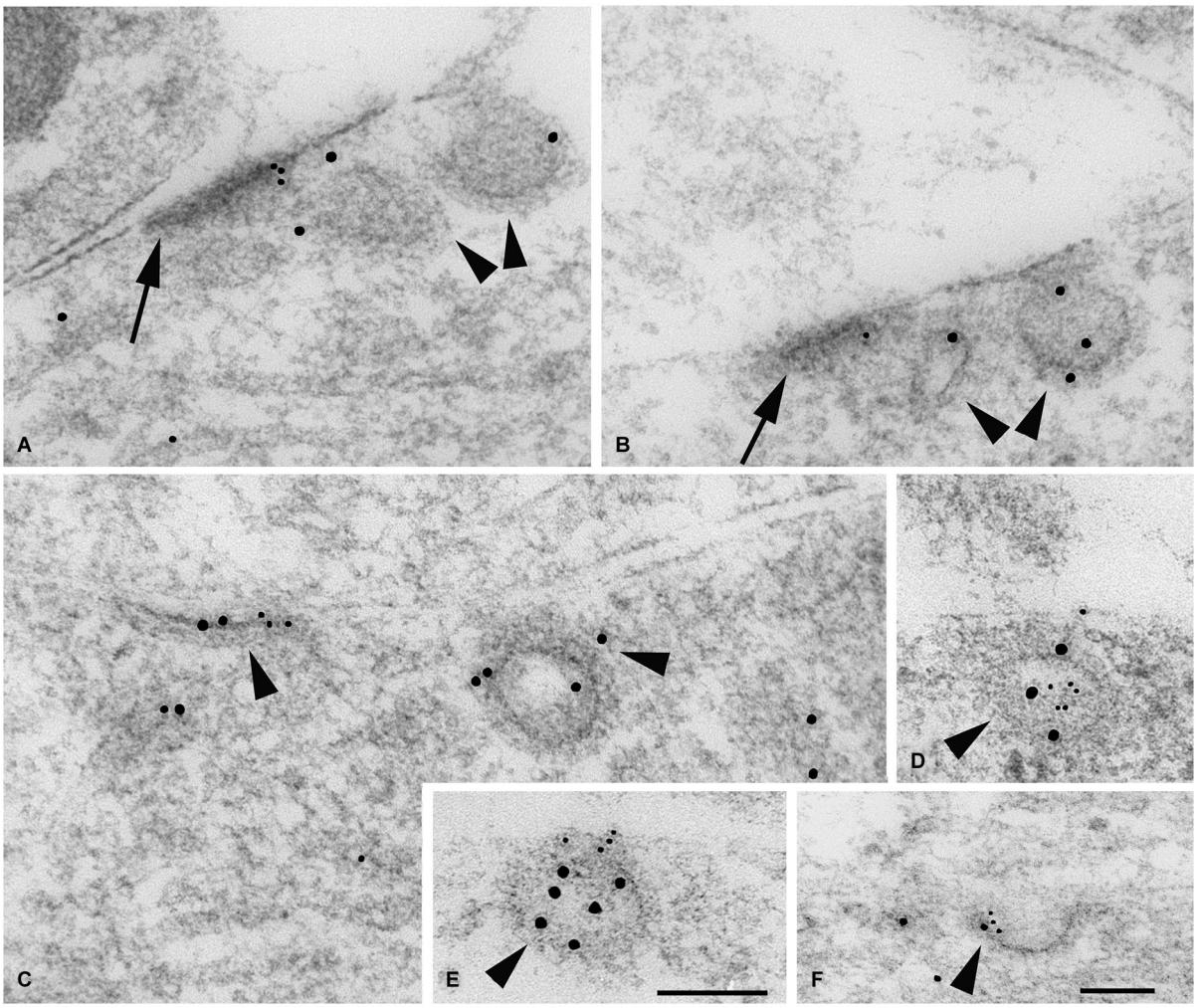


FIGURE 5 | Double immunogold labeling of clathrin-coated pits/vesicles (CCP/Vs; arrowheads) associated with bare densities (A,B; arrows) and extrasynaptic membrane regions (C–F) in the P2 hippocampus CA1 stratum radiatum with GluN1 (A–C,F) or GluN2A/B (D,E) antibody (5 nm gold), and clathrin (A–E) or adaptin α (F) antibody (10 nm gold). **(A,B)** These two "bare" densities on dendrites actually show fairly close associations with adjacent processes. In both micrographs, a definitive CCP/V is seen in the vicinity of the density, and a second probable CCP/V is evident closer to the density. **(C,F)** In C, GluN1 and clathrin antibodies label an early, flat CCP/V adjacent to a CCP/V that is pinching off, and GluN1 and adaptin α label a better-developed CCP/V in F (both are dendrites). **(D,E)** GluN2A/B and clathrin antibodies label a newly formed CCV in **(E)**, and another CCV in **(D)** in a process at a point where the latter is contacted by another process. Scale bars, 100 nm. Scale in **(E)** is valid for micrographs **(A–E)**. Figure is a reprint of Figure 3 from Petralia et al., 2003.

2014) and Scribble1 has also been shown to be implicated in the retromer localization to endosomes in epithelial cells (de Vreede et al., 2014). The retromer functions as a well-known complex involved in the retrograde transport from endosomes to the Golgi (Collins, 2008; Seaman et al., 2013) and is highly expressed in the hippocampus. Von Zastrow et al. recently showed that it is essential for functional surface expression of AMPARs and NMDARs at synapses (Choy et al., 2014). This function also may involve a complex of retromer and SNX27, which contains an N-terminal PDZ domain (Wang et al., 2013; Gallon et al., 2014). Moreover, many other proteins can affect NMDAR membrane expression differently and influence or change the ratio between GluN2A and GluN2B. These include PDZ proteins (i.e., MAGUKs) as already discussed (Losi et al., 2003; Sans et al., 2003, 2005; Chung et al., 2004; Mauceri et al., 2004; Howard et al., 2010), SNARE-related proteins (Lau et al., 2010; Suh et al., 2010) and kinases (Prybylowski et al., 2005; Sanz-Clemente et al., 2010, 2013a).

The mechanisms involved seem to be a bit different for GluN3 subunits. Endocytosis of GluN3A-containing NMDARs is mediated by PACSIN1 (protein kinase C and casein kinase substrate in neurons protein 1)/syndapin1, a neuron-specific accessory protein controlling clathrin-mediated endocytosis; PACSIN1 binds to the C-terminus of GluN3A via PACSIN1's NPF motif (Pérez-Otaño et al., 2006). A novel endocytic motif (YWL) located within the cytoplasmic C-terminal tail of GluN3A is

involved in the binding to the clathrin adaptor AP-2 (Chowdhury et al., 2013).

DEVELOPMENTAL CHANGES IN SYNAPTIC NMDAR NUMBER AND COMPOSITION

As we noted above, there are changes in NMDAR composition during development; NMDARs in early postnatal development mainly contain GluN2B or GluN2D, while GluN2A and GluN2C are more prevalent in adults. A downregulation of GluN2D-containing NMDARs in CA1 hippocampal pyramidal neurons may explain the great decrease in sensitivity to magnesium block beginning at P4 (Kirson et al., 1999). But especially there is a major switch from NMDARs with GluN2B to those with GluN2A. In the hippocampus, GluN2B is high at synapses at P2 (postnatal day 2) and there is a gradual decrease of GluN2B with age, as GluN2A increases; adults still show some NMDARs with GluN2B but GluN2A dominates (**Figure 6**); this also is accompanied by a similar switch in MAGUKs from mainly SAP102 to mainly PSD-95 plus some SAP102 (Sans et al., 2000; Petralia et al., 2005). This suggests that GluN2A-containing NMDARs are necessary for many functions found in the adult. In contrast, motoneurons in areas of the brain associated with the suckling reflex needed immediately after birth may already have high levels of GluN2A early in development (Oshima et al., 2002). The switch from GluN2B- to GluN2A-containing NMDARs is a conserved phenomenon among mammals and has been shown to occur in human development (Jantzie et al., 2013). In the rat hippocampus, activity induces a rapid change from GluN2B- to GluN2A-containing NMDARs, and this is bidirectional depending on activity (Bellone and Nicoll, 2007); in fact, studies of single synapses indicate that inactivity in silenced spine synapses enhances NMDAR currents and increases the number of GluN2B-containing NMDARs (Lee et al., 2010). At least in some cases, the switch is controlled by learning experiences such as with vision (light vs. dark rearing; Quinlan et al., 1999a,b). Stimuli that induce LTP induce the switch from GluN2B- to GluN2A-containing NMDARs in young animals (Bellone and Nicoll, 2007); this effect is not seen in older animals. As expected, regulation of the switch is controlled by calcium and phosphorylation. For example, casein kinase 2 (CK2) phosphorylates

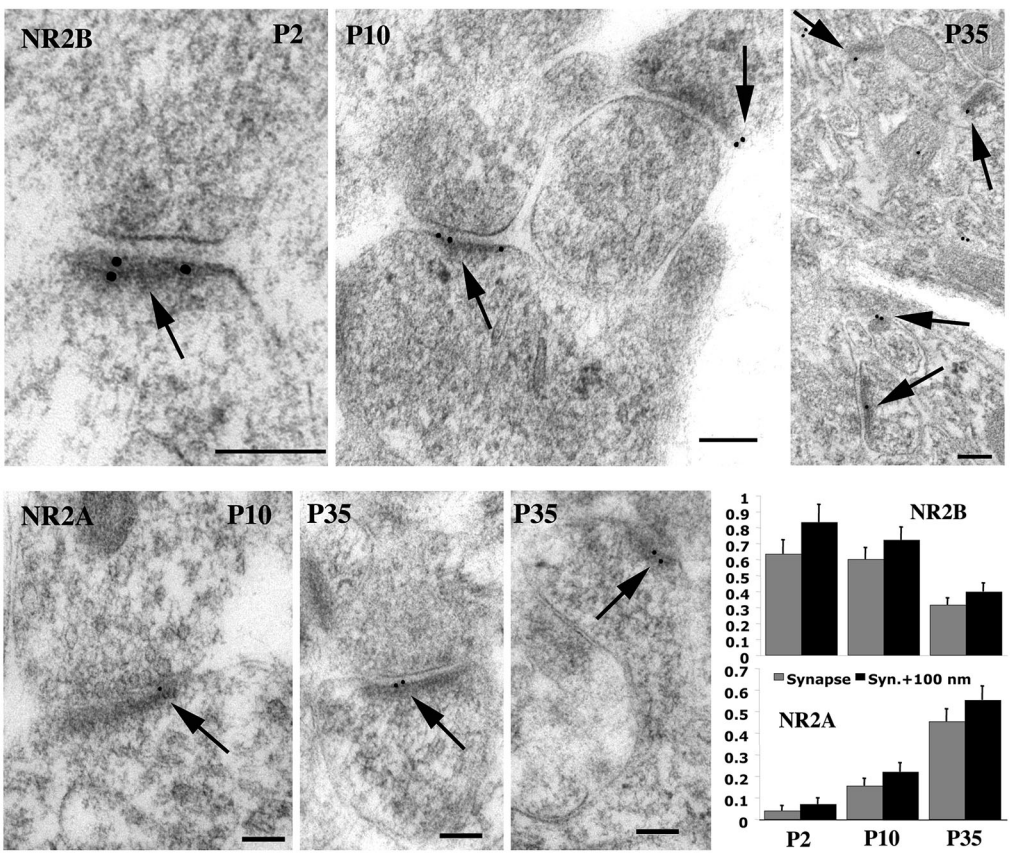


FIGURE 6 | Immunogold labeling for GluN2A (= NR2A) and GluN2B (= NR2B) in synapses during postnatal development of hippocampus CA1 stratum radiatum. Micrographs illustrate the decrease in GluN2B and increase in GluN2A at synapses during development. For GluN2B, there was a significant decrease from P2 to P35 and from P10 to P35; 30%, 33%, and 23% of synapses were labeled for P2, P10, and P35, respectively. The Y-axis indicates gold per synapse (= synaptic cleft plus postsynaptic density) or per synapse + 100 nm (= 100 nm below the postsynaptic membrane). Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the postsynaptic density, and histograms show values plus standard errors. Figure is a reprint of Figure 6 from Petralia et al., 2005.

synaptic GluN2B to drive its endocytosis and replacement by GluN2A in cortical and hippocampal neuron cultures (Sanz-Clemente et al., 2010). The switch in hippocampal neurons actually involves many components, including activation of NMDARs and mGluR5, PLC, PKC, and calcium-release from IP3 receptor-dependent stores (Matta et al., 2011). Experience-dependent epigenetic remodeling associated with the GluN2B to GluN2A switch is mediated by a transcription factor called REST (repressor element 1 silencing transcription factor); when activated, REST is recruited to the promoter of the gene for GluN2B, where it binds to a 23-base pair motif in the promoter, recruiting co-repressors that can remodel the chromatin (Rodenas-Ruano et al., 2012).

Also as noted above, GluN3A is prevalent in early development and is lost or reduced with age. GluN3A is believed to prevent the stabilization of premature synapses, and its downregulation is necessary for synapse maturation (Wong et al., 2002; Pérez-Otaño et al., 2006; Roberts et al., 2009). GluN3A can be found in both the pre- and postsynaptic processes, and during development of the visual cortex, presynaptic NMDARs appear to switch from GluN1/2B/3A to GluN1/2B (Larsen et al., 2011). In this case, the GluN3-containing presynaptic NMDARs promote glutamate release and spike timing-dependent LTD in the juvenile visual cortex, probably important for developing the early receptive field properties. After maturation, the now GluN3A-lacking NMDARs may be active under strongly depolarizing conditions to promote the facilitation of repetitive stimuli. Possible differences in the development and distribution of GluN3A between rodents and humans have been reported (Eriksson et al., 2007; Nilsson et al., 2007). Indeed, human GluN3A contains a proline rich motif in the C-terminus that is not found in rat GluN3A; this domain may bind SH3 domains to affect trafficking, although apparently not the SH3 domain of PSD-95 (Eriksson et al., 2007). The developmental switch from GluN2B to GluN2C-containing NMDARs in cerebellar granule cells is mediated by innervation from mossy fibers that release neuregulin, activating ErbB2 and ErbB4 receptors on the granule cells (Ozaki et al., 1997; Garcia et al., 2000; Hahn et al., 2006). During this time, surface delivery of GluN2C-containing NMDARs depends on its phosphorylation by protein kinase B and its subsequent association with protein 14-3-3 (Chen and Roche, 2009).

CHANGES IN NMDAR NUMBER AND COMPOSITION IN MATURE SYNAPSES

Generally, it has been thought that change in NMDAR composition at synapses is a developmental phenomenon accompanying the increase of AMPARs to adult levels (Sans et al., 2000; Petralia et al., 2005), and that adult plasticity involves changes in number and composition of AMPARs but not NMDARs (reviewed in Paoletti et al., 2013). Indeed, Bellone and Nicoll (2007) found no evidence of NMDAR plasticity in the CA1 region in hippocampal slices in 3 weeks postnatal Sprague-Dawley rats. A number of studies looking at NMDAR changes in the hippocampus of maturing rodents have concentrated on the period up to this point—from 2 to 3 weeks postnatal. Studies have shown evidence of bidirectional control of NMDAR GluN2A/GluN2B ratio in the Schaffer collateral/CA1 spine synapses of the hippocampus in slices from 2–3 week old Sprague-Dawley rats (Xu et al., 2009; Peng et al., 2010), i.e., looking at the period just prior to the 3 week limit indicated by Bellone and Nicoll (2007). Zhao et al. (2008) also provided evidence that stimulation can induce extrasynaptic NMDARs, mainly containing GluN2B (**Figure 4**), to move laterally into the synapse in 3 week-old hippocampal slices. In contrast to these studies showing changes in NMDARs only within 3 weeks postnatal, Grosshans et al. (Grosshans et al., 2002) found that LTP involves rapid PKC and Src-family dependent surface expression of NMDARs in the CA1 region of hippocampal slices from 6–8 week old rats. The reason for the difference is not clear but it may be that the latter study noted changes in extrasynaptic receptors. In support of this, studies of the visual cortex have indicated that there are later changes in extrasynaptic or presynaptic NMDARs (Yashiro et al., 2005; Larsen et al., 2011). Also, Harney et al. (2008) suggest that in the hippocampal dentate gyrus of 3–4 weeks old rats, extrasynaptic GluN2D-containing NMDARs are recruited to synapses during LTP. They presume that these receptors may be perisynaptic and that addition of these receptors to the synapse is a transient phenomenon; this is in contrast to other studies suggesting that GluN2D-containing NMDARs are exclusively extrasynaptic, as discussed above. Other studies in the 3–4 week period have shown the PKC-dependent insertion of NMDARs into mossy fiber synapses on CA3 hippocampal neurons during mossy fiber LTP (Kwon and Castillo, 2008) and orexin-induced, PLC/PKC-dependent insertion of NMDARs in synapses of dopaminergic neurons in the ventral tegmental area (Borgland et al., 2006). The latter synapses undergo the GluN2B to GluN2A switch in the first postnatal week, and interestingly, after this (and possibly even in adult mice), cocaine can evoke a switch to quasi-calcium impermeable NMDARs containing GluN3A and GluN2B (along with a switch to calcium-permeable AMPARs; Yuan et al., 2013). Subsequent recovery appears to be mediated by mGluR1, replacing the GluN3A-containing NMDARs with ones containing GluN2A again.

CONCLUDING REMARKS

In this brief review, we have highlighted some of the recent work on NMDAR assembly, ER exit to membrane and synapse trafficking of NMDARs. Because of its involvement in a critical function such as neurotransmission in a complex cell like a neuron, it is reasonable to propose that an NMDAR will interact with tens or maybe even hundreds of different proteins during its lifetime. Most of the identified interactions involve the cytoplasmic C-terminus of the GluN subunits. It is clear that more protein partners of NMDARs, including those that bind to extracellular and transmembrane domains (TMDs), await identification because they are not readily detectable using current assays, but may prove to be important to the trafficking and/or function of NMDARs. Furthermore, it is obvious that most studies dealing with the NMDARs have been performed using rat/mouse genes and thus the potential differences in trafficking of rodent and human NMDARs have been mostly neglected. However, human NMDARs exhibit similar functional and pharmacological properties to rodent NMDARs, consistent with the critical role that these receptor play in excitatory synapses (Hedegaard et al., 2012).

Therefore, we expect that future studies will identify novel protein partners of the NMDARs as well as will address where the protein interactions of the NMDARs occur and how these interactions are regulated. Indeed, this knowledge will shed new light on our understanding of the different stages of processing, synaptic delivery, synaptic retention, and degradation of NMDARs and will also enable us to find new strategies to treat some human brain disorders.

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Modulation of the glutamatergic transmission by Dopamine: a focus on Parkinson, Huntington and Addiction diseases

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Dopamine (DA) plays a major role in motor and cognitive functions as well as in reward processing by regulating glutamatergic inputs. In particular in the striatum the release of DA rapidly influences synaptic transmission modulating both AMPA and NMDA receptors. Several neurodegenerative and neuropsychiatric disorders, including Parkinson, Huntington and addiction-related diseases, manifest a dysregulation of glutamate and DA signaling. Here, we will focus our attention on the mechanisms underlying the modulation of the glutamatergic transmission by DA in striatal circuits.

Keywords: Dopamine, NMDA receptor, AMPA receptors, Addiction, Parkinson disease, Huntington disease

INTRODUCTION

Dopamine (DA) is a catecholamine that acts as neuromodulator by playing an important role in motor and cognitive functions as well as in reward processing.

Our major understanding of the DA transmission derives from studies of the midbrain DA system that comprehend both Substantia Nigra pars compacta (SNc-A9) and Ventral Tegmental Area (VTA—A10). The former is at the origin of the nigrostriatal pathway where DA neurons project to the dorsal striatum and play a central role in controlling fine motor functions. Instead DA neurons within the VTA form the mesostriatal pathway and project to the ventral striatum (or Nucleus accumbens, NaC) exerting an important role in reward processing (Paillé et al., 2010; Tritsch and Sabatini, 2012). How does DA shape all these different functions in the brain? In both circuitries, DA acts as a neuromodulator regulating the glutamatergic inputs onto the principal neurons and therefore controlling the striatal output. More than 95% of striatal neurons are represented by Medium Spiny Neurons (MSNs; Kreitzer, 2009) that form asymmetric synapses with glutamatergic projections and symmetric contacts at the DA inputs. Therefore, the activity of DA neurons and the consequent release of DA in the proximity of the synaptic cleft rapidly influences synaptic transmission, intrinsic excitability and dendritic integration (Tritsch and Sabatini, 2012), partially explaining the different functions of DA in the brain. Importantly DA can modulate glutamatergic transmission by the convergence effect onto MSNs, by acting on D2-R located presynaptcally on Glutamatergic inputs or by modulating excitatory inputs onto GABAergic and Cholinergic interneurons.

Interestingly, several neurodegenerative and neuropsychiatric disorders, including Parkinson, Huntington and addiction-related diseases, manifest a dysregulation of glutamate and DA signaling within the striatum. In this review, we will focus our attention on the mechanisms underlying the modulation of the glutamatergic transmission by DA in the nigrostriatal and mesostriatal circuitries (**Figure 1**).

NIGROSTRIATAL CIRCUIT

DA neurons of the SNc project to the dorsal striatum. This structure is mainly populated by MSNs that are classified in two populations according to their axonal projections and DA receptor expression. DA receptor type 1 (D1R)-containing MSNs form the direct pathway and send their axons to the GABAergic output nuclei of the basal ganglia, the internal segment of the Globus Pallidus (GPi) and the Substantia Nigra pars reticulata (SNr), which in turn send their afferences to the motor nuclei of the thalamus. DA receptor type 2 (D2R)-containing MSNs constitute the indirect pathway and send their axons to the external segment of the Globus Pallidus (GPe), which in turn project to the glutamatergic neurons of the Sub-Thalamic Nucleus (STN). STN neurons then send their axons to the basal ganglia output nuclei (GPi and SNr) where they form excitatory synapses on the inhibitory output neurons. Activation of the direct and indirect pathway exerts an opposite effect on movement: activation of the direct pathway disinhibits the thalamocortical projections and leads to activation of the cortical premotor circuits facilitating movements. The activation of the indirect pathway instead inhibits the thalamocortical projection neurons reducing the premotor drive and inhibiting movements (Kreitzer and Malenka, 2008). Interestingly this model has been

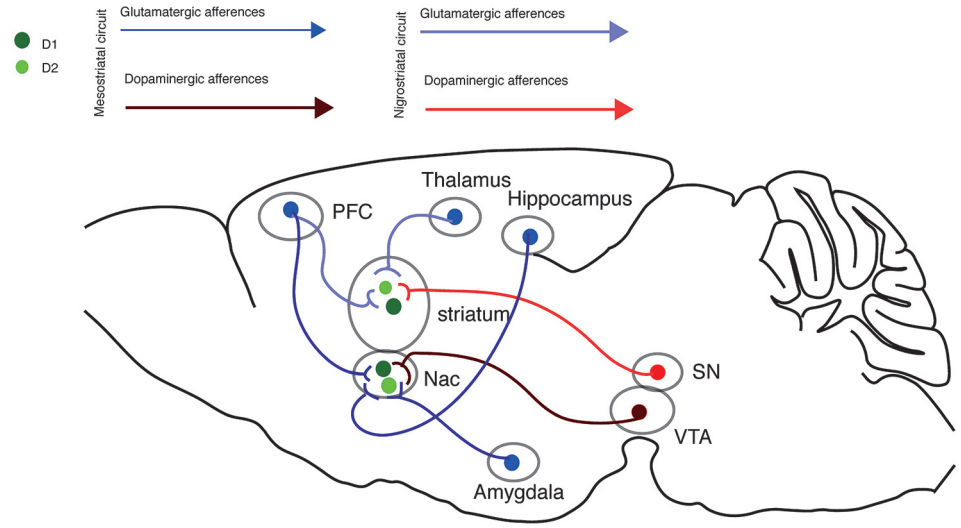

FIGURE 1 | Nigrostriatal and Mesostriatal circuits. Sagittal view of the excitatory inputs onto the nigrostriatal and mesostriatal circuits.

recently challenged and it has been proposed that the two pathways are structurally and functionally intertwined (Dunah and Standaert, 2001; Calabresi et al., 2014).

By acting on D1R or D2R, DA differently modulates the activity of the direct and indirect pathway both controlling the excitability of MSNs in the striatum and governing synaptic plasticity at different glutamatergic inputs. The majority of glutamatergic afferents onto the dorsal striatum originates in the cortex and thalamus. While corticostriatal afferences may carry motor and cognitive information, thalamostriatal ones convey information for the reward saliency and the wakefulness (Huerta-Ocampo et al., 2014). Despite this view, both corticostriatal and thalamostriatal terminals form synaptic contacts with D1 and D2 MSNs and the convergence of their inputs suggests that they are similarly involved in activation of the MSNs.

Profound functional differences in these pathways have been found, suggesting input-dependent differences in synaptic functions (Smeal et al., 2008). Future studies are needed to investigate the input segregation onto the direct and indirect striatal pathways and their functional implications.

MESOSTRIATAL CIRCUIT

This circuit originates in the VTA where DA neurons project to D1 and D2 MSNs of the ventral striatum. Although the presence of D1 and D2 MSNs in the ventral striatum is well established, there are several evidences showing that projections from the NAc may not be so segregated as for the dorsal striatum. Indeed, it has been shown that both D1 and D2 MSNs project to the ventral pallidum, while D1 MSNs can also directly project to the VTA (Lu et al., 1998; Zhou et al., 2003; Smith et al., 2013). Despite these differences, it is well established that D1 and D2 MSNs in the NAc exhibit different electrophysiological properties (Paillé et al., 2010; Pascoli et al., 2011b, 2014b) and respond differently to VTA stimulation (Grueter et al., 2010; Paillé et al., 2010). Despite this clear segregation of D1 and D2 containing MSN, it should be mention the existence of a small population of neurons containing both D1Rs and D2Rs (Matamales et al., 2009).

Similarly to the nigrostriatal circuit, DA modulates and integrates glutamatergic synaptic inputs from the prefrontal cortex, the amygdala and the hippocampus. Interestingly, different forms of synaptic plasticity have been described at different excitatory inputs onto D1 and D2 MSNs suggesting that specific pattern of neuronal activity coinciding with DA signal are needed for specific reward-related behavioral outcomes (Paillé et al., 2010; Pascoli et al., 2014b).

DA RECEPTORS AND SIGNALING PATHWAYS

DA transmission is mediated by Guanine nucleotide binding Protein Coupled Receptors (GPCRs). They are metabotropic receptors with seven transmembrane domains coupled to G-proteins that lead to the formation of second messengers and the activation or inhibition of subsequent signaling cascades. Although five different DA receptors have been cloned so far, it is possible to classify them in two major populations according to their structures and their pharmacological properties: (a) D1-like receptors (D1 and D5) which stimulate cAMP production; and (b) D2-like receptors (D2, D3 and D4) which reduce the intracellular cAMP levels. The ability of D1-like and D2-like receptors to modulate in opposite directions the concentration of cAMP, and thus the downstream signal transduction, depends on their interaction with specific G proteins.

D1-like receptors are the most highly expressed DA receptor in the brain, are mainly localized within the forebrain and, compared to the D2-like family, have a highly conserved sequence (Tritsch and Sabatini, 2012). Binding of DA with D1-like receptors leads to an increase in the adenylyl cyclase activity and a consequent rise in cAMP levels. This pathway induces the activation of protein kinase A (PKA) and the

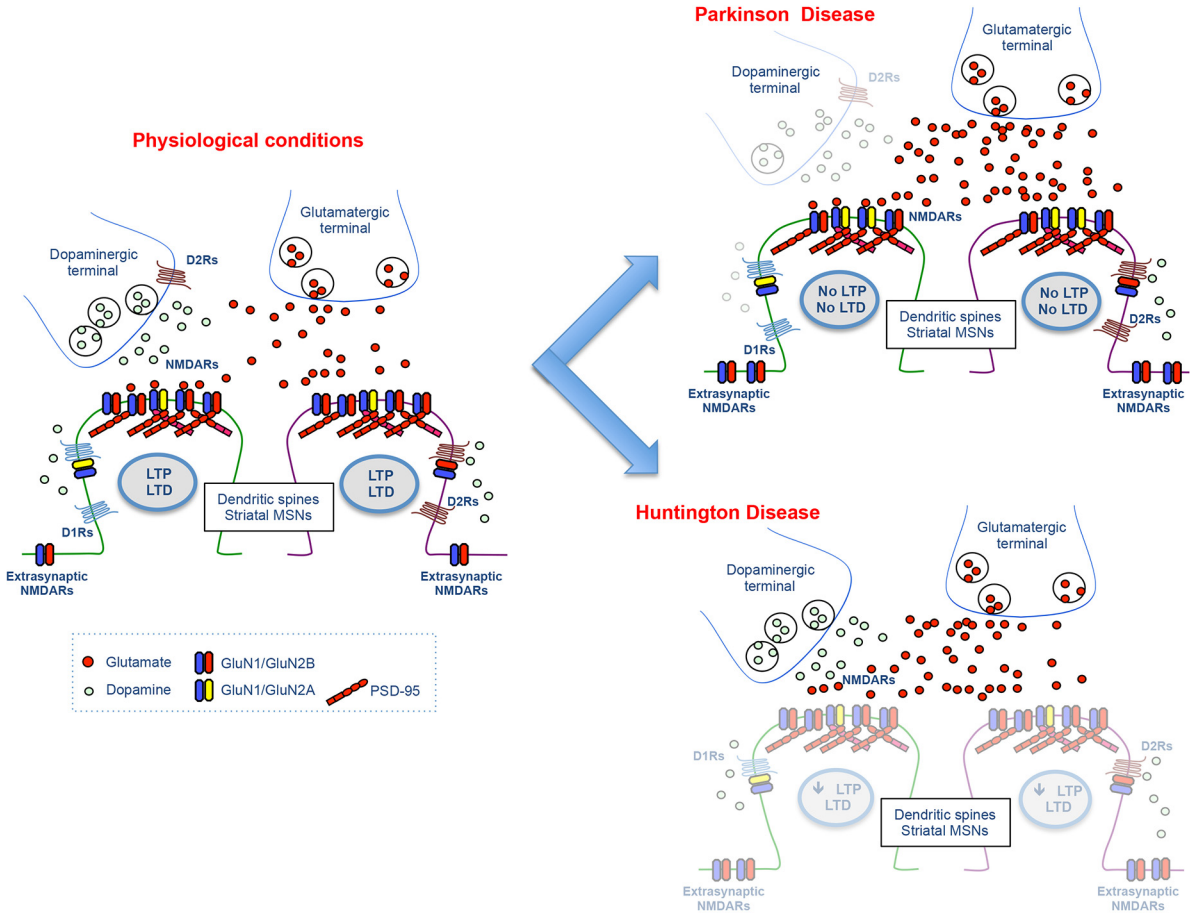

FIGURE 2 | Molecular and functional changes at the glutamatergic synapse in Parkinson and Huntington disease. The cartoon illustrates the physiological glutamatergic corticostriatal synapse (left panel) and the molecular and functional alterations at DA and NMDA receptor level observed in experimental models of Parkinson and Huntington disease (right panels).

phosphorylation of different substrates as well as the induction of immediate early gene expression that contribute to the overall D1R response (Beaulieu and Gainetdinov, 2011). DARPP-32 (DA and cAMP-regulated phosphoprotein, 32kDa) is one of the most studied PKA substrates activated by DA and provides a mechanism for integrating information at dopaminoceptive neurons (Svenningsson et al., 2004). Via the control of Protein Phosphatase-1 (PP-1), DARPP-32 regulates neuronal excitability as well as glutamatergic transmission. Activation of the cAMP/PKA/DARPP-32 pathway indeed increases the opening of the L-type Ca2+ channels promoting the transition of MSNs to a higher level of excitability (Vergara et al., 2003). At the same time, the activation of this pathway promotes the phosphorylation of both AMPARs and NMDARs providing a mechanism for the direct control of glutamatergic transmission by DA signaling (Snyder et al., 1998, 2005).

There are multiple modulatory effects following D2R activation. First of all, these receptors are coupled with Gi/o proteins and their activation negatively modulates cAMP signaling, reducing the phosphorylation of the downstream proteins (PKA targets), such as DARPP-32. At the same time, activation of D2R, via the Gβγ subunits, inhibits L-type Ca^{2+} channels and activates G-protein-coupled Inwardly Rectifying potassium (K^{+}) channels (GIRK) causing a decrease of neuronal excitability and a reduction in the synthesis and release of DA (Kebabian and Greengard, 1971). Moreover D2Rs are also located presynatpically onto the excitatory inputs where influence glutamate release and on ChaT interneurons in the striatum where contribute to reducing Ach release (Surmeier et al., 2007).

Interestingly, DA has a lower affinity for D1Rs compared to D2Rs, pointing at a different effect on the direct and indirect pathway during tonic or phasic DA release. Indeed, it has been suggested that phasic release activates D1Rs to facilitate limbic inputs while tonic release bidirectional activates D2Rs on PFC inputs (Floresco et al., 2003; Goto and Grace, 2005; Goto et al., 2007). It is important to consider the different effects of DA change the functions of the brain regions that receive DA inputs. Indeed, an altered DA modulation of the excitatory inputs onto these regions plays an important role in the pathophysiology of many neurological disorders (Goto et al., 2007).

DA MODULATION OF NMDARs AND AMPARs

DA modulates the functioning of the glutamatergic synapse by acting at different levels. The classical view indicates that DA can regulate the activity of ionotropic glutamate receptors with a reduction of AMPAR-evoked responses and an increase of NMDAR-evoked responses (Cepeda et al., 1993; Levine et al., 1996; Cepeda and Levine, 1998; Graham et al., 2009). In particular, activation of D1R usually leads to potentiation of NMDAR-dependent currents, while activation of D2R induces a decrease of AMPAR-dependent responses. This view has a key relevance in the striatum where dopaminergic terminals form synaptic contacts at the neck of MSN spines, while the head receives inputs from glutamatergic terminals (Surmeier et al., 2007).

Interestingly, NMDARs in the corticostriatal synapse show peculiar features. Indeed, even if GluN2B represents the predominant regulatory subunit expressed in this brain area (Dunah and Standaert, 2001), it has been proposed that GluN2A- but not GluN2B-containing NMDARs induce a depression of synaptic transmission that does not involve activation of corticostriatal neurons but it is rather mediated NMDARs at MSN synapses (Schotanus and Chergui, 2008a). Interestingly, recent reports have suggested that GluN2A and GluN2B subunits differentially contribute to the glutamatergic transmission in striatal MSNs (Paoletti et al., 2008; Jocoy et al., 2011). While genetic deletion or pharmacological blockade of GluN2A increase D1R-mediated potentiation of NMDAR-dependent responses, inhibition of GluN2B reduces this potentiation, suggesting a counterbalance of their respective functions. Moreover, it has shown that GluN2A subunits contribute mainly to NMDA responses in D1-MSNs, whereas GluN2B subunits is more involved in NMDA responses in D2R cells (Paoletti et al., 2008; Jocoy et al., 2011).

Several studies have investigated the effect of D1R stimulation on NMDAR subunit trafficking at the synaptic membrane. Pharmacological activation of D1R enhances NMDARs surface levels (Hallett et al., 2006; Paoletti et al., 2008) and NMDAR localization in the synaptosomal membrane fraction through stimulation of the tyrosine kinase Fyn (Dunah et al., 2004; Tang et al., 2007). In more detail, it has been shown that treatment with D1R agonist (SKF38393) leads to a significant decrease of GluN2A-containing NMDARs and to a concomitant increase in spine head width (Vastagh et al., 2012). Interestingly, co-treatment of corticostriatal slices with GluN2A antagonist (NVP-AAM077) and D1R agonist augmented the increase of dendritic spine head width observed with SKF38393 alone. Conversely, GluN2B antagonist (ifenprodil) blocked any morphological effect induced by D1 activation (Vastagh et al., 2012). However, further studies are still needed for a comprehensive understanding of the specific role of GluN2A- *vs*. GluN2B-containing NMDARs in the modulation of dendritic spine morphology at striatal MSNs.

BAC transgenic mice expressing EGFP in D1R- and D2R-positive cells (Valjent et al., 2009) has recently been used to carefully analyze DA-dependent modulation of MSNs within the direct and indirect pathways (Cepeda et al., 2008). In agreement with previous studies, D1R-dependent modulation of glutamate-evoked responses was correlated with the activation of direct pathway neurons. On the contrary, D2R-dependent reduction of glutamate-evoked responses was specific to the indirect pathway (André et al., 2010). Moreover, recent and advanced tools such as optogenetics and sophisticated Ca^{2+} imaging have shown that activation of D2 receptors decrease NMDAR-induced responses by presynaptic modulation of glutamate release (Higley and Sabatini, 2010).

Notably, several studies describing the co-existence of D1Rs and NMDARs at striatal MSN synapses indicate the presence of a possible direct molecular interaction between the two receptor systems (Kung et al., 2007; Heng et al., 2009; Kruusmägi et al., 2009; Jocoy et al., 2011; Vastagh et al., 2012). A direct interaction between these two receptors was originally proposed by Lee et al. (2002), who showed co-immunoprecipitation of D1R with GluN1/GluN2A subunits of the NMDAR. This interaction is not static, but is decreased by D1R activation (Lee et al., 2002; Luscher and Bellone, 2008). In addition, disruption of D1R interaction with GluN2A-containing NMDARs by interfering peptides is sufficient to induce a modulation of NMDAR currents thus suggesting a direct role for this receptor-receptor binding in NMDA-transmission (Lee et al., 2002; Brown et al., 2010). However, the issue is more complicated since in both striatal neurons and transfected HEK293 cells, D1R directly interacts with GluN1 subunit to form a constitutive oligomeric complex that is recruited to the plasma membrane by the presence of GluN2B subunit (Fiorentini et al., 2003). Moreover, this interaction abolishes D1R internalization, a crucial adaptive response that normally occurs upon agonist stimulation (Fiorentini et al., 2003).

More recent studies have applied high-resolution single nanoparticle live-imaging techniques to investigate the role of the dynamic interaction between D1R and NMDAR at hippocampal synapses (Ladepeche et al., 2013a). The prevention of the physical interaction between D1R and GluN1 by interfering peptide is able to fully abolished the synaptic stabilization of D1R, thus suggesting that D1Rs are dynamically retained at glutamatergic synapses through a mechanism requiring the interaction with NMDAR (Ladepeche et al., 2013a). Moreover, disruption of D1R/NMDAR complex increases NMDAR synaptic content through a fast lateral redistribution of the receptors, and favors long-term synaptic potentiation (Ladepeche et al., 2013b). In particular, D1R activation reduces D1R/GluN1 interaction at perisynaptic sites and allows the lateral diffusion of NMDARs into the postsynaptic density where they support the induction of Long-term potentiation (LTP; Argilli et al., 2008; Ladepeche et al., 2013b).

D2-type DA receptors also interact with NMDARs. At the postsynaptic density, D2Rs form a specific complex with the NMDARs through the C-terminal domain of GluN2B subunit (Liu et al., 2006). Interestingly, DA stimulation by cocaine (i) enhances the D2R/GluN2B interaction; (ii) reduces the association of CaMKII with GluN2B; (iii) lowers the CaMKII-dependent phosphorylation of GluN2B (Ser1303); and (iv) inhibits NMDA receptor-mediated currents in MSNs (Liu et al., 2006).

DA can also modulate the activity of AMPARs leading to a reduction of AMPAR-evoked responses (Cepeda et al., 1993;

Levine et al., 1996; Cepeda and Levine, 1998; Bellone and Lüscher, 2006; Engblom et al., 2008; Mameli et al., 2009; Brown et al., 2010). Early studies performed in cultured neurons showed that activation of D1R in striatal MSNs promotes the phosphorylation of AMPARs by PKA as well as the potentiation of current amplitude (Price et al., 1999). D2Rs antagonists increase the phosphorylation of GluR1 at Ser845 without affecting the phosphorylation at Ser831 (Håkansson et al., 2006). The same effect is observed using eticlopride, a selective D2R antagonist. On the contrary, D2R agonist quinpirole decreased GluR1 phosphorylation at Ser845 (Håkansson et al., 2006). Modulation of DA receptors is also able to regulate AMPAR trafficking at the synaptic membranes. In particular, treatment with D1R agonist leads to an increase of AMPA receptor subunits surface expression (Snyder et al., 2000; Gao et al., 2006; Vastagh et al., 2012).

DA MODULATION OF SYNAPTIC PLASTICITY

DA plays an important role in modulating long-term changes in synaptic strength. One of the best-characterized forms of synaptic plasticity in the striatum is the long-term depression (LTD). In the dorsal and ventral striatum this form of plasticity requires the concomitant activation of mGluR5 and voltage-gated calcium channels and it is expressed by the release of endocannabinoids (eCBs). eCBs act retrogradly onto their CB receptors and decrease the probability of glutamate release (Robbe et al., 2002; Kreitzer and Malenka, 2005).

Interestingly, this form of LTD depends upon the activation of D2Rs, but whether it is controversial whether is only expressed at glutamatergic inputs onto MSNs of the indirect pathway of the dorsal striatum. Indeed, while eCB-LTD has been first characterized in D2R MSNs of the dorsal striatum (Kreitzer and Malenka, 2007), this form of plasticity has been described in both D1R and DR2 striatal neurons of the direct and indirect pathways in BAC transgenic mice (Wang et al., 2006). One possible explanation for the expression of this form of LTD at MNS synapses that do not express D2Rs is that, in both cell types, D2R-dependence of LTD induction is not direct, but it rather depends upon the activation of D2Rs in cholinergic interneurons (Wang et al., 2006).

Long-term potentiation (LTP) at excitatory inputs onto MSNs in the dorsal and ventral striatum is less characterized, and the information that are available so far is even more controversial compared to striatal LTD because of the variety of protocols used to induce this form of plasticity by different laboratories. In the dorsal striatum, LTP induction onto D1 MSNs depends on D1Rs, while, in D2 MSNs, the same form of synaptic plasticity requires the activation of adenosine A2R (Shen et al., 2008; Pascoli et al., 2014a). In both the direct and indirect pathways, the activation of D1Rs and A2Rs, and the concomitant activation of NMDARs leads to the phosphorylation of DARPP-32 and MAPKs that are involved in the expression of LTP (Calabresi et al., 1992, 2000; Kerr and Wickens, 2001; Surmeier et al., 2014). In the ventral striatum, a protocol of High Frequency Stimulation (HFS) induces a form of LTP that relies on the activation of D1Rs but not D2Rs (Schotanus and Chergui, 2008b). Interestingly, previous work showed that LTP is impaired by both D1 and D2 antagonists suggesting that this form of LTP depends upon DA concentration (Li and Kauer, 2004). A recent study, using cell identification, reported that while HFS-LTP is induced in both D1 and D2 MSNs, this form of LTP is blocked by cocaine treatment only in the direct pathway (Pascoli et al., 2011b). The authors characterized the induction and expression mechanisms of this LTP which was reported to be NMDA and ERK pathway-dependent. Future studies are required to investigate the mechanisms underlying LTP in the indirect pathway, and to characterize this form of synaptic plasticity in an input specific manner.

The role of DA in governing striatal plasticity has been addressed by analyzing the mechanisms of Spike Time Dependent Plasticity (STDP) in the dorsal striatum. In both D1 and D2 MSNs, synaptic plasticity follows Hebbian rules. LTP is indeed induced when postsynaptic spiking follows synaptic activity (positive timing), while LTD is favored when the order is reversed (negative timing). Compared to other synapses, in the dorsal striatum, DA plays important roles in determining the sign of synaptic plasticity. In the direct pathway, positive timing gives rise to LTP only when D1 are stimulated, otherwise it leads to LTD. Instead, negative timing induces LTD when D1Rs are not stimulated. In the indirect pathway, D2 signal is necessary for LTD when the postsynaptic spiking is followed by synaptic stimulation. When D2Rs are blocked and A2Rs are stimulated, the same pairing protocol induces LTP (Shen et al., 2008). Therefore, DA modulation in the dorsal striatum ensures that the bidirectional synaptic plasticity follows the Hebbian rules. Further investigation is needed to determine whether these rules apply to all glutamatergic inputs and to the ventral striatum too.

PARKINSON DISEASE

Parkinson's disease (PD) physiopathology is linked to a widespread degeneration of DA-releasing neurons of the Substantia Nigra pars compacta (SNpc), with the loss of DA reaching striatal projecting neurons (Obeso et al., 2010). The degeneration of the nigrostriatal dopaminergic pathway leads to significant morphological and functional changes in the striatal neuronal circuitry, including modifications of the corticostriatal glutamatergic synaptic architecture (Sgambato-Faure and Cenci, 2012; Mellone and Gardoni, 2013) and the consequent loss of striatal synaptic plasticity (Calabresi et al., 2014). A very elegant study demonstrated the asymmetry of the effect of DA denervation on the connectivity of striatonigral and striatopallidal MSNs (Day et al., 2006). In particular, DA depletion leads to a profound decrease in dendritic spines and glutamatergic synapses on striatopallidal MSNs but not on striatonigral MSNs (Day et al., 2006).

It was recently shown that distinct degrees of DA denervation differentially affect the induction and the maintenance of two distinct and opposite forms of corticostriatal synaptic plasticity (Paillé et al., 2010). An incomplete (approximately 75%) nigral denervation does not affect corticostriatal LTD in MSNs, which is however abolished by a complete lesion. This result indicates that a low although critical level of DA is required for this form of synaptic plasticity. Conversely, an incomplete DA denervation dramatically alters the maintenance of LTP in MSNs, demonstrating a critical role of this form of synaptic plasticity

in the early motor parkinsonian symptoms (Paillé et al., 2010). In two different models of PD Shen et al. (2008) showed that in D2R-expressing MSNs, LTP was induced not only by the usual pairing protocol but also with a validated protocol known to induce LTD. Conversely, in D1R-expressing MSNs a protocol normally inducing LTP produces a robust form of LTD that was sensitive to CB1 receptor block (Shen et al., 2008). Imbalances between neural activity in the direct vs. the indirect pathway have been indicated as a major event underlying severe motor deficits observed in PD (Calabresi et al., 2014). In models of PD, eCB-mediated LTD is absent but is rescued by treatment with D2R receptor agonist or with inhibitors of eCB degradation (Kreitzer and Malenka, 2007), thus indicating eCB-mediated depression of indirect-pathway synapses as a critical player in the control of motor behavior in PD.

Alterations of NMDAR subunit composition at MSNs synapses have been reported to sustain this altered expression of plasticity (Sgambato-Faure and Cenci, 2012; Mellone and Gardoni, 2013). It is known that NMDARs are characterized by GluN2A and GluN2B regulatory subunits in MSNs, being GluN2B the most abundant (Dunah and Standaert, 2001). Notably, changes in synaptic NMDAR GluN2A/GluN2B subunit ratio in striatal MSNs correlate with the motor behavior abnormalities observed in a rat model of PD (Picconi et al., 2004; Gardoni et al., 2006; Mellone and Gardoni, 2013). In particular, levels of GluN2B were specifically reduced in synaptic fractions from fully-lesioned 6-OHDA rats when compared to sham-operated rats in the absence of GluN2A alterations in the same samples (Picconi et al., 2004; Gardoni et al., 2006; Paillé et al., 2010). In addition, in the 6-OHDA model of PD, rats with a partial lesion of the nigrostriatal pathway (about 75%) showed a dramatic increase in the GluN2A immunostaining at the synapse without any modifications of GluN2B (Paillé et al., 2010). Overall these data indicate an increased GluN2A/GluN2B ratio at MSNs synapses at different stages of DA denervation in experimental rat models of PD. Accordingly, a cell-permeable peptide that interfers with the interaction between GluN2A and the scaffolding protein PSD-95 is able to reduce the synaptic levels of GluN2A-containing NMDARs and to rescue the physiological NMDAR composition and synaptic plasticity in MSNs (Paillé et al., 2010). Moreover, stimulation of D1Rs by systemic administration of SKF38393 normalizes NMDAR subunit composition and improves motor behavior in a model of early PD establishing a critical link between a specific subgroup of DA receptors and NMDARs and motor performances (Paillé et al., 2010).

Altogether, the emerging pathophysiological picture shows that the strength of glutamatergic signals from the cortex to the striatum might be dynamically regulated by the different degree of DA denervation during the progression of the disease (**Figure 2**). In fact, bidirectional changes in corticostriatal synaptic plasticity are critically controlled by the degree of nigral denervation that influences the endogenous DA levels and the assembly of striatal NMDARs (Sgambato-Faure and Cenci, 2012).

HUNTINGTON DISEASE

Huntington's disease (HD) is a progressive neurodegenerative disease which is characterized by chorea, cognitive decline, and psychiatric disturbances. Alterations in DA and DA receptor levels in the brain contribute to the clinical symptoms of HD (Spokes, 1980; Richfield et al., 1991; Garrett and Soares-da-Silva, 1992; van Oostrom et al., 2009). In particular, time-dependent modifications of DA signaling are correlated to biphasic alterations of the activity of the glutamatergic synapse (Cepeda et al., 2003; Joshi et al., 2009; André et al., 2011a). In agreement with this biphasic activity, Graham et al. (2009) demonstrated that susceptibility to NMDAR-dependent excitotoxicity in HD mouse models was correlated to the severity of their symptomatic stage. On the one hand, HD mice at an early age display enhanced sensitivity to excitotoxic NMDAR-dependent events compared to wild-type animals. On the other hand, old symptomatic HD mice are more resistant to NMDA-dependent neurotoxicity (Graham et al., 2009).

Dysfunction and loss of striatal MSNs represent the major neuropathological feature of the disease (Martin and Gusella, 1986). Although the mechanisms explaining a selective degeneration of MSNs in HD have not been addressed, several reports correlated an abnormal functioning of both dopaminergic and glutamatergic transmission to the induction of striatal MSNs death (Charvin et al., 2005; Fan and Raymond, 2007; Tang et al., 2007).

A decrease of D1R and D2R in striatum from postmortem HD brains has been reported in several studies (Joyce et al., 1988; Richfield et al., 1991; Turjanski et al., 1995; Suzuki et al., 2001). In addition, a significant alteration of both D1R and D2R density and function in the striatum has been described in HD mouse models (Bibb et al., 2000; Ariano et al., 2002; Paoletti et al., 2008; André et al., 2011b). Studies performed in knock-in HD striatal cells showed that mutant huntingtin enhances striatal cell death through activation of D1R but not D2R (Paoletti et al., 2008). Particularly, pretreatment with NMDA increased D1R-induced cell death of mutant but not wild-type cells thus suggesting that NMDARs potentiate the vulnerability of HD striatal cells to DA toxicity (Paoletti et al., 2008). Interestingly, an aberrant Cdk5 activity is involved in the augmented sensitivity of HD striatal cells to DA and glutamate inputs (Paoletti et al., 2008). In agreement with these data, Tang et al. (2007) reported that glutamate and DA act synergistically to induce elevated Ca^{2+} signals and to induce apoptosis of MSNs in HD mice. Again, these effects are selectively mediated by D1R and not by D2Rs (Tang et al., 2007). However, a role for D2R in mediating MSN degeneration has been put forward (Charvin et al., 2005, 2008), thus raising the hypothesis that both activation of D1R and D2R might contribute to glutamate/DA dependent toxicity. More recently, André et al. (2011b) showed that, at the early stage, glutamate release was increased onto D1R cells while it was unaltered onto D2R cells in HD mice. Notably, at the late stage, glutamate transmission was decreased onto D1R cells only. Overall, this study suggests that more changes occur in D1R cells than in D2R cells, at both presymptomatic and symptomatic ages. Finally, in agreement with this study, Benn et al. (2007) showed that the percentage of D2R-positive cells are not modified with the phenotype or with age. However, it must be taken into account that these results represent a clear discrepancy with early studies indicating a higher vulnerability of

D2R in HD (Reiner et al., 1988; Albin et al., 1992). Accordingly, further studies are needed for a complete characterization and understanding of D1R vs. D2R alterations in HD.

Changes in synaptic vs. extra-synaptic localization of NMDARs are also crucial for neuronal survival in HD (Levine et al., 2010). In particular, a selective increase of striatal GluN2B-containing NMDARs in association with an early increase in extrasynaptic NMDAR signaling has been described in different HD animal models (Zeron et al., 2004; Milnerwood et al., 2010). In addition, excitotoxicity mediated by GluN2B-containing NMDARs exacerbated selective MSNs degeneration in a knockin HD model (Heng et al., 2009).

DA and glutamate cross-talk seems to have a key role also in aberrant synaptic plasticity which is observed in HD animal models. DA-dependent LTP, but not LTD, in the dorsal striatum is reduced in the R6/2 mouse model of HD (Kung et al., 2007; **Figure 2**). Interestingly, the deficits in LTP and short-term plasticity observed in animal models of HD are reversed by treatment with the D1R agonist SKF38393 (Dallérac et al., 2011).

ADDICTION

Drug-evoked synaptic plasticity of glutamatergic synapses in the mesocorticolimbic system has been largely implicated in addictive behaviors (Luscher and Bellone, 2008) and DA neurons of the VTA are the point of convergence at which addictive drugs can alter the brain circuits (Brown et al., 2010). Drug-evoked synaptic plasticity has been characterized at excitatory input onto DA neurons of the VTA 24 h after a single injection of addictive drugs (Ungless et al., 2001; Bellone and Lüscher, 2006; Mameli et al., 2007; Yuan et al., 2013). Interestingly, it is induced by activation of D1/D5Rs and NMDARs (Ungless et al., 2001; Argilli et al., 2008) and it is expressed by insertion of GluN3A-containing NMDARs (Yuan et al., 2013) and GluA2-lacking AMPARs (Bellone and Lüscher, 2006). Moreover, it has been shown that the redistribution of glutamatergic receptors induced by cocaine in the VTA depends upon the action of cocaine on DA transporter (DAT) and that DA neurons activity itself is sufficient to induce drug-evoked synaptic plasticity at glutamatergic synapses (Brown et al., 2010). D1 signaling in the VTA is necessary for these adaptations suggesting that the convergence of DAergic/glutamatergic signaling in the VTA modifies the circuit at the synaptic level.

Interestingly, redistribution of glutamatergic transmission in the VTA is permissive for the expression of drug-evoked plasticity in the NAc and subsequent addictive behaviors. Indeed, deletion of GluN1 selectively in the DA neurons of the VTA abolishes both cocaine-evoked plasticity in the NAc (Engblom et al., 2008) and prevent reinstatement of self-administration (Mameli et al., 2009).

In the NAc, the convergence of DA and glutamate after cocaine exposure contributes to addictive behaviors by the facilitation of AMPAR trafficking at certain glutamatergic inputs. Early studies have found that D1R stimulation increases GluA1 surface expression via PKA activation promoting further NMDA-dependent synaptic plasticity (Sun et al., 2005, 2008; Gao et al., 2006). Recently, the role of AMPAR trafficking in drug-evoked synaptic plasticity and its link to behavioral adaptation has been demonstrated. Indeed, insertion of GluA2-lacking (GluA1 homomeric) AMPARs has been shown both after incubation of cocaine craving and cocaine self-administration at excitatory input onto MSNs in the NAc (Conrad et al., 2008; Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014b; **Figure 3**). Although these studies show some discrepancies regarding the cell- and input-specificity of Ca^{2+} permeable AMPAR insertion, the removal

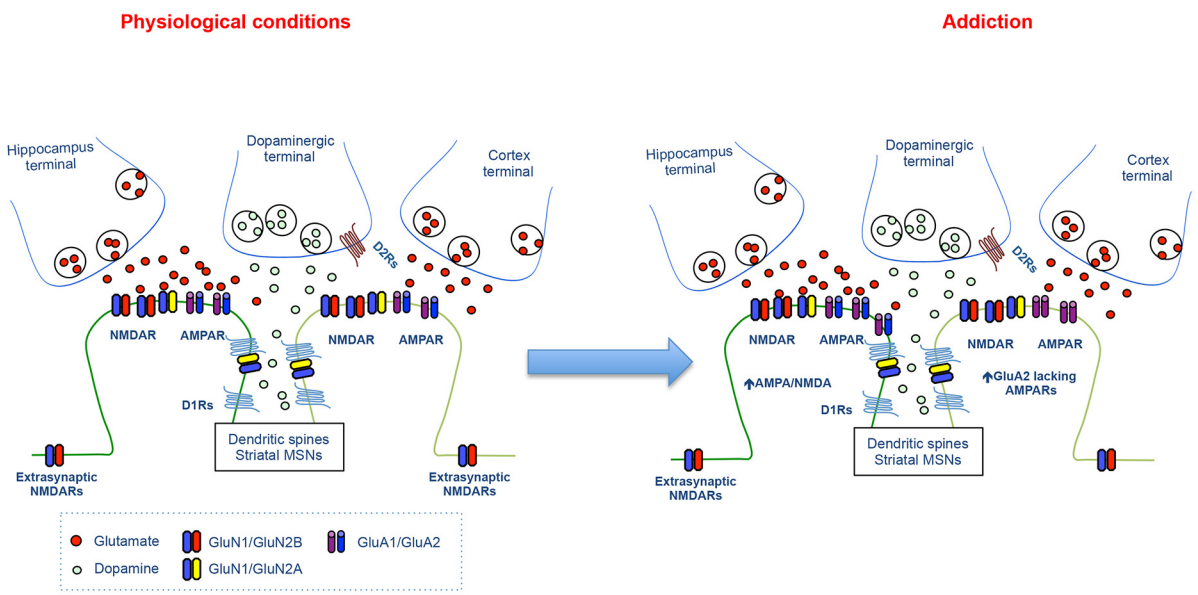

FIGURE 3 | Synaptic changes at the glutamatergic synapses during cocaine seeking. The cartoon illustrates the physiological glutamatergic corticostriatal and hippocampastriatal synapses (left panel) and the synaptic alterations at excitatory synapses onto MSNs during cocaine seeking (Right panel).

of these receptors is an efficient method to revert addictive behaviors (Loweth et al., 2014; Pascoli et al., 2014b). Altogether, these studies indicate that the expression of addictive behaviors depends upon the convergence of DA/glutamate signal and the consequent changes in the efficacy and quality of excitatory synaptic transmission.

Which are the mechanisms underlying the interactions between the glutamate and the DA system in the NAc in drug addiction? Many studies have shown that different behavioral and molecular responses induced by cocaine rely on the D1R-NMDAR interaction that regulates the activity of ERK pathways and control gene expression, plasticity and behavior (Girault et al., 2007; Bertran-Gonzalez et al., 2008; Pascoli et al., 2014a). Interestingly, cocaine-induced activation of the ERK pathway is restricted to D1 MSNs and depends upon the concomitant activation of D1 and NMDARs. Moreover, direct blockade of ERK signaling induced by cocaine prevents the expression of conditioned place preference (CPP; Valjent et al., 2000), locomotor sensitization (Valjent et al., 2006) and drug-evoked synaptic plasticity (Pascoli et al., 2011b; Cahill et al., 2014). To confirm the role of DA/glutamate interaction in cocaine-induced ERK activation, it has also been shown that indirect inhibition of the ERK pathway blocks addictive behaviors. Cocaine activates the tyrosine kinase Fyn that, via phosphorylation of GluN2B, potentiates Ca^{2+} influx through NMDARs and activates ERK signaling. Interestingly, the inhibition of Fyn inhibits cocaine-induced ERK activation while inhibition of GluN2B-containing NMDAR impairs locomotor sensitization and CPP (Pascoli et al., 2011a). Moreover, the blockade of the D1/GluN1 downstream pathways, although it preserves the individual signaling, blocks both the D1-induced potentiation of Ca^{2}+ influx via NMDARs and the ERK activation. As a consequence, behavioral sensitization is impaired (Cahill et al., 2014).

CONCLUSIONS

Functional interactions between DA and glutamate receptors modulate an incredible variety of functions in the brain and, when abnormal, they contribute to numerous central nervous system disorders. In particular, an integrated cross-talk between DA and glutamate receptors plays a key role in motor control, cognition and memory, neurodegenerative disorders, schizophrenia and addictive behaviors. Accordingly, a huge number of studies, described in the present review, have been performed aiming at understanding the molecular and functional mechanisms coordinating functions of glutamate and DA receptors. Hopefully, a complete knowledge of dysregulation of glutamate and DA signaling as in Parkinson, Huntington and addiction-related diseases, could represent the first step for the identification and setting up of novel therapeutical approaches for these brain disorders.

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Distinct regions within the GluN2C subunit regulate the surface delivery of NMDA receptors

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N-methyl-D-aspartate (NMDA) receptors mediate fast excitatory synaptic transmission in the mammalian central nervous system. The activation of NMDA receptors plays a key role in brain development, synaptic plasticity, and memory formation, and is a major contributor to many neuropsychiatric disorders. Here, we investigated the mechanisms that underlie the trafficking of GluN1/GluN2C receptors. Using an approach combining molecular biology, microscopy, and electrophysiology in mammalian cell lines and cultured cerebellar granule cells, we found that the surface delivery of GluN2C-containing receptors is reduced compared to GluN2A- and GluN2B-containing receptors. Furthermore, we identified three distinct regions within the N-terminus, M3 transmembrane domain, and C-terminus of GluN2C subunits that are required for proper intracellular processing and surface delivery of NMDA receptors. These results shed new light on the regulation of NMDA receptor trafficking, and these findings can be exploited to develop new strategies for treating some forms of neuropsychiatric disorders.

Keywords: glutamate receptor, ion channel, intracellular trafficking, electrophysiology, cerebellar granule cells, endoplasmic reticulum

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors are ionotropic glutamate receptors that play a key role in glutamatergic neurotransmission. NMDA receptors are heterotetramers composed of GluN1, GluN2, and/or GluN3 subunits. The GluN1 subunit is encoded by a single gene that expresses eight splice variants. GluN2 subunits are encoded by four different genes, giving rise to GluN2A, GluN2B, GluN2C, and GluN2D subunits; finally, GluN3 subunits are encoded by two genes, giving rise to GluN3A and GluN3B subunits (Lau and Zukin, 2007; Petralia et al., 2009; Traynelis et al., 2010). The current consensus is that functional NMDA receptors are composed primarily of two GluN1 subunits and two GluN2 subunits, and their activation requires both glutamate and the co-agonist glycine (Traynelis et al., 2010). All NMDA receptor subunits share the following structural features: (i) four membrane-spanning segments (M1–M4), which help form the channel's pore; (ii) an extracellular N-terminus and an extracellular loop between M3 and M4; and (iii) an intracellular C-terminus (Madden, 2002; Traynelis et al., 2010).

The GluN2A through GluN2D subunits have expression patterns that vary widely both in time (i.e., during development) and in space (i.e., among various brain regions; Paoletti, 2011). For example, in cerebellar granule cells (CGCs), GluN2B subunits are expressed early in development but disappear almost entirely by postnatal day 21; in contrast, GluN2A and GluN2C subunits are expressed later in development (Akazawa et al., 1994; Monyer et al., 1994). In addition to its expression in the cerebellum, low levels of GluN2C mRNA have also been found in the hippocampus (Pollard et al., 1993). GluN2C-containing NMDA receptors have distinct functional properties, including reduced magnesium affinity and reduced conductance, and these properties are conferred upon the receptor's synaptic currents (Lu et al., 2006; Paoletti, 2011). Interestingly, the GRIN2C gene, which encodes the GluN2C subunit, has several splice variants (Rafiki et al., 2000), and its expression is perturbed in some neurological disorders (Marianowski et al., 1995; Kadotani et al., 1998).

It is generally believed that the number and type of NMDA receptors present at the cell surface are regulated at multiple levels, including their synthesis, subunit assembly, processing in the endoplasmic reticulum (ER), intracellular trafficking, and degradation. Studies have shown that before a functional NMDA receptor heterotetramer is formed in the ER, GluN1, and GluN2 monomers form an intermediate complex, for example GluN1-GluN1 and/or GluN1-GluN2 dimers (Atlason et al., 2007; Schuler et al., 2008). These intermediate complexes likely employ specific ER retention mechanisms, as they are not trafficked from the ER (with the exception of certain GluN1 splice variants; Mcllhinney et al., 1998; Okabe et al., 1999; Fukaya et al., 2003). Although distinct regions within the GluN1, GluN2A, and GluN2B subunits regulate ER processing and the trafficking of functional NMDA receptors (Standley et al., 2000; Meddows et al., 2001; Mu et al., 2003; Hawkins et al., 2004; Horak et al., 2008; Horak and Wenthold, 2009; Kenny et al., 2009; Qiu et al., 2009), subunit-dependent differences in early NMDA receptor processing (e.g., between GluN1/GluN2A-B and GluN1/GluN2C-D receptors) have not yet been studied in detail.

Here, we determined which structural features of the GluN2C subunit regulate the surface expression of GluN2C-containing NMDA receptors. By combining microscopy and electrophysiology recordings of heterologous cells and cultured CGCs that express recombinant GluN subunits, we found that

the surface expression of GluN1/GluN2C receptors is reduced compared to GluN1/GluN2A and GluN1/GluN2B receptors. Furthermore, using a panel of truncated and otherwise mutated GluN2C subunits, we identified three distinct regions in the GluN2C subunit—specifically, within the N-terminus, M3 domain, and C-terminus—that regulate the surface expression of GluN2C-containing NMDA receptors. Interestingly, trafficking of GluN1/GluN2A receptors is also regulated by the N-terminal and M3 domain–mediated mechanisms; however, the C-terminal–mediated mechanism appears to be specific to GluN2C-containing receptors. We conclude that the GluN2C subunit uses several regulatory mechanisms to control the early processing of functional NMDA receptors.

MATERIALS AND METHODS

MOLECULAR BIOLOGY

The following cDNAs encoding full-length or truncated NMDA receptor subunits were used: extracellular-tagged yellow fluorescent protein (YFP)-GluN1-1a and extracellular-tagged green fluorescent protein (GFP)-GluN2A, GFP-GluN2B, and GFP-GluN2C (Luo et al., 2002; Horak et al., 2008; Chen and Roche, 2009). Untagged versions of the GluN1-1a, GluN2A, and GluN2C subunits were also used (Horak et al., 2006). Point mutations were generated using the Quick-Change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. The amino acid residues are numbered as published (Ishii et al., 1993). All constructs were verified by DNA sequencing.

HETEROLOGOUS CELL CULTURE

African green monkey kidney fibroblast (COS-7) cells were cultured in Minimum Essential Medium with Earle's salts (MEM) containing 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). COS-7 cells were used for microscopy experiments because they remain attached to glass coverslips during extensive washing procedures. Human embryonic kidney 293 (HEK293) cells were cultured in Opti-MEM I (Invitrogen) containing 5% FBS (v/v); these cells were used for electrophysiology.

For transfection, equal amounts of the various cDNAs (0.9 μg in total) were added to 2 μl Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions, and the DNA–Lipofectamine complexes were added to COS-7 or HEK293 cells for 5 h as described previously (Horak et al., 2008). The transfected cells were cultured in Opti-MEM I containing 1% FBS (v/v) supplemented with 20 mM $MgCl_2$, 1 mM DL-2-amino-5-phosphonopentanoic acid, and the NMDA receptor antagonist kynurenic acid (3 mM) to prevent cytotoxicity caused by NMDA receptor activation. All experiments were performed within 24–48 h of transfection.

PRIMARY CEREBELLAR GRANULE CELLS

Cerebellar granule cells were prepared from postnatal day 6–8 rats as described previously (Prybylowski et al., 2005). In brief, cells were cultured in Basal Eagle's Medium (Invitrogen) supplemented with 10% FBS (v/v), 2 mM glutamine, and 25 mM KCl. After 5 days in culture (DIV5), the CGCs were transfected using the calcium phosphate technique as described previously (Prybylowski et al., 2002). Microscopy experiments were performed within 48–72 h of transfection. All experimental procedures involving animals were performed in accordance with the guidelines of our institute's Animal Care Committee.

MICROSCOPY

To surface-label the NMDA receptor subunits, COS-7 cells and CGCs were washed in phosphate-buffered saline (PBS), then incubated on ice for 15 min in a blocking solution containing PBS and 10% (v/v) normal goat serum (NGS) as described previously (Horak et al., 2008). The cells were then incubated for 30 min in blocking solution containing polyclonal rabbit anti-GFP antibody (Merck Millipore, Darmstadt, Germany; 1:1000). Next, the cells were washed twice in PBS, then incubated for 30 min in blocking solution containing the following fluorescent secondary antibodies: Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen) for the COS-7 cells, or Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen) for the CGCs. The cells were then washed twice in PBS and fixed for 20 min in PBS containing 4% paraformaldehyde (w/v) for 20 min. The COS-7 cells were then mounted using ProLong Antifade reagent (Invitrogen). The CGCs were processed further for intracellular labeling of the total pool of NMDA receptor subunits. In brief, the cells were permeabilized for 5 min in PBS containing 0.1% Triton X-100 (w/v), blocked for 1 h with blocking solution containing 0.1% Triton X-100, and incubated in the primary (anti-GFP) and secondary (Alexa Fluor 488 goat anti-rabbit IgG; Invitrogen) antibodies for 1 h each.

For the internalization studies, live cells were washed in PBS, then incubated on ice for 30 min in the primary (anti-GFP) antibody to label the surface receptors. The cells were then washed in PBS, and the coverslips were returned to conditioned medium for 30 min at 37°C. The cells were washed in PBS, incubated in an unconjugated goat anti-rabbit antibody (Invitrogen), fixed, permeabilized, incubated with a fluorescent secondary antibody, washed, and mounted using ProLong Antifade reagent (Lavezzari et al., 2004; Scott et al., 2004). To visualize both the surface and total pools of NMDA receptors, z-stack images were scanned using an Olympus scan® fluorescence microscope (COS-7 cells) or a Leica SPE confocal microscope (CGCs); the images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). For the microscopy experiments, ≥45 transfected COS-7 cells from ≥3 independent experiments and ≥20 transfected CGCs (unless stated otherwise) were used for analysis as described previously (Horak et al., 2008). All summary data are expressed as mean ± SEM. Differences were analyzed using the unpaired Student's *t*-test or one-way ANOVA followed by the Dunn's test.

ELECTROPHYSIOLOGY

Whole-cell voltage-clamp recordings were performed using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Union City, CA, USA) with compensation for both capacitance and series resistance. The extracellular solution contained (in mM): 160 NaCl, 2.5 KCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose, 0.7 $CaCl_2$, 0.2 EDTA, and 10 μM glycine (pH adjusted to 7.3 with NaOH. Glass patch pipettes

(3–5 MΩ tip resistance) were filled with an intracellular solution containing (in mM): 125 gluconic acid, 15 CsCl, 5 EGTA, 10 HEPES, 3 $MgCl_2$, 0.5 $CaCl_2$, and 2 ATP-Mg salt (pH adjusted to 7.2 with CsOH). A microprocessor-controlled multi-barrel rapid-perfusion system (the time constant of solution exchange in the vicinity of the cells was ∼20 ms) was used to apply the test solutions (Kaniakova et al., 2012a). The experiments were performed at room temperature. Glutamate-induced responses were low-pass filtered at 2 kHz with an eight-pole Bessel filter, digitally sampled at 5 kHz, and analyzed using pCLAMP version 9 (Molecular Devices). All summary data are expressed as mean ± SEM. Differences were analyzed using the unpaired Student's *t*-test or one-way ANOVA followed by the Dunn's test.

RESULTS

THE IDENTITY THE SPECIFIC GluN2 SUBUNIT TYPE DETERMINES SURFACE DELIVERY OF THE NMDA RECEPTOR

Previous studies examined the molecular mechanisms that underlie the early trafficking of GluN1/GluN2A and GluN1/GluN2B receptors; however, the trafficking of other NMDA receptor types—including GluN1/GluN2C receptors—has been largely neglected. Therefore, the aim of this study was to examine the role that various regions within the GluN2C subunit play in delivering GluN1/GluN2C receptors to the surface membrane of mammalian cell lines and cultured CGCs. We first measured the surface expression of NMDA receptors comprised of GluN1-1a together with GFP-GluN2A, GFP-GluN2B, or GFP-GluN2C subunits in transfected COS-7 cells (**Figures 1A,B**). Our data show that GluN1-1a/GFP-GluN2A receptors were expressed at the cell surface at significantly higher levels than GluN1-1a/GFP-GluN2B receptors; this finding is consistent with previous results (Chen et al., 1999). Interestingly, however, the surface expression of GluN1-1a/GFP-GluN2C receptors was lower than both GluN1-1a/GFP-GluN2A and GluN1-1a/GFP-GluN2B receptors, even though all three GluN2 subunits were expressed at similar levels (**Figures 1A,B**). Similar results were obtained when we examined the surface expression of YFP-GluN1-1a/GluN2A and YFP-GluN1-1a/GluN2C receptors in COS-7 cells (Figure S1). Finally, consistent with its strict requirement for delivering the receptor to the surface membrane, when the GluN1 subunit was not co-transfected, none of the GluN2 subunits (i.e., GFP-GluN2A, GFP-GluN2B, or GFP-GluN2C) reached the cell surface (Figure S2).

Next, we used cultured CGCs to further examine whether the surface expression of GluN2C subunits is reduced compared to GluN2A and GluN2B subunits. Cultured CGCs are an ideal model system for these experiments, as these neurons are relatively homogeneous, thus allowing us to detect relatively small changes in the surface and total expression of GluN subunits; moreover, the subunits are expressed in their native environment (Prybylowski et al., 2002; Traynelis et al., 2010). We found that compared to GluN2A and GluN2B subunits, GluN2C subunits are expressed at the surface at significantly lower levels; as with the heterologous cells, the total expression levels were similar among all three GluN2 subunits (**Figures 1C,D**). Together, these results suggest that the GluN2C subunit contains unique structural element(s) that regulate the surface delivery of NMDA receptors.

The reduced surface expression of GluN1/GluN2C receptors may be due to a faster internalization rate compared to GluN1/GluN2A and GluN1/GluN2B receptors. To test this possibility, we performed an internalization assay for GluN1-1a/GluN2A and GluN1-1a/GluN2C receptors expressed in COS-7 cells (**Figures 1E–G**). We found that GluN1-1a/GluN2C receptors internalize more slowly than GluN1-1a/GluN2A receptors; thus, the presence of the GluN2C subunit must regulate forward trafficking of the receptor rather than decreasing the receptor's surface stability.

DISTINCT REGIONS WITHIN THE GluN2C SUBUNIT REGULATE THE FORWARD TRAFFICKING OF NMDA RECEPTORS

Previous studies identified several regions within NMDA receptor subunits—including the N-terminus, membrane domains, and C-terminus—as key elements for controlling the delivery of NMDA receptors to the cell surface (Stephenson et al., 2008; Traynelis et al., 2010). Moreover, the N-terminal domain of the GluN2A subunit—but not the GluN2B subunit—contains an ER retention signal (Horak et al., 2008; Qiu et al., 2009). We first confirmed that a GluN2A subunit that is truncated after the M1 domain (GFP-GluN2A-M1stop), but not the equivalent truncated GluN2B subunit (GFP-GluN2B-M1stop), is retained in the intracellular compartment (**Figures 2A,B**). We next examined the trafficking of GFP-GluN2C-M1stop and found that this truncated GluN2C protein is retained in the intracellular compartment; thus, the N-terminal domains of both GluN2A and GluN2C regulate their intracellular processing, perhaps via a similar mechanism (**Figures 2A,B**). We also generated two new GFP-GluN2C-M1stop constructs that lack either the A2 segment (GFP-GluN2C-M1stop-Δ159–292) or the region immediately downstream of the A2 segment (GFP-GluN2C-M1stop-Δ293–556). As we expected, deleting the A2 segment prevented the intracellular retention of the truncated GluN2C subunit, similar to a previous study using the GluN2A subunit (Qiu et al., 2009); moreover, the GFP-GluN2C-M1stop-Δ293–556 subunit was still retained intracellularly (**Figures 2A,B**). Using confocal microscopy, we found that GFP-GluN2A-M1stop and GFP-GluN2C-M1stop subunit co-localized closely with an ER marker, but not a Golgi apparatus (GA) marker, supporting the notion that these constructs are not targeted to a different subcellular compartment such as the lysosomes (Figure S3).

A previous study also found that deleting the A2 segment reduces the surface expression of GluN1/GluN2A receptors (Qiu et al., 2009). Therefore, we deleted the A2 segment from the GluN2C subunit (GFP-GluN2C-Δ159–292). Expressing this construct together with the GluN1-1a subunit in COS-7 cells significantly reduced the surface expression of the receptors (**Figures 3A,B**). Next, we used electrophysiology to confirm these microscopy findings. We performed whole-cell voltage-clamp recordings of HEK293 cells expressing GluN1-1a/GFP-GluN2C receptors or GluN1-1a/GFP-GluN2C-Δ159–292 receptors. Applying 1 mM glutamate (for 5 sec at a membrane potential of −60 mV) elicited receptor-mediated currents in cells expressing GluN1/GluN2C channels, and these currents were

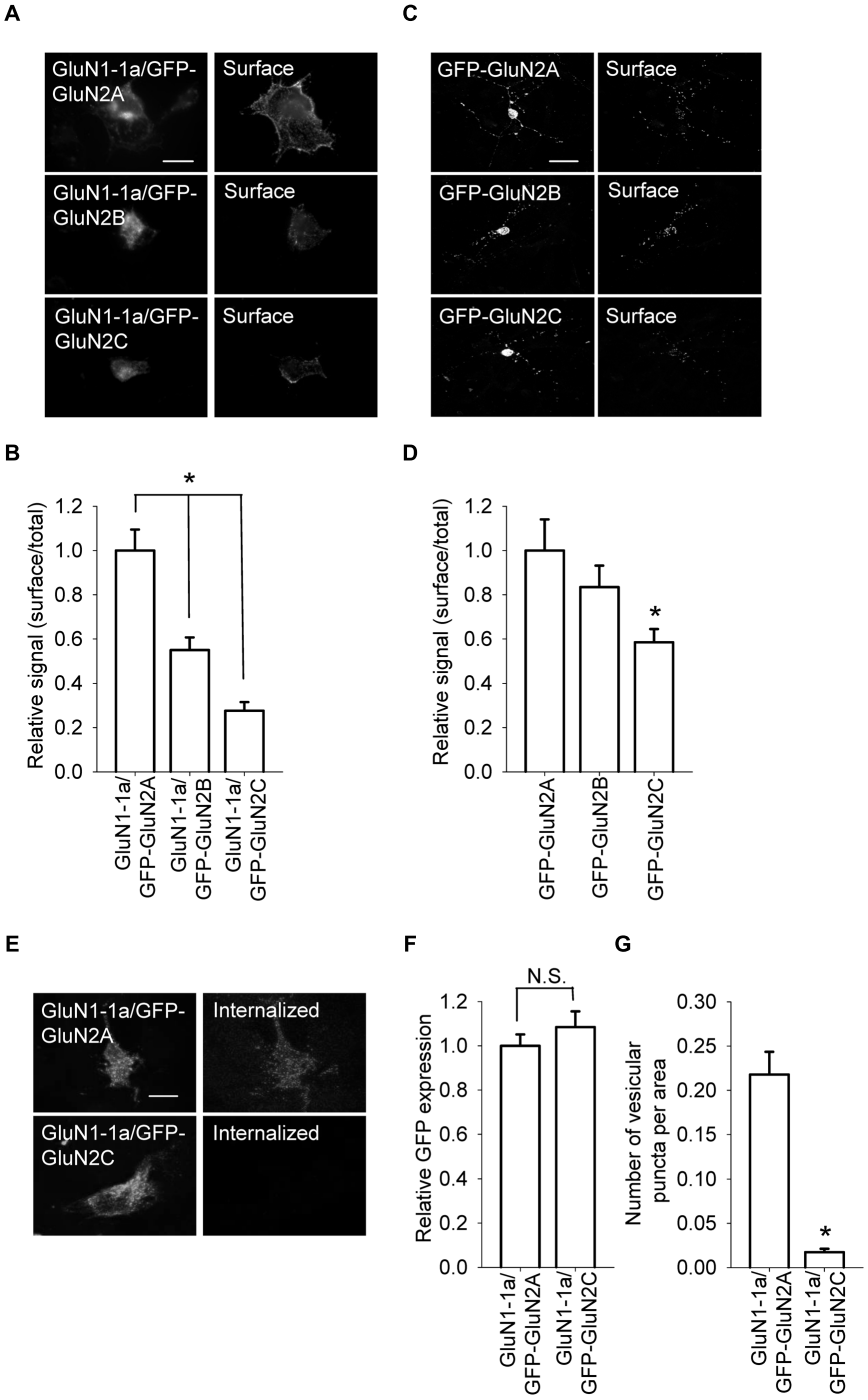

FIGURE 1 | GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2C receptors are differentially expressed at the cell surface.
(A) Representative images of the total (left panel) and surface (right panel) pools of GluN1/GluN2A, GluN1/GluN2B, and GluN1/GluN2C receptors expressed in COS-7 cells. Scale bar, 20 μm. **(B)** Summary of the normalized intensity ratios of surface and total NMDA receptors expressed in COS-7 cells and visualized using immunofluorescence. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2A); ANOVA. **(C)** Representative images of total (left panel) and surface (right panel) NMDA receptor pools in cerebellar granule cells (CGCs). Scale bar, 20 μm. **(D)** Summary of the ratio of surface and total expression of NMDA receptors visualized using confocal microscopy. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2A); ANOVA. **(E)** Internalization of GluN1/GluN2 receptors in transfected COS-7 cells. Live cells were incubated for 30 min at 37°C with an anti-GFP antibody; the cells were then fixed and incubated with a fluorescent secondary antibody. Representative images of the GFP signal (left) and internalized receptors (right) in transfected cells are shown. Scale bar, 20 μm. **(F,G)** Summary ($n \geq 40$ from three independent experiments) of GFP expression **(F)** and the average number of vesicular puncta per area **(G)** for the indicated NMDA receptors. $*p < 0.05$ (relative to GFP-GluN2A); Student's *t*-test.

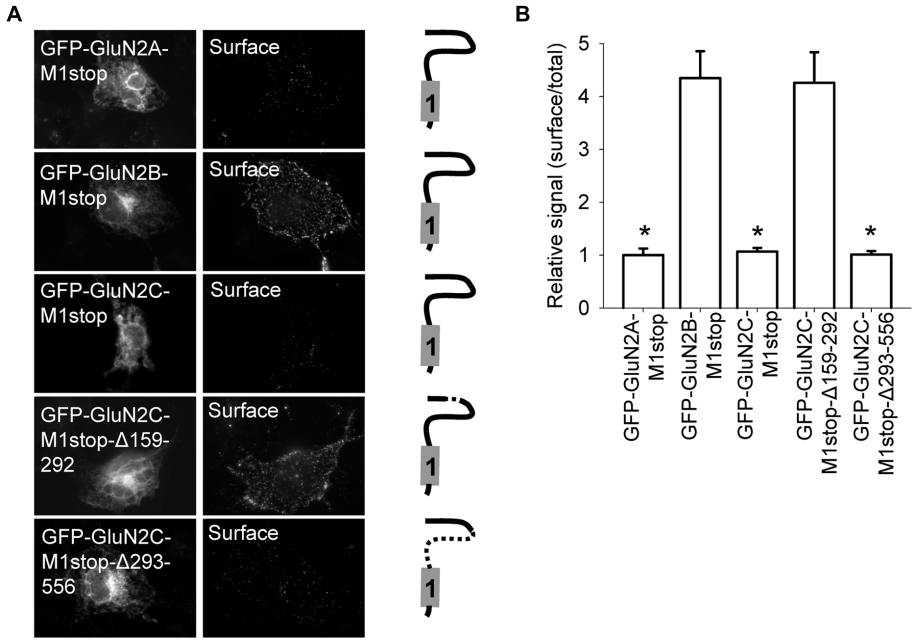

FIGURE 2 | Surface delivery of truncated GluN2 subunits. **(A)** Schematic drawings of the membrane topology of the indicated truncated GluN2 subunits and representative images of total (left panel) and surface (right panel) pools of NMDA receptors expressed in COS-7 cells. **(B)** Summary of the normalized ratios of surface and total expression of the indicated NMDA receptor subunits measured using fluorescence microscopy. $*p < 0.05$ (relative to GFP-GluN2A-M1stop); ANOVA.

significantly smaller in cells expressing the GluN2C-Δ159–292 subunit (**Figures 3C,D**). To identify the intracellular compartment to which the mutant GluN1-1a/GFP-GluN2C-Δ159–292 receptors trafficked (given that they were not present at the cell surface), we performed immunofluorescence experiments using COS-7 cells that expressed GluN1-1a/GluN2C or GluN1-1a/GluN2C-Δ159–292 receptors and co-stained the cells with antibodies against ER and GA markers. These experiments revealed that these receptor combinations (including wild-type receptors) clearly co-localized with the ER, but not with the GA (Figures S4 and S5). Finally, we overexpressed the GFP-GluN2C and GFP-GluN2C-Δ159–292 subunits in cultured CGCs and compared their surface delivery using confocal microscopy. We found that the GFP-GluN2C-Δ159–292 subunit was significantly reduced at the cell surface (**Figures 3E,F**). In all cases, the total expression of the mutant GluN2C subunit did not differ significantly from the corresponding control (i.e., full-length) GluN2C subunit. Based on previously published results obtained from GluN1/GluN2A receptors (Qiu et al., 2009), we hypothesized that although the structural differences within the N-terminal regions do not likely account for the observed differences in surface expression between the various GluN2-containing receptors, the N-terminus clearly plays an important role in delivering GluN1/GluN2C receptors to the cell surface.

We recently reported that specific residues within the M3 domains of both the GluN2A and GluN2B subunits are essential for delivering functional NMDA receptors to the cell surface (Kaniakova et al., 2012a). Based on this finding, we asked whether the M3 domain in GluN2C plays a similar role in the delivery of NMDA receptors to the cell surface. We therefore generated three constructs in which the amino acid residue at position 645, 656, or 657 (within the M3 domain) was replaced with an alanine residue (yielding constructs GFP-GluN2C-W645A, GFP-GluN2C-Y656A, and GFP-GluN2C-T657A, respectively); these residues are homologous to previously identified key residues in the GluN2A and GluN2B receptors (Kaniakova et al., 2012a; **Figure 4A**). When co-expressed with the GluN1-1a subunit in COS-7 cells, each mutant GluN2C subunit had reduced surface expression compared to wild-type GluN2C (**Figures 4B,C**). Consistent with these results, HEK293 cells expressing GluN1-1a/GFP-GluN2C-W645A or GluN1-1a/GFP-GluN2C-T657A receptors had reduced glutamate-induced currents (**Figures 4D,E**); similar results were obtained using cultured CGCs transfected with GFP-GluN2C-W645A, GFP-GluN2C-Y656A, or GFP-GluN2C-T657A subunits (**Figures 4F,G**). Together, these results support our conclusion that the M3 domain in GluN2C is essential for delivering the receptor to the cell surface. Lastly, co-localization experiments revealed that GluN1-1a/GFP-GluN2C-W645A and GluN1-1a/GFP-GluN2C-T657A receptors are present mostly in the ER (Figures S4 and S5).

Finally, we asked whether structural differences in the M3 domains among the various GluN2 subunits can explain the observed differences in surface expression. Because the M3 domains of GluN2A and GluN2C differ by only one amino acid residue (Figure S6), we generated a full-length GluN2A subunit containing the GluN2C M3 domain (GFP-GluN2A-S632L)

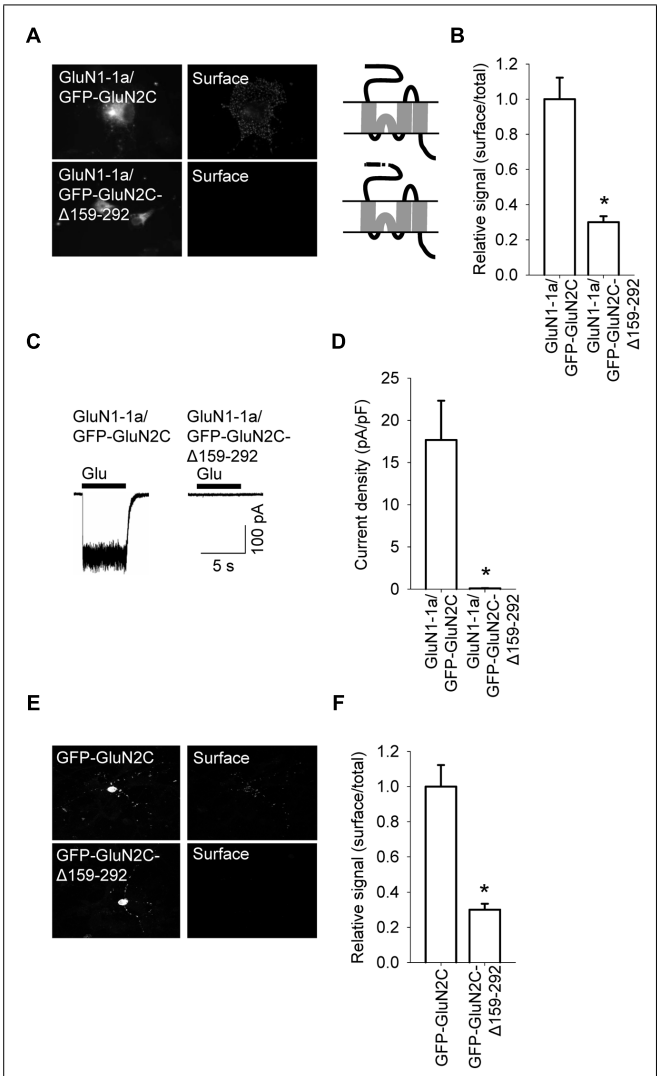

FIGURE 3 | A specific region within the N-terminus of GluN2C is essential for the surface delivery of NMDA receptors. (A) Schematic drawings of the membrane topology of the indicated full-length GluN2 subunits and representative images of total (left panel) and surface (right panel) NMDA receptor pools in transfected COS-7 cells. **(B)** Summary of the normalized ratios of surface and total expression of the indicated NMDA receptor subunits measured using fluorescence microscopy. $^*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA. **(C)** Whole-cell voltage-clamp recordings were performed in HEK293 cells expressing the indicated NMDA receptor subunit combinations. Currents were elicited by applying a 5-sec pulse of 1 mM glutamate (indicated by the filled bar); representative traces are shown. **(D)** Quantitative analysis of peak current density (pA/pF) mediated by the indicated NMDA receptors; $n \geq 21$. $^*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); Student's *t*-test. **(E)** Representative images of total (left panel) and surface (right panel) NMDA receptor pools in CGCs. **(F)** Summary of the normalized ratios of surface and total expression of NMDA receptors measured using confocal microscopy. $^*p < 0.05$ (relative to GFP-GluN2C); Student's *t*-test.

and a full-length GluN2C subunit containing the GluN2A M3 domain (GFP-GluN2C-L634S). When co-expressed with GluN1-1a subunits in COS-7 cells, these mutant subunits did not differ significantly from their respective controls in terms of surface expression (Figure S6). Thus, these data suggest that although the M3 domain in GluN2C is essential for delivering GluN2C-containing NMDA receptors to the cell surface, other regions in the GluN2C subunit are likely responsible for the differences in surface delivery of GluN2A-, GluN2B-, and GluN2C-containing receptors.

The C-terminal region of GluN subunits was previously implicated in regulating the delivery of NMDA receptors to the cell surface (Traynelis et al., 2010; Sanz-Clemente et al., 2012). To determine whether the C-terminus of the GluN2C subunit regulates the surface delivery of GluN2C-containing NMDA receptors, we first generated a GluN2C subunit that lacks the C-terminal domain (GFP-GluN2C-855stop); the protein was truncated at a similar position as the truncated versions of GluN2A and GluN2B in previous studies (Vissel et al., 2001; Horak et al., 2008). Interestingly, when co-expressed in COS-7 cells with the GluN1-1a subunit, receptors containing the truncated GluN2C failed to traffic to the cell surface (**Figures 5A,B**). Therefore, we generated a series of C-terminal truncated GluN2C subunits in order to determine whether a specific structural element is essential for the forward trafficking of GluN2C-containing NMDA receptors. We found that the surface expression of seven truncated versions of the GluN2C subunit (truncated from residue 872 through residue 889) is significantly reduced; in contrast, five truncated GluN2C subunits (truncated from residue 890 through residue 1241) had normal levels of surface expression (**Figures 5A,B**). These results suggest that the region adjacent to residue 889 is critically involved in regulating the surface expression of GluN2C-containing receptors. To test this idea, we generated a full-length pentamutant GFP-GluN2C subunit in which the SLPSP sequence (amino acid residues 885–889) was replaced with alanines, yielding GFP-GluN2C-SLPSP/AAAAA (**Figure 6A**). We then co-expressed this construct together with GluN1-1a and used immunofluorescence to measure surface NMDA receptors. The GluN1-1a/GFP-GluN2C-SLPSP/AAAAA receptors were delivered to the cell surface at significantly lower levels than control receptors (**Figures 6B,C**). Similar results were obtained when the GluN1-1a and GFP-GluN2C-SLPSP/AAAAA subunits were expressed in HEK293 cells (**Figures 6D,E**) or cultured CGCs (**Figures 6F,G**); however, in both expression systems, the GFP-GluN2C-855stop subunit caused even less surface expression (**Figures 6D–G**).

Because the SLPSP motif contains two serine residues, we asked whether surface delivery of the GluN2C-containing receptors is regulated by phosphorylation at these sites. We therefore generated two mutant GluN2C constructs; one construct has alanines substituted for both serines (GFP-GluN2C-S885A,S888A), and the other construct has both serines replaced with the phosphomimetic residue glutamate (GFP-GluN2C-S885E,S888E). We then expressed these mutant subunits together with GluN1-1a in COS-7 cells and measured the surface expression of the receptors. We found that both GluN1-1a/GFP-GluN2C-S885A,S888A and GluN1-1a/GFP-GluN2C-S885E,S888E receptors had reduced surface expression (**Figures 6B,C**). Taken together with our co-localization studies using GluN1-1a/GFP-GluN2C-855stop receptors (Figures S4 and S5), we propose that the proximal C-terminus of the GluN2C subunit—and

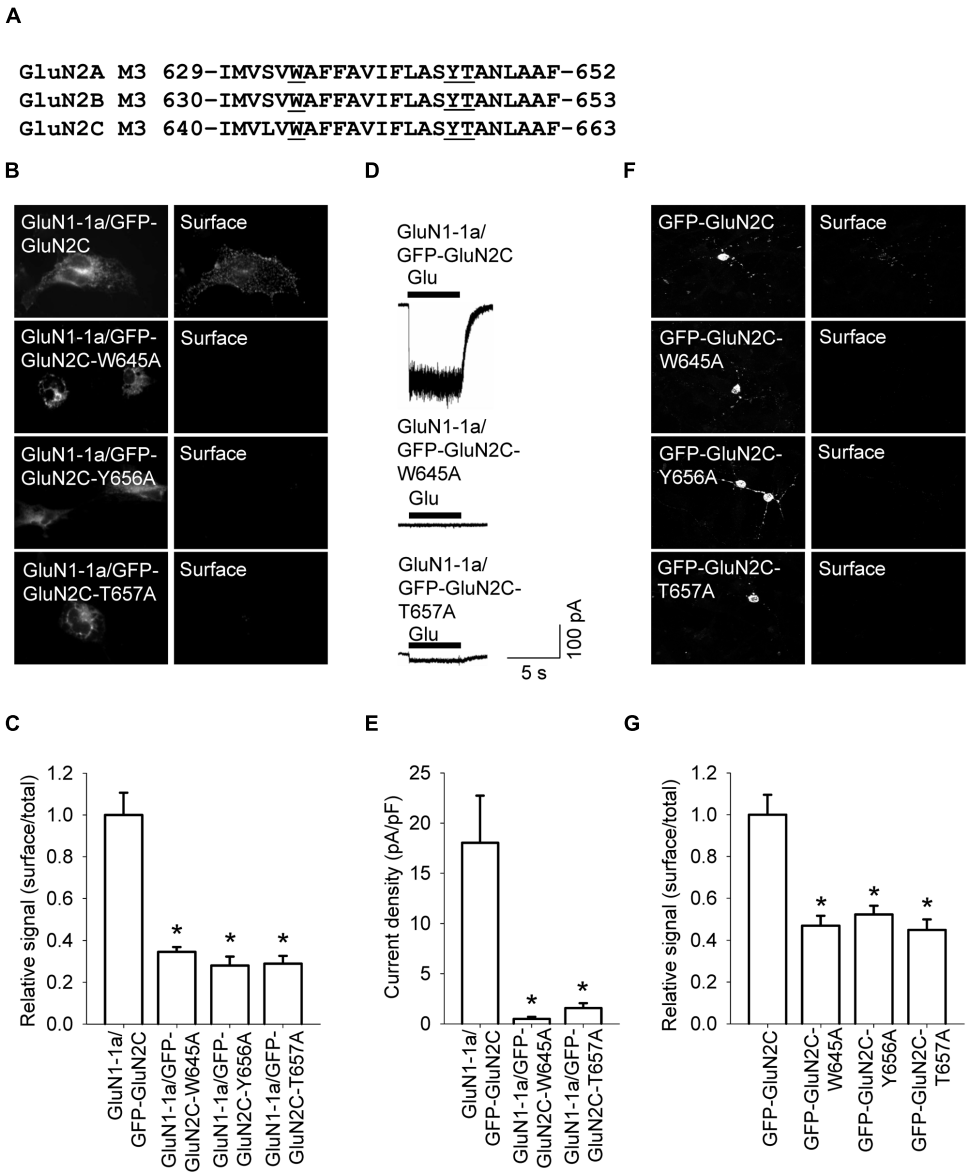

FIGURE 4 | Three amino acid residues within the M3 domain of the GluN2C subunit are essential for delivering full-length NMDA receptors to the cell surface. (A) The sequences of the M3 domains of the GluN2A, GluN2B, and GluN2C subunits are shown; the three residues that were replaced with alanine residues are underlined. **(B)** Representative images of total (left panel) and surface (right panel) pools of NMDA receptors expressed in COS-7 cells. **(C)** Summary of the normalized ratios of surface and total expression of the indicated NMDA receptor subunits measured using fluorescence microscopy. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA. **(D)** Whole-cell voltage-clamp recordings were performed in HEK293 cells expressing the indicated NMDA receptor subunits. Currents were elicited by applying a 5-sec pulse of 1 mM glutamate (indicated by the filled bar); representative traces are shown. **(E)** Quantitative analysis of peak current density (pA/pF) mediated by the indicated NMDA receptors; $n \geq 21$. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA. **(F)** Representative images of total (left panel) and surface (right panel) GluN2 pools in CGCs. **(G)** Summary of the normalized ratios of surface and total expression of NMDA receptor subunits measured using confocal microscopy. $*p < 0.05$ (relative to GFP-GluN2C); ANOVA.

the SLPSP motif in particular—is a critical structural element that regulates the surface delivery of GluN1/GluN2C receptors (see also Discussion). We also suggest that the C-terminus of GluN2C is the most likely structural element underlying the decreased surface expression of GluN1/GluN2C receptors compared to GluN1/GluN2A and GluN1/GluN2B receptors.

DISCUSSION

The early processing and intracellular transport of NMDA receptors to the cell surface is regulated by specific mechanisms that ensure that only properly assembled receptors containing the appropriate subunits are released from the ER and delivered to the cell surface. Here, we investigated the mechanism by which the GluN2C subunit regulates the surface

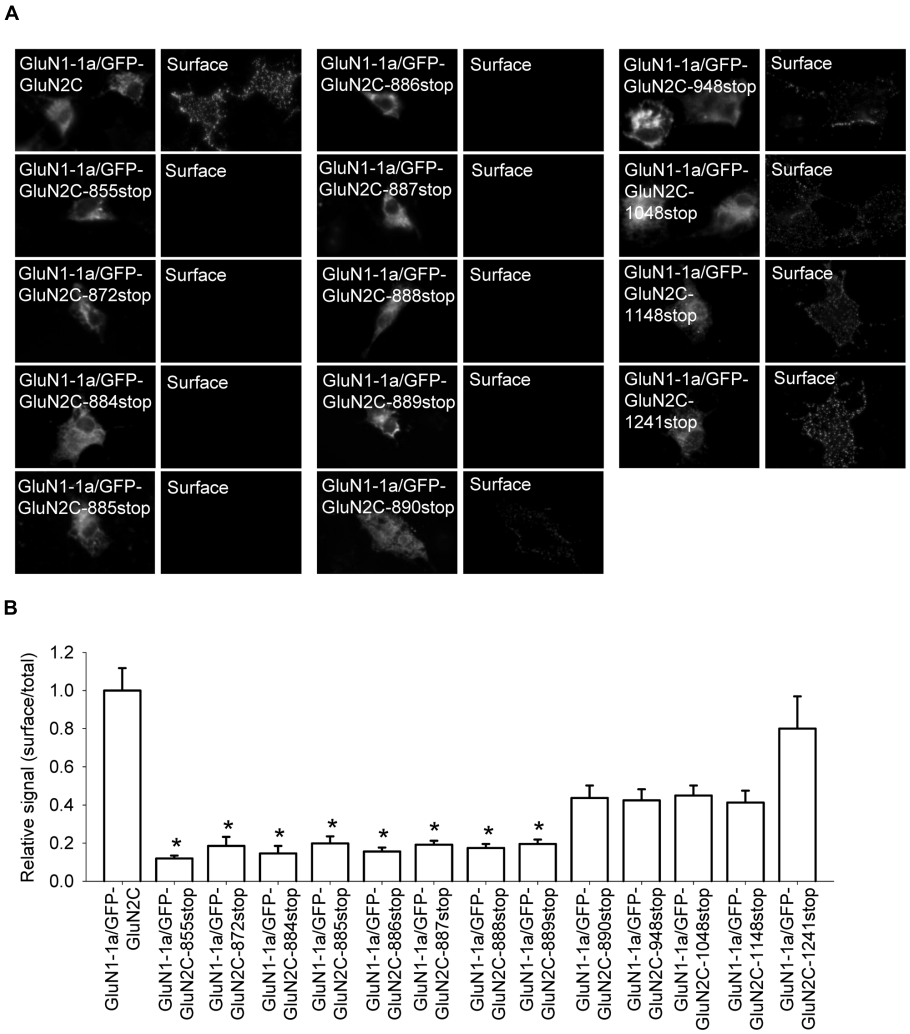

FIGURE 5 | The intracellular C-terminal domain of GluN2C is essential for the surface delivery of NMDA receptors. (A) Representative images of total (left panel) and surface (right panel) pools of labeled NMDA receptor subunits expressed in COS-7 cells. **(B)** Summary of the normalized ratios of surface and total expression of NMDA receptors measured using fluorescence microscopy. *$p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA.

delivery of NMDA receptors. Using a combination of molecular biology, microscopy, and electrophysiology in mammalian cell lines and CGCs expressing recombinant NMDA receptors, we found that the delivery of GluN1/GluN2C receptors to the cell surface is reduced considerably compared to both GluN1/GluN2A and GluN1/GluN2B receptors. Furthermore, we identified three regions within different domains of the GluN2C subunit that play a key role in the surface expression of GluN2C-containing NMDA receptors. We conclude that the GluN2C subunit regulates the forward trafficking of NMDA receptors by a unique mechanism that differs from other NMDA receptor types.

ROLE OF THE GluN2C SUBUNIT IN THE FORWARD TRAFFICKING OF NMDA RECEPTORS

Our finding that GluN1/GluN2A and GluN1/GluN2B receptors are differentially targeted to the cell surface of mammalian cell lines is consistent with previously published results (Chen et al., 1999). However, in cultured CGCs, we found no difference in surface expression between GluN1/GluN2A and GluN1/GluN2B receptors. This discrepancy may be due to the presence of endogenous NMDA receptor subunits in cultured neurons; these endogenous subunits can form multiple NMDA receptor complexes, including triheteromeric receptors (Hansen et al., 2014). The finding that surface targeting of GluN2C-containing receptors is reduced compared to GluN2A- and GluN2B-containing receptors—which was observed in both mammalian cell lines and cultured CGCs—indicates that the GluN2C subunit contains critical structural elements that control the trafficking of GluN2C-containing receptors. These GluN2C-specific elements are likely recognized by specific protein-protein binding partners, including sorting nexin 27 (SNX27) and 14-3-3-epsilon (Chen and Roche, 2009; Cai et al., 2011), as well as other unidentified proteins. Indeed, when expressed alone (i.e., without

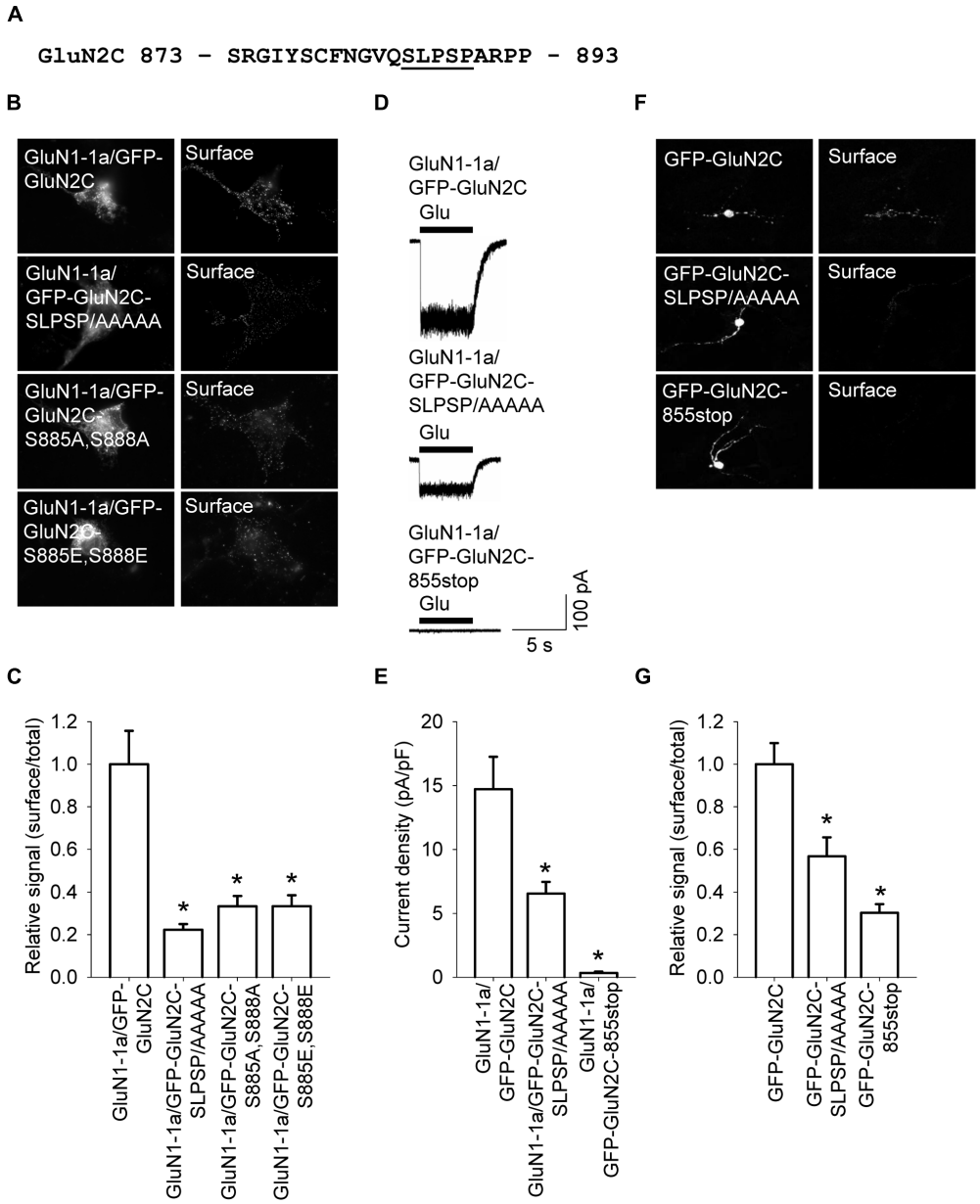

FIGURE 6 | A short amino acid sequence in the proximal C-terminus of GluN2C is essential for surface delivery of NMDA receptors. (A) The amino acid sequence of the proximal part of the C-terminus of GluN2C; the residues that were replaced with alanines are underlined. **(B)** Representative images of total (left panel) and surface (right panel) pools of NMDA receptors expressed in COS-7 cells. **(C)** Summary of the normalized ratios of surface and total expression of the indicated NMDA receptor subunits measured using fluorescence microscopy. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA. **(D)** Whole-cell voltage-clamp recordings were performed in HEK293 cells expressing the indicated NMDA receptor subunits. Currents were elicited by applying a 5-sec pulse of 1 mM glutamate (indicated by the filled bar); representative traces are shown. **(E)** Quantitative analysis of the peak current density (pA/pF) mediated by the indicated NMDA receptors; $n \geq 19$. $*p < 0.05$ (relative to GluN1-1a/GFP-GluN2C); ANOVA. **(F)** Representative images of total (left panel) and surface (right panel) GluN2 pools in CGCs. **(G)** Summary of the normalized ratios of surface and total expression of NMDA receptor subunits measured using confocal microscopy. $*p < 0.05$ (relative to GFP-GluN2C); ANOVA.

GluN1), the GluN2C subunit was retained in the intracellular compartment, as shown previously for GluN2A and GluN2B (Mcllhinney et al., 1998; Horak et al., 2008). This finding suggests that co-assembly of the GluN1 subunit in the receptor is essential for the release of all GluN2 subunit types from the ER.

ROLE OF DISTINCT REGIONS IN THE GluN2C SUBUNIT IMPLICATED IN THE FORWARD TRAFFICKING of NMDA RECEPTORS

We identified three distinct regions within the GluN2C subunit that are essential for driving the surface delivery of GluN1/GluN2C receptors. First, truncating the GluN2C subunit immediately downstream of the M1 domain caused the protein to be retained

in the intracellular compartment, and this retention was released by deleting the N-terminal A2 segment. However, deleting the N-terminal A2 segment from the full-length (i.e., non-truncated) GluN2C subunit reduced the surface expression of GluN1/GluN2C receptors. Interestingly, a similar phenomenon was reported previously for GluN2A, but not GluN2B (Qiu et al., 2009). Given that the A2 segment is relatively well conserved among GluN2 subunits, it is currently unclear why the A2 segment regulates GluN2A and GluN2C differently than GluN2B. It is possible that the A2 segment of some GluN2 subunits can interact with specific binding partner(s); alternatively, the presence of a specific A2 segment is required to enable the GluN1/GluN2 heterotetramer to pass the intracellular quality control checkpoints (with the ER serving as the most likely checkpoint). The latter possibility may be supported by the finding that the GluN1 homodimer must dissociated in order to form the GluN1/GluN2 heterotetramer (Farina et al., 2011).

Second, our experiments revealed the identity of three amino acid residues within the M3 domain of GluN2C—specifically, W645, Y656, and T657—that are important for delivering GluN2C-containing NMDA receptors to the cell surface. We previously reported that the presence of identical residues within the GluN1, GluN2A, and GluN2B subunits is essential for surface delivery of the NMDA receptors; therefore, a shared mechanism likely underlies the M3 domain's ability to regulate the surface expression of NMDA receptors (Kaniakova et al., 2012a). In contrast to previous data with GluN1, GluN2A, and GluN2B subunits with mutations in the M3 domain, we observed extremely small currents in GluN1/GluN2C receptors with a mutation in the GluN2C subunit's M3 domain. One explanation may be that the reduced surface expression of wild-type GluN2C-containing receptors—compared to GluN1/GluN2A and GluN1/GluN2B receptors—is further reduced by the same mechanisms, as in the case of GluN1/GluN2A and GluN1/GluN2B receptors. Alternatively, the GluN1/GluN2C receptors may be regulated via their membrane domains more tightly than other receptor types, and this may be reflected in their reduced surface localization. The membrane domains of GluN subunits were found to be essential for regulating NMDA receptors (Ren et al., 2007; Salous et al., 2009). Moreover, the presence of the M4 domain in GluN1 and GluN2 was found to be essential for forming functional receptors (Schorge and Colquhoun, 2003). Thus, based on these previous data and our observations, specific inter-membrane domain interactions are clearly essential for mediating the delivery of NMDA receptors to the cell surface; however, it is currently not clear whether their effect is mediated during ER processing and/or downstream intracellular transport (Greger et al., 2003; Cao et al., 2011; Salussolia et al., 2011; Kaniakova et al., 2012b).

Finally, we found that deleting the entire C-terminus of GluN2C significantly reduces the number of functional NMDA receptors at the cell surface. Interestingly, deleting the C-terminus of GluN2A or GluN2B does not have such a profound effect (Vissel et al., 2001; Horak et al., 2008). Our series of deletions and mutations revealed a critical structural element within the proximal C-terminal region of the GluN2C subunit; this 5-residue motif (SLPSP) regulates the surface expression of GluN1/GluN2C receptors. Whether the SLPSP motif interacts with a specific binding partner remains unclear. Indeed, we cannot exclude the possibility that additional structural elements within the C-terminus of GluN2C—aside from the SLPSP motif—also regulate the transport of GluN1/GluN2C receptors. This view is supported by a compelling study that identified the RHASLP motif in the C-terminus of GluN2C as a 14-3-3 binding motif (Chen and Roche, 2009). Moreover, we found that deleting the PDZ-binding motif in the GluN2C subunit (i.e., our GluN2C-1241stop construct) did not affect the surface expression of NMDA receptors, which is consistent with a previous study that found that phosphorylation of the serine residues adjacent to the PDZ-binding motif does not regulate the trafficking of GluN1/GluN2C receptors (Chen et al., 2006). Based on this large body of data, we propose that the C-terminus of GluN2C subunits—including the SLPSP motif—plays a unique and specific role in regulating the delivery of GluN1/GluN2C receptors to the cell surface. At this time, our data cannot be used to determine whether the GluN2C C-terminus—including the SLPSP motif—plays a role in ER processing and/or intracellular transport of NMDA receptors. Nevertheless, the C-terminus of GluN2C has relatively low homology with the C-termini of GluN2A and GluN2B (Ishii et al., 1993); therefore, the SLPSP motif within the C-terminus of GluN2C does not have a corresponding motif (i.e., located the same distance from the M4 domain) in the GluN2A and GluN2B subunits.

PHYSIOLOGICAL IMPLICATIONS

Our results clearly demonstrate that multiple structural elements within the GluN2C subunit regulate the transport of GluN2C-containing NMDA receptors. Although it is currently not clear why cells use multiple mechanisms to regulate the trafficking of various NMDA receptor types, it is possible that this strategy ensures that only properly folded GluN2C-containing receptors are transported to the cell surface. Indeed, specific regulatory mechanisms may be used under specific circumstances (e.g., during the activity-driven stimulation of synapses); thus, having several regulatory options available enables the cell to react appropriately under different conditions. Interestingly, mice that express a GluN2C subunit that lack the C-terminus have clear deficits in motor coordination (Sprengel et al., 1998); this observation is consistent with our finding that the C-terminus of GluN2C is an essential element for delivering NMDA receptors to the cell surface. Given that the proper regulation of NMDA receptors is essential for many processes, including excitatory neurotransmission, synaptic plasticity, learning, and memory consolidation, our results provide key insight into the molecular mechanisms that underlie the function of NMDA receptors. These results may also facilitate the development of new therapeutic strategies for treating a wide variety of diseases that are associated with aberrant NMDA receptor trafficking.

AUTHOR CONTRIBUTIONS

Martin Horak, Katarina Lichnerova, Kristyna Skrenkova, Ladislav Vyklicky, and Martina Kaniakova performed the experiments,

Martin Horak, Kristyna Skrenkova, Ladislav Vyklicky, and Martina Kaniakova analyzed the data, and Martin Horak wrote the manuscript with input from the co-authors. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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DYRK1A-mediated phosphorylation of GluN2A at Ser^{1048} regulates the surface expression and channel activity of GluN1/GluN2A receptors

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N-methyl-D-aspartate glutamate receptors (NMDARs) play a pivotal role in neural development and synaptic plasticity, as well as in neurological disease. Since NMDARs exert their function at the cell surface, their density in the plasma membrane is finely tuned by a plethora of molecules that regulate their production, trafficking, docking and internalization in response to external stimuli. In addition to transcriptional regulation, the density of NMDARs is also influenced by post-translational mechanisms like phosphorylation, a modification that also affects their biophysical properties. We previously described the increased surface expression of GluN1/GluN2A receptors in transgenic mice overexpressing the Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A), suggesting that DYRK1A regulates NMDARs. Here we have further investigated whether the density and activity of NMDARs were modulated by DYRK1A phosphorylation. Accordingly, we show that endogenous DYRK1A is recruited to GluN2A-containing NMDARs in the adult mouse brain, and we identify a DYRK1A phosphorylation site at Ser^{1048} of GluN2A, within its intracellular C-terminal domain. Mechanistically, the DYRK1A-dependent phosphorylation of GluN2A at Ser^{1048} hinders the internalization of GluN1/GluN2A, causing an increase of surface GluN1/GluN2A in heterologous systems, as well as in primary cortical neurons. Furthermore, GluN2A phosphorylation at Ser^{1048} increases the current density and potentiates the gating of GluN1/GluN2A receptors. We conclude that DYRK1A is a direct regulator of NMDA receptors and we propose a novel mechanism for the control of NMDAR activity in neurons.

Keywords: GluN2A, DYRK1A, phosphorylation, NMDA receptor, trafficking, Down syndrome

INTRODUCTION

N-methyl-D-aspartate receptors (NMDARs) belong to the ionotropic class of glutamate receptors, playing critical roles in neural development and survival, as well as in synaptic plasticity and memory processes (Traynelis et al., 2010; Hunt and Castillo, 2012; Paoletti et al., 2013). Moreover, impairment of NMDAR activity has been associated with certain pathological conditions (reviewed in Lau and Zukin, 2007). *N*-methyl-D-aspartate receptors are heterotetramers composed of two obligatory GluN1 subunits and two GluN2 (A–D) or GluN3 (A–B) subunits (Paoletti et al., 2013). The biosynthetic pathway of NMDARs leads them to the plasma membrane, where they act as cation-permeable channels gated simultaneously by co-agonists binding and membrane depolarization (reviewed in Traynelis et al., 2010). *N*-methyl-D-aspartate receptor activity is dictated by their location and density at the cell surface, their GluN2 subunit composition, and their post-translational modifications (Barria and Malinow, 2002; Lavezzari et al., 2004; Chen and Roche, 2007; Storey et al., 2011). Non-genomic mechanisms are particularly important in regulating the assembly, trafficking, docking and internalization of NMDARs. Of these, the phosphorylation status of the C-terminal domain of NMDARs regulates activity-dependent NMDAR levels and activity (Salter and Kalia, 2004; Chen and Roche, 2007; Sanz-Clemente et al., 2012). A number of kinases may preferentially phosphorylate a given GluN2 subunit isoform, linking NMDAR composition and activity to intracellular signaling (Sanz-Clemente et al., 2012; Ryan et al., 2013). However, the complete picture of NMDAR phosphorylation, including the kinases and amino acid residues involved, and the functional output of phosphorylation events, is still to be fully defined.

We previously revealed a link between the Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) and NMDARs, evident through the increased GluN1/GluN2A content of synaptosomes obtained from transgenic TgDyrk1A mice (Altafaj et al., 2008). Dual specificity tyrosine-phosphorylation-regulated kinase 1A is a protein kinase found in both the nucleus and cytosol of many different cell types. Upon self-activation by tyrosine autophosphorylation, this kinase phosphorylates serine and threonine residues in exogenous substrates that are involved in a wide variety of cellular functions, including intracellular signaling and synaptic remodeling (reviewed in Aranda et al., 2011). Dual specificity tyrosine-phosphorylation-regulated kinase 1A has received a lot of attention because of its cytogenetic location in the Down syndrome (DS) critical region on human chromosome 21 (HSA21) and its overexpression in DS individuals (Guimerà et al., 1996). Consistent with its potentially etiological role in DS, Dyrk1A overexpression in mice provokes DS-like neurodevelopmental, visual, motor and cognitive phenotypic alterations (reviewed in Park and Chung, 2013), some of which can be rescued through Dyrk1A normalization (Ortiz-Abalia et al., 2008; Altafaj et al., 2013; Laguna et al., 2013).

Here we show that DYRK1A interacts functionally with GluN2A to post-translationally regulate the biophysical properties and the surface expression of NMDARs. We found that DYRK1A physically interacts with GluN1/GluN2A complexes and that it phosphorylates the C-terminal domain of the GluN2A subunit at serine residue 1048 (S^{1048}). DYRK1A-mediated phosphorylation of this residue hinders the internalization of GluN1/GluN2A receptors, provoking increased cell surface expression of these receptors. Moreover, GluN2A S^{1048} phosphorylation not only increases the peak current density but also the GluN1/GluN2A channel opening rate. Together, these findings suggest that DYRK1A is a novel regulator of GluN1/GluN2A receptors.

MATERIALS AND METHODS

PLASMIDS

The expression plasmids for rat GluN1 and GFP-GluN2A were kindly provided by Dr. Vicini (Georgetown University Medical Center, Washington, USA; Vicini et al., 1998). The plasmids to express HA-tagged rat GluN1 in mammalian cells and for the bacterial expression of rat GluN2A C-terminal domain fragments (C1: 897–1117, C2: 1102–1409 and C3: 1409–1464) fused at the N-terminal to glutathione S-transferase (GST) were kindly provided by Dr. Nakanishi and Dr. Nakazawa, respectively (Institute of Medical Science, University of Tokyo, Japan; Tezuka et al., 1999; Taniguchi et al., 2009). The rat GluN2A C-terminal domain fragments (C-term: 897–1464, C1Δ1: 839–1076, C1Δ2: 839–1016) were digested with EcoRI/XhoI and ligated into the digested pGEX-5X-2 vector (Promega). Nucleotide changes (the mutation of serine codons to alanine) were achieved by oligonucleotide-directed mutagenesis, using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). All the plasmids generated by PCR or site-directed mutagenesis, as well as all the in-frame fusions, were verified by DNA sequencing. The expression plasmids encoding the human DYRK1A variants, both the wild type and the kinase-inactive (KD) mutant, have been described previously: HA-tagged variants (pHA derivatives), N-terminal GST fusions (pGST derivatives), and DYRK1A fused to the enhanced green fluorescent protein (described in Alvarez et al., 2007).

GST FUSION PROTEIN EXPRESSION IN BACTERIA

Expression constructs for GST fusion proteins were transformed into *Escherichia coli* BL21(DE3)pLysS and protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for either 3 h at 37°C for the unfused GST, GST-C1, GST-C2 and GST-C3 fusion proteins, or for 8 h at 20°C for GST-CterGluN2A and GST-DYRK1A. The recombinant proteins were bound to glutathione beads (GE Healthcare Life Sciences) and when required, they were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl [pH 8], and dialyzed against a buffer containing 50 mM HEPES [pH 7.4], 150 mM NaCl and 2 mM EDTA. Protein concentrations were determined with a colorimetric assay (the bicinchoninic acid protein assay kit, BCA: Pierce) and/or by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels in which they were compared with standards.

IMMUNOPRECIPITATION

For immunoprecipitation of the endogenous GluN1 and DYRK1A complexes, brains from 2- to 4-month-old mice were dissected out and mechanically homogenized with 10 up-and-down strokes at 700 rpm of a glass-Teflon homogenizer in 10 vol of cold sucrose buffer (320 mM sucrose, 10 mM HEPES [pH 7.4], 1 mM EDTA and Halt™ Protease and Phosphatase Inhibitor Cocktail [PPIC; Pierce]). The homogenate was centrifuged at 4°C for 10 min at 800×*g* to remove the nuclei and large debris. Subsequently, the supernatant was centrifuged for 15 min at 14,000×*g* to obtain the membrane-associated fraction, which was further solubilized in lysis buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 1% Nonidet P40 [NP-40]) for immunoprecipitation with anti-DYRK1A and RIPA buffer for immunoprecipitation with anti-GluN1 supplemented with PPIC. The homogenates were clarified by centrifugation at 4°C for 10 min at 16,000×*g*. After preclearing the soluble lysates for 1 h at 4°C with equilibrated protein G-Sepharose, they were incubated overnight at 4°C with 10 μg of either an anti-GluN1 mouse monoclonal antibody (mAb; Millipore Cat# 05-432 RRID:AB_10015247), or an anti-DYRK1A mAb (Abnova Corporation Cat# H00001859-M01 RRID:AB_534844). Non-specific mouse immunoglobulin G (IgG; Sigma-Aldrich Cat# I5381 RRID:AB_1163670) was used as a control for specificity. The immunocomplexes were incubated with protein G-Sepharose for 2 h at 4°C, and the beads were then washed twice with lysis buffer and once with phosphate-buffered saline (PBS). The bound proteins were eluted in Laemmli's buffer (LB) and analyzed in Western blots.

WESTERN BLOT ANALYSIS

For protein extraction, cells were washed with PBS and scraped off the plate in 400 μl of lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40 and PPIC).

After 10 min incubation at 4°C, the cell debris was pelleted at 15,000×*g*, the solubilized proteins were collected and the protein concentration was determined using a BCA assay. Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Amersham), which were then blocked with 10% skimmed milk in 10 mM Tris-HCl (pH 7.5)/100 mM NaCl (TBS) plus 0.1% Tween 20 (TBS-T). The membranes were probed overnight at 4°C with the primary Ab of interest (diluted in TBS-T/ + 5% skimmed milk) directed against: GluN1, GluN2A (Sigma-Aldrich Cat# M264 RRID:AB_260485), DYRK1A, the HA epitope (Covance, Inc. Cat# MMS-101R-500 RRID:AB_10063630), and GFP (Clontech Cat# 632381). Protein loading was monitored by assessing β-Actin (Sigma-Aldrich Cat# A2228 RRID:AB_476697). Antibody binding was detected with an anti-mouse or anti-rabbit Ab coupled to horseradish peroxidase (Dako, Cat. No. P0447 and P0448, respectively) for 1 h at room temperature (RT) and the immunocomplexes were visualized by chemiluminescence (ECL detection system: Pierce), following the manufacturer's instructions. Immunosignals were analyzed densitometrically with Image *J* software (National Institutes of Health, USA).

IN VITRO KINASE ASSAYS

The *in vitro* kinase (IVK) assays were performed with either GluN1 immunocomplexes from mouse brain or bacterially expressed recombinant GST fusion proteins as the substrates, and bacterially expressed GST-DYRK1A (wt or KD version) as the kinase, purified as described previously (Alvarez et al., 2007). For IVK assays with anti-GluN1 immunocomplexes, the immobilized proteins were washed twice with kinase buffer (50 mM HEPES [pH 7.4], 0.5 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM $MnCl_2$), and incubated for 20 min at 30°C in 30 μl of kinase buffer with a final concentration of 50 μM ATP and [γ-^{32}P]ATP (1×10^{-2} μCi/pmol). For GST fusion proteins, eluted GST-GluN2A C-terminal fragments (0.5–1 μg) were incubated for 20 min at 30°C in 40 μl of kinase buffer with 50 μM ATP and [γ-^{32}P]ATP (1×10^{-3} μCi/pmol). Reactions were stopped by adding 6× LB, the samples were resolved by SDS-PAGE and then stained with Coomassie blue. The incorporation of ^{32}P was detected by autoradiography of the dried gels.

CELL CULTURE AND TRANSFECTION

HEK-293T and COS-7 cell lines were obtained from the American Type Culture Collection and maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Furthermore, D-2-amino-5-phosphonopentanoic acid (D-AP5, 200 μM for HEK-293T and 500 μM for COS-7: Abcam Biochemicals) was added to the medium to avoid excitotoxicity in cells co-transfected with GluN1 and GFP-GluN2A (1:1 ratio). Transient transfection of HEK-293T cells was achieved by the calcium phosphate method and the cells were analyzed 48 h after transfection. COS-7 cells were transiently transfected with Lipofectamine 2000 (Life Technologies), according to the manufacturer's recommendations, and the cells were fixed 20 h after transfection.

To prepare dissociated cortical neuron cultures, embryonic day (E)18 mouse embryos were obtained from pregnant females, the cerebral cortex was isolated and maintained in cold Hank's Balanced Salt Solution (HBSS, Gibco) supplemented with 0.45% glucose (HBSS-Glucose). After carefully removing the meninges, the cortical tissue was digested mildly with trypsin for 17 min at 37°C and dissociated. The cells were washed three times in HBSS and resuspended in Neurobasal medium supplemented with 2 mM Glutamax (Gibco) before filtering in 70 μm mesh filters (BD Falcon). The cells were then plated onto glass coverslips (5×10^4 cells/cm^2) coated with 0.1 mg/ml poly-L-lysine (Sigma) and 2 h after seeding, the plating medium was substituted by complete growth medium, Neurobasal medium supplemented with 2% B27 (Invitrogen) and 2 mM Glutamax, and the coverslips were incubated at 37°C in a humidified 5% CO_2 atmosphere. Every 3–4 days, half of the conditioned medium was removed and replaced by fresh growth medium. Primary cultures were transfected with Lipofectamine 2000 on day 8 *in vitro* (div8), according to the manufacturer's, instructions and the cells were fixed 48 h after transfection. All the experimental procedures were carried out according to European Union guidelines (Directive 2010/63/EU) and following protocols that were approved by the Ethics Committee of the Bellvitge Biomedical Research Institute (IDIBELL).

IMMUNOFLUORESCENCE ANALYSIS OF SURFACE NMDA RECEPTORS

The surface-to-total expression of NMDARs was analyzed in COS-7 cells that were washed twice with PBS before they were fixed with 4% paraformaldehyde (PFA). Surface expression of GFP-GluN2A was detected using an antibody against GFP (1:1000, Life Technologies Cat# A11122 RRID:AB_10073917) that recognizes the extracellular epitope of heterologously expressed receptors and that was visualized with an Alexa 647-conjugated goat anti-rabbit Ab (1:1000, Molecular Probes (Invitrogen) Cat# A21245 RRID:AB_141775). The total pool of receptors was detected by the fluorescent signal emitted by the GFP-GluN2A transfected. HA-DYRK1A positive cells were identified after permeabilizing the cells with 0.1% Triton X-100 and labeling with anti-HA (1:1000) that was visualized with an Alexa 555-conjugated donkey anti-mouse Ab (1:2000, Molecular Probes, Cat# A31570).

To analyze the surface expression of the transfected NMDARs in primary neuronal cultures, cells were washed twice with PBS and fixed with 4% PFA in PBS containing 4% sucrose. The surface expression of GFP-GluN2A was detected with anti-GFP (1 h) and visualized with an Alexa 488-conjugated goat anti-rabbit Ab (1:1000, Life Technologies Cat# A11034 RRID:AB_10562715). The intracellular pool of receptors was identified by permeabilizing cells with 0.1% Triton X-100 and labeling them with a rabbit anti-GFP-Alexa 555-conjugated Ab (1:250, Invitrogen Cat# A31851 RRID:AB_1500154). HA-DYRK1A positive neurons were identified by labeling them with mouse anti-HA that was visualized with an Alexa 647-conjugated donkey anti-mouse Ab (1:1000, Molecular Probes (Invitrogen) Cat# A31571 RRID:AB_162542).

Fluorescence was visualized with a Leica TCS-SL spectral confocal microscope (Leica Microsystems, Wetzlar, Germany) using a Plan-Apochromat 63×/1.4 N.A. immersion oil objective (Leica Microsystems) and a pinhole aperture of 114.54 μm or 202 μm (for surface receptors). To excite the different fluorophores, the confocal system is equipped with three excitation laser beams at 488 nm, 546 nm and 633 nm. In each experiment, the fluorescence intensity was measured in 10–15 cells per condition (COS-7) and in 10–15 dendrites from at least two or three pyramidal neurons per condition. Fluorescence was quantified using Adobe Photoshop CS5 software (Adobe Systems Inc.) and the results are represented as the mean ± standard errors of the means (SEM) of the ratio of surface/total (COS-7 cells) or surface/intracellular (primary cultures) GluN2A immunosignal, analyzing at least three independent experiments.

ENDOCYTOSIS ASSAYS

N-methyl-D-aspartate glutamate receptor internalization was assessed using an "antibody feeding" technique in living COS-7 cells that expressed wild-type or mutant NMDARs (transiently co-transfected with GluN1 and GFP-tagged GluN2A constructs), both in the presence or absence of transfected HA-DYRK1A. To measure the rate of NMDAR internalization, cells were labeled for 30 min at 4°C with rabbit anti-GFP Ab (1:1000), which binds to the GFP tag of membrane-anchored GFP-GluN2A/GluN1 receptors. The medium containing the antibody was removed, the cells were washed and they were incubated for 30 min at 37°C to allow NMDAR internalization. The cells were then washed twice with PBS, fixed with 4% PFA/2% sucrose and washed three times with PBS. After blocking, surface NMDARs were labeled with an anti-rabbit Alexa 647-conjugated secondary Ab (1:500) and following washing of the secondary Ab, the cells were permeabilized and the internalized NMDARs were labeled with an Alexa 555-conjugated anti-rabbit Ab (1:4000, Molecular Probes (Invitrogen) Cat# A21429 RRID:AB_141761). Cells co-transfected with HA-DYRK1A were also labeled with anti-HA (1:1000) that was visualized with an Alexa 488-conjugated anti-mouse secondary Ab (1:1000, Life Technologies Cat# A21202 RRID:AB_10049285). The cells immunofluorescence was then analyzed as described above and the results are represented as the mean ± SEM of the ratio of internalized/surface GluN2A subunits.

ELECTROPHYSIOLOGICAL RECORDINGS OF WHOLE-CELL NMDA CURRENTS IN HEK293T CELLS

Electrophysiological recordings were obtained 24 h after transfection, perfusing the cells continuously at RT with the external bath solution (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 11 glucose, and 10 HEPES, adjusted to pH 7.3 with Tris and supplemented with the open-channel non-reversible antagonist MK-801 (5 μM, Tocris). *N*-methyl-D-aspartate (1 mM, Tocris), in the presence of glycine (50 μM, Tocris) and MK-801 (5 μm), was applied for 3 s using a fast perfusion system ($VC^3$8 Focal Perfusion System, ALA Scientific Instruments). Such system features solenoid valves and a Quartz MicroManifold (QMM) that are optimized for high-speed solution exchange, with valve opening speeds of 1–2 ms. The activated currents were recorded in the whole-cell mode at a holding potential of −60 mV, acquired at 10 kHz and filtered at 1 kHz. Electrodes with open-tip resistances of 2–5 MΩ were used, and the data were acquired and analyzed using pClamp 8 software (Axon Instruments) and a D-6100 Darmstadt amplifier (List Medical, Germany). The internal pipette solution contained (in mM): 140 CsCl, 1 EGTA, 4 Na_2ATP, 0.1 Na_3GTP and 10 HEPES, adjusted to pH 7.25 with CsOH.

Statistical analysis

Comparison between experimental groups was evaluated using InStat Software (GraphPad Software, Inc.), applying an One Way Analysis of Variance (ANOVA) followed by a Bonferroni *post hoc* test or a Student's *t* test. Data are presented as the means ± SEM, at least, three independent experiments.

RESULTS

DYRK1A INTERACTS WITH GluN2A AND PHOSPHORYLATES ITS CYTOSOLIC DOMAIN AT SERINE 1048

Having previously established a functional connection between the GluN2A NMDAR subunit and the protein kinase DYRK1A in animal models of overexpression (Altafaj et al., 2008), we evaluated the possibility that DYRK1A had a direct effect on GluN2A-containing NMDARs. The ability of a kinase to phosphorylate its substrate requires a physical interaction—even if it is indirect, weak and/or transient—between the enzyme and its potential substrate (GluN1/GluN2A). To test this possibility, and given that a fraction of DYRK1A is present in non-nuclear lysates of the mouse brain (Martí et al., 2003; Aranda et al., 2008), as are NMDARs, we assessed the proteins that associate with this kinase by immunoprecipitating it from adult mouse brain lysates with an antibody that specifically recognizes DYRK1A. In Western blots, a GluN2A-immunoreactive protein was eluted from the beads that pulled down DYRK1A but not from the non-specific IgG control beads (**Figure 1A**), indicating that GluN2A and DYRK1A were present in the same protein complexes.

To assess whether NMDAR subunits are substrates of the DYRK1A serine/threonine kinase, complexes containing the GluN1 subunit were immunopurified from adult mouse brain lysates. These complexes contained both GluN1 and GluN2A, as witnessed in Western blots (**Figure 1B**, left panel), and they were used as substrate in the IVK assay with purified GST-DYRK1A. Weak phosphorylation of two protein bands with molecular weights around 170 kDa and 130 kDa was evident when the IVK was performed in the presence of GST alone (**Figure 1B**). Based on the electrophoretic mobility of the NMDAR subunits, these labeled bands could correspond to GluN2A (165 kDa) and GluN1 (115 kDa), suggesting that the NMDAR complexes were able to recruit endogenous kinases. However, radiolabeled ATP was incorporated more intensely into these proteins when purified DYRK1A was included in the IVK assay (**Figure 1B**), an indication that both subunits are targets of DYRK1A. While the assignment of GluN2A phosphorylation was clear, we could not be completely confident about the phosphorylation of GluN1, since the molecular weight of the putative radilolabeled GluN1 has a similar

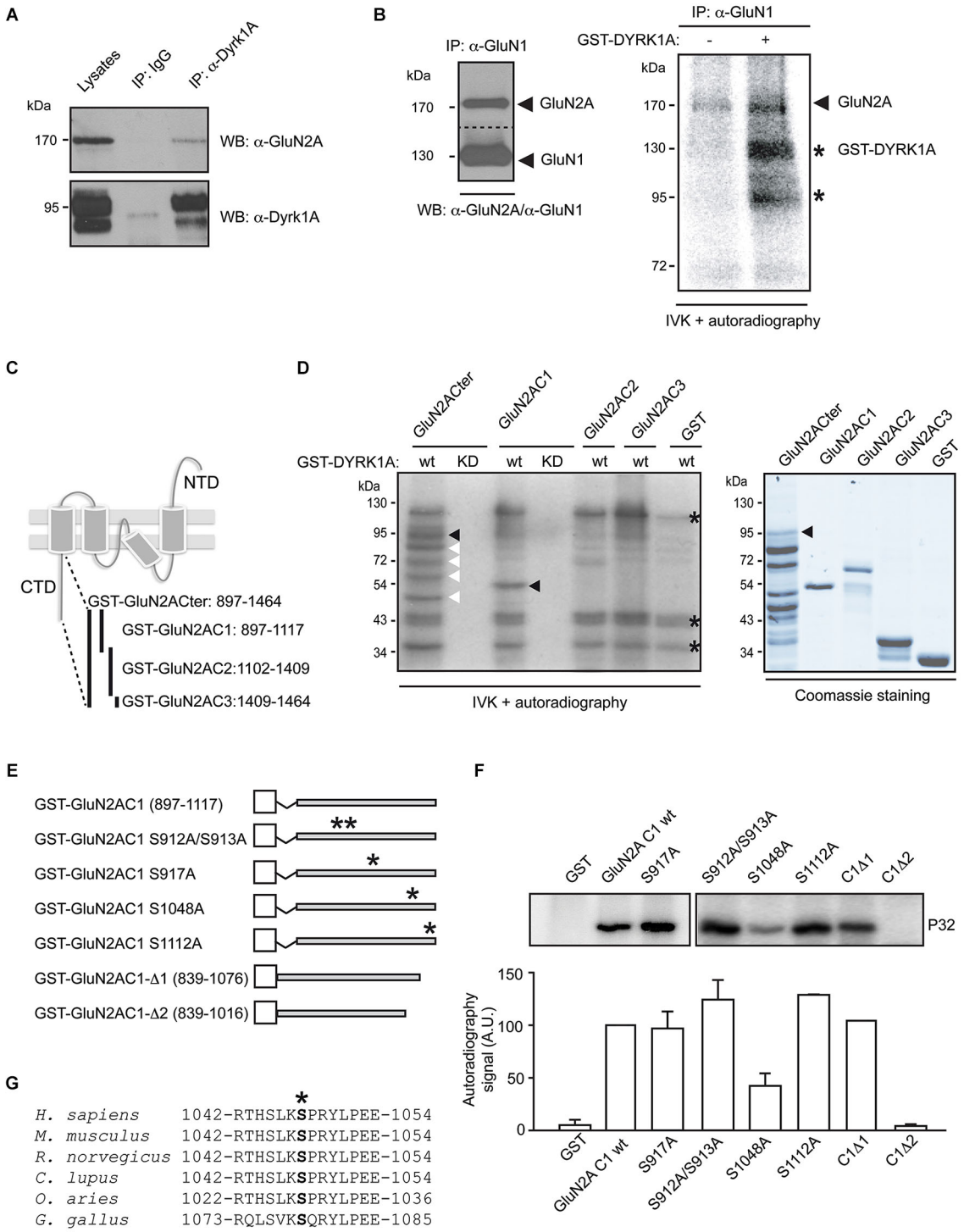

FIGURE 1 | Dual specificity tyrosine-phosphorylation-regulated kinase 1A interacts with GluN2A and phosphorylates the GluN2A subunit at S^{1048}. (A) Solubilized proteins from the adult mouse brain (input lane; 10% of lysates) were immunoprecipitated with either a mouse IgG or an anti-DYRK1A antibody, and both the lysates and the immunoprecipitates were analyzed in Western blots probed with anti-GluN2A and anti-DYRK1A antibodies as indicated. **(B)** Equivalent aliquots of anti-GluN1 purified immunocomplexes obtained from the adult mouse brain were analyzed in Western blots probed with an anti-GluN2A antibody (left panel) or they were used as the substrate in a radioactive *in vitro* phosphorylation assay in the presence or absence of purified recombinant GST-DYRK1A. The radiolabeled proteins were then fractionated by SDS-PAGE and detected by autoradiography (right panel). The arrows indicate the phosphorylated GluN2A and possibly, the phosphorylated GluN1, and the stars indicate the labeled bands resulting from GST-DYRK1A autophosphorylation. **(C)** Schematic representation of the GluN2A subunit topology and the GST fusion proteins covering the cytoplasmic tail of GluN2A used in the assays. **(D)** As indicated, bacterially purified GluN2A GST-fusions

(*Continued*)

FIGURE 1 | Continued
or unfused GST were examined in an IVK assay, in the presence of a GST fusion protein of the wild-type (wt) or kinase-deficient DYRK1A (KD). The substrates were analyzed by Coomassie staining (left panel) and the phosphorylated bands indicated by black arrows represent the full-length recombinant proteins, while the white arrows refer to the GluN2ACter truncated products and the asterisks indicate the GST-DYRK1Awt autophosphorylated bands (full-length or truncated products). **(E)** Schematic representation of the different mutant variants of the GST-GluN2AC1 fragment in which the asterisks indicate the position of the corresponding Ser to Ala mutants. **(F)** The GST-GluN2AC1 fragment or the indicated mutants were used as substrates in IVK assays with GST-DYRK1A. The panel shows a representative experiment and the histogram corresponds to the average ^{32}P incorporation ± SEM (n = 2–3) calculated by densitometry (*$p < 0.05$). **(G)** The amino acid sequences of GluN2A from human (NP_000824), mouse (NP_032196), rat (NP_036705), dog (XP_005621613), sheep (XP_004020812) and chicken (XP_425252) were aligned to show the conserved region surrounding S^{1048} (marked with an asterisk). The numbers indicate the first and last amino acids listed.

electrophoretic mobility as autophosphorylated GST-DYRK1A (**Figure 1D**).

The phosphorylation of the GluN2A subunit described in the literature (Sanz-Clemente et al., 2012) and deposited in the Phosphosite database[1] occurs mostly in its intracellular C-terminal domain. Therefore, to further confirm the ability of DYRK1A to phosphorylate GluN2A, we focused on the GluN2A region between amino acids 839 and 1464, corresponding to its cytosolic tail. The GluN2A C-terminal domain (Cter), and three different non-overlapping fragments (C1, C2 and C3; **Figure 1C**), were expressed as GST fusion proteins in bacteria (**Figure 1D**) and assayed as substrates in IVKs with bacterially expressed GST-DYRK1A wild-type or GST-DYRK1A^{KD}, a kinase-deficient mutant as a negative control. As expected, no phosphorylation of these substrates was detected in the presence of GST-DYRK1A^{KD}, confirming the incapacity of this mutant to autophosphorylate or to phosphorylate exogenous substrates (**Figure 1D**). By contrast, GST-DYRK1Awt was autophosphorylated in the IVK, as evident by the presence of the signals at 130 kDa (full-length GST-DYRK1Awt), 36 kDa and 29 kDa (GST-DYRK1A truncated products). In the case of the GluN2A cytoplasmic tail (GluN2A-Cter), the complete fragment was phosphorylated in the assay (**Figure 1D**), indicating the ability of DYRK1A to phosphorylate the cytosolic domain of GluN2A. However, only the GluN2A C1 fragment (897–1117) proved to be a DYRK1A substrate, in contrast to the other truncated Cter fragments, C2 and C3, (**Figure 1D**). The Cter region putatively phosphorylated by DYRK1A, was further narrowed down by generating two deletion mutants (GST-GluN2AC1Δ1 [839–1076] and GST-GluN2AC1Δ2 [839–1016]; **Figure 1E**). Eliminating residues 1076–1117 did not alter DYRK1A phosphorylation, while the deletion of amino acids 1017–1076 completely abolished DYRK1A-mediated phosphorylation of GluN2AC1 (**Figure 1F**), suggesting that DYRK1A targets residues between amino acids 1017–1076 of GluN2A.

[1]http://www.phosphosite.org

Dual specificity tyrosine-phosphorylation-regulated kinase 1A has been described as a proline-directed protein kinase, showing preference for serine and threonine residues followed by a proline, and with an arginine at position -3 (Himpel et al., 2000). Close inspection of the GluN2A Cter suggested the presence of a unique putative DYRK1A-phosphorylation site at serine residue 1048 (S^{1048}), which is conserved in GluN2A receptors from several species (**Figure 1G**). Mutation of this serine residue to the non-phosphorylatable amino acid alanine significantly reduced DYRK1A-mediated phosphorylation. Such a reduction was not observed when other serine residues within the GluN2A-C1 fragment were mutated (S912, S913, S917, S1112; **Figure 1F**), confirming the importance of S1048 for DYRK1A phosphorylation of GluN2A.

DYRK1A ENHANCES THE SURFACE EXPRESSION OF GluN1/GluN2A RECEPTORS

Phosphorylation of NMDA receptors is a mechanism regulating their trafficking and endocytosis, which in turn modulates their surface density (Lin et al., 2006; Sanz-Clemente et al., 2010; Zhang et al., 2012; Chowdhury et al., 2013). Thus, we hypothesized that DYRK1A phosphorylation of GluN2A might regulate the surface expression of NMDARs. To test this hypothesis, we first assessed the surface density of GluN2A in COS7 cells exogenously expressing GluN1 and GFP-GluN2A alone (GluN1-GluN2A), or in the presence of wild-type or KD HA-DYRK1A. Immunofluorescence analysis showed a significant increase in the surface:total ratio of GFP-GluN2A when DYRK1A was co-expressed with this subunit (100 ± 3.7% for GluN1-GluN2A cells *vs.* 126 ± 5.0% for DYRK1A-expressing cells; n = 130 and 111, respectively; $p < 0.001$; **Figure 2**). No such enhancement was detected when the kinase inactive DYRK1A^{KD} was co-expressed with the NMDAR subunits (99 ± 4.6% for cells co-expressing KD DYRK1A^{KD}; n = 45; **Figure 2**). We then generated GFP-fusions of GluN2A in which S^{1048} was mutated to a non-phosphorylatable amino acid (Ser1048Ala) or to an amino acid that mimics the phosphorylated status of this residue (Ser1048Glu). The surface density of GFP-GluN2A^{S1048A} showed a surface:total ratio similar to wild type GluN2A (98 ± 6.5%; n = 38; **Figure 2**) and moreover, DYRK1A failed to increase the surface levels of the phospho-deficient GFP-GluN2A^{S1048A} (98 ± 6.5% for GluN1-GluN2A^{S1048A} *vs.* 110 ± 6.8 for cells co-expressing DYRK1A; n = 38 and 47, respectively). Conversely, the surface:total ratio of the phospho-mimetic GFP-GluN2A^{S1048E} mutant was higher (100 ± 3.7 for control cells *vs.* 128 ± 6.8 for DYRK1A-expressing cells; n = 130 and 59, respectively; $p < 0.001$), even in the absence of HA-DYRK1A (**Figure 2**). The increase in the surface expression of GluN2A^{S1048E} was not enhanced by co-transfection of DYRK1A (128 ± 6.8 for GluN1-GluN2A^{S1048E} *vs.* 119 ± 6.9 for DYRK1A-expressing cells; n = 59 and 52, respectively), suggesting that if other potential GluN2A secondary phosphosites of DYRK1A existed, they would not be involved in the increased GluN2A surface expression.

To translate the observed cell surface enrichment of GluN1/GluN2A in the presence of DYRK1A to a neuronal context, we evaluated the surface density of transiently transfected

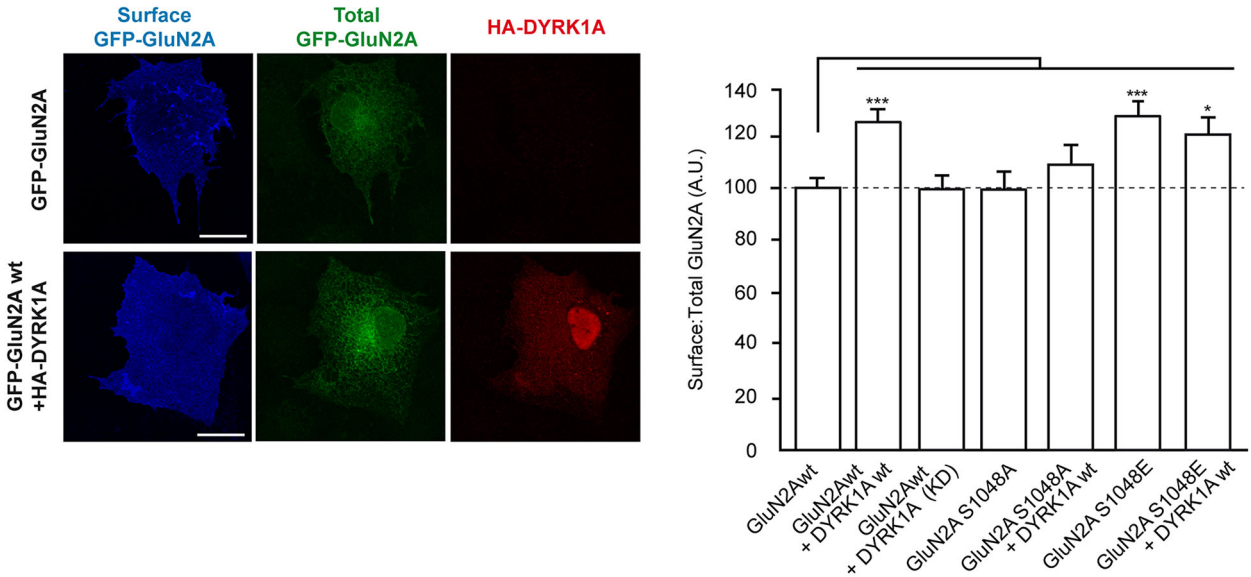

FIGURE 2 | Dual specificity tyrosine-phosphorylation-regulated kinase 1A mediated phosphorylation of GluN2A at S^{1048} increases the surface expression of GluN2A. COS-7 cells were transiently co-transfected with plasmids to express GluN1 and wild-type (wt), or mutant versions of GFP-GluN2A (S1048A, phospho-deficient; S1048E, phospho-mimetic), in the presence or absence of HA-DYRK1A (wt, wild-type; KD, kinase-inactive). After fixing, the cells were incubated with an anti-GFP antibody to label the surface receptors (blue). Direct green fluorescence was used to measure total GFP-GluN2A expression (green). HA-DYRK1A expressing cells were identified by anti HA-immunostaining (red). Scale bar = 10 μm. The histogram represents the mean ± SEM of the GluN2A surface expression normalized to the total GFP-GluN2A signal, with the values for transfections GluN1+GFP-GluN2Awt considered as 100 (n = 38–130 cells from, at least, three independent experiments per condition; $*p < 0.05$, $***p < 0.001$, ANOVA).

GFP-GluN2A in primary cortical neurons established from mouse embryos. In primary cortical neurons, the intracellular levels of transfected GluN2A subunit was neither affected by the presence of a mutation on Ser1048 (96.7 ± 10.0% of control for $GluN2A^{S1048A}$ and 97.9 ± 5.7% for $GluN2A^{S1048E}$; n = 31–52) nor by the presence of HA-DYRK1A (110.5 ± 8.9% of control; n = 48). As observed in COS7 cells, the presence HA-DYRK1A increased the surface expression of GFP-GluN2A in the dendrites of primary cortical neurons (surface:intracellular GFP ratio = 116 ± 4% of control; n = 43–48, control or DYRK1A transfected; $p < 0.01$) but not that of the phospho-deficient GFP-$GluN2A^{S1048A}$ construct (106.4 ± 3.5% of control; n = 31; **Figure 3**). Likewise, the phospho-mimetic mutant GFP-$GluN2A^{S1048E}$ showed a robust increase in surface expression compared with GFP-GluN2A (135.4 ± 2.7% of control; n = 52; $p < 0.001$). Collectively, these results indicate that S^{1048} phosphorylation is necessary and sufficient for DYRK1A to increase the levels of GluN2A at the plasma membrane.

DYRK1A REDUCES THE INTERNALIZATION OF SURFACE GluN1/GluN2A HETERODIMERS

The density of NMDARs at the cell surface reflects the balance between delivery/docking mechanisms ("*on*" mechanisms) and receptor internalization ("*off*" mechanisms), processes critical for synaptic maturation (Barria and Malinow, 2002). *N*-methyl-D-aspartate glutamate receptors traffic between the plasma membrane and intracellular compartments through vesicle-mediated membrane delivery and endocytosis. Phosphorylation of NMDARs regulates the clathrin-mediated endocytosis of these receptors (Chung et al., 2004; Lavezzari et al., 2004; Scott et al., 2004; Prybylowski et al., 2005; Sanz-Clemente et al., 2010; Chowdhury et al., 2013), a mechanism that possibly explains the increase in surface NMDAR expression provoked by DYRK1A. Therefore, we performed antibody-feeding assays in living cells to quantify the internalization rate of GFP-GluN2A in the presence or absence of DYRK1A. Unlike the kinase inactive version, wild type HA-DYRK1A significantly decreased the relative amount of GFP-GluN2A internalized compared with cells that did not express DYRK1A (100 ± 4.2% for GluN1-GluN2A *vs.* 73 ± 4.5% for DYRK1Awt co-expressing cells [$p < 0.001$] or 94 ± 10.1% for cells co-expressing $DYRK1A^{KD}$ [non-significant]; n = 115, 97 and 33, respectively; **Figure 4**). By contrast, the internalization of the phospho-deficient $GluN2A^{S1048A}$ was not affected when co-expressed with DYRK1A (100 ± 7.2 for GluN1-$GluN2A^{S1048A}$ cells *vs.* 90 ± 7.3 for DYRK1A-expressing cells; n = 52 and 53, respectively). Furthermore, the rate of internalization of the phospho-mimetic $GluN2A^{S1048E}$ mutant was also significantly reduced, irrespective of the presence of DYRK1A (43 ± 4.8% for cells expressing GluN1-$GluN2A^{S1048E}$ construct and 45 ± 6.7% for cells co-expressing DYRK1A and $GluN2A^{S1048E}$; n = 31 and 20, respectively; $p < 0.001$). Overall, these results indicate that the phosphorylation of GluN2A at S^{1048} is critical for DYRK1A to dampen NMDAR endocytosis. Moreover, the

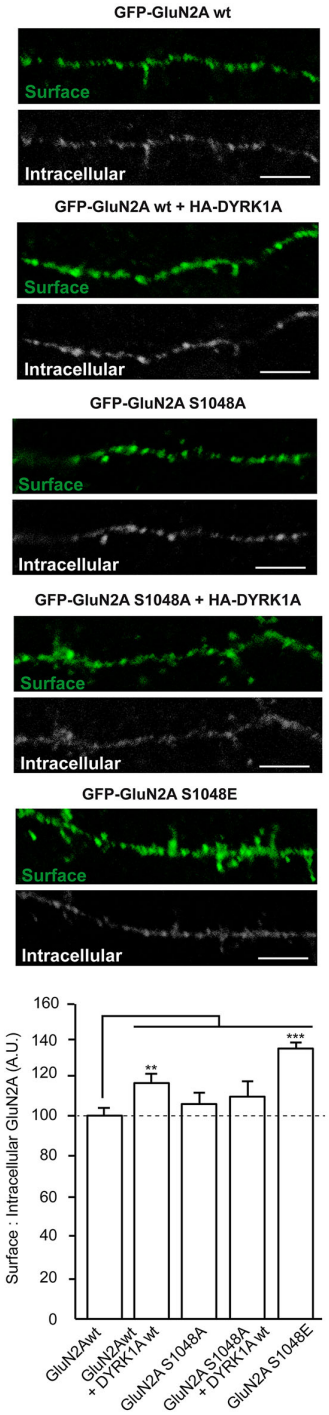

FIGURE 3 | Dual specificity tyrosine-phosphorylation-regulated kinase 1A mediated phosphorylation of GluN2A at S^{1048} increases its surface expression in primary cortical neurons. Primary cultures of mouse embryo cortices were transiently transfected with GFP-GluN2A (wt, wild-type; S1048A, phospho-deficient mutant; S1048E, phospho-mimetic mutant) on day *in vitro* 8 (DIV8), in the presence or absence of heterologous HA-DYRK1A. The effect of DYRK1A on the surface:intracellular ratio of GluN1/GluN2A in primary mouse cortical neurons was evaluated by immunofluorescence. Prior to permeabilization,

(*Continued*)

FIGURE 3 | Continued
anti-GFP/Alexa488 was used to detect the surface chimeric receptors (represented in green), whereas intracellular GFP-GluN2A receptors were visualized after permeabilizing the cells, using an anti-GFP/Alexa555 antibody. Scale bar = 5 μm. The histogram represents the mean ± SEM GluN2A surface expression normalized to the intracellular GFP-GluN2A signal (n = 31–52 dendrites from, at least, three independent experiments per condition, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ANOVA).

inhibitory effect on the rate of receptor internalization could contribute to the observed increase in NMDAR surface density in DYRK1A-expressing cells.

DYRK1A MODULATES GluN1/GluN2A-MEDIATED NMDA CURRENTS

In addition to the cellular consequences resulting from NMDAR phosphorylation, this post-translational modification could also potentially affect the biophysical properties of NMDARs. We assessed the potential regulatory effects of GluN2A S^{1048} phosphorylation by DYRK1A by studying the electrophysiological properties of NMDA elicited currents in HEK-293T cells exogenously expressing the GluN1 and GluN2A subunits. Currents elicited by NMDA were recorded in the whole-cell configuration 24 h after transfection, in the presence of the essentially irreversible NMDAR open-channel blocker MK-801. As the channel must enter the open state to be blocked by MK-801, the decay time constant of the current in the presence of both NMDA and MK-801 can be used to determine the opening rate (Jahr, 1992; Rosenmund et al., 1995). In cells expressing GluN1/$GluN2A^{wt}$, the peak NMDA elicited whole-cell current density increased significantly in the presence of DYRK1A (from 64.74 ± 16.43 pA/pF for control cells to 149.2 ± 29.76 pA/pF for DYRK1A-expressing cells; n = 13 in both conditions, $p < 0.05$; **Figures 5A,E**). However, the phospho-deficient $GluN2A^{S1048A}$ mutant variant did not respond to the presence of DYRK1A with an alteration in the peak current density (58.63 ± 10.07 pA/pF for cells expressing $GluN2A^{S1048A}$ and 79.5 ± 12.51 pA/pF for DYRK1A co-expressing cells, n = 12 and 13, respectively; **Figures 5C,E**).

To gain insight into the effect of DYRK1A on NMDA channel gating, we analyzed the opening rate constant of NMDARs. Agonist-evoked currents were normalized to the same peak amplitude to compare the time course of decay (**Figures 5B,D**). The faster decay of the NMDA current observed in the presence of DYRK1A indicated a higher rate of channel opening. Indeed, the statistical analysis showed that the presence of DYRK1A significantly increased the opening rate of GluN1/$GluN2A^{wt}$ receptors (from 2.91 ± 0.62 s^{-1} in control cells to 6.63 ± 0.89 s^{-1} in DYRK1A co-expressing cells, n = 13 in both conditions, $p < 0.001$; **Figures 5B,F**). By contrast, DYRK1A had no effect on the opening rate of phospho-deficient GluN1/$GluN2A^{S1048A}$ receptors (from 3.85 ± 0.48 s^{-1} for $GluN2A^{S1048A}$ expressing cells to 4.06 ± 0.44 s^{-1} for DYRK1A co-expressing cells, n = 12 and 13, respectively; **Figures 5D,F**). Together, these data indicate that phosphorylation of GluN2A at S^{1048} by DYRK1A not only increases the NMDA elicited peak current density, which is consistent with the increased surface expression of GluN1/GluN2A receptors, but it also affects receptor function by potentiating NMDAR gating.

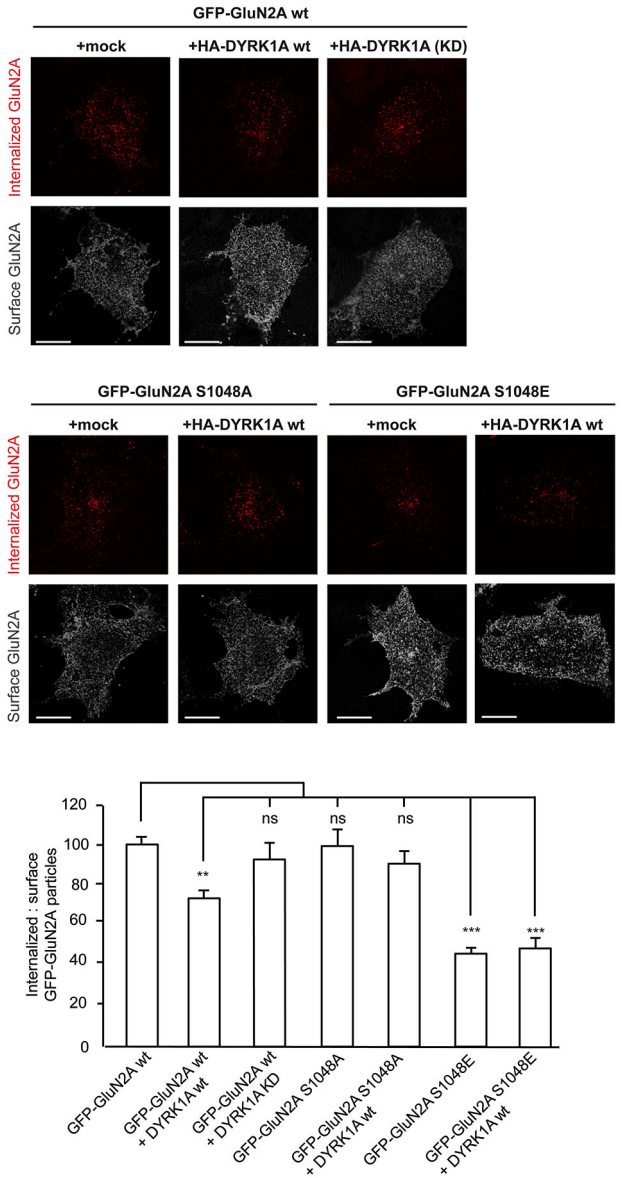

FIGURE 4 | Reduction in the internalization rate of GluN2A in the presence of DYRK1A. Live transiently co-transfected COS-7 cells were incubated with an anti-GFP antibody for 30 min at 4°C. After the rapid removal of the excess antibody, the cells were placed in the incubator for additional 30 min at 37°C to allow the GluN2A-labeled particles to be internalized. Membrane receptors were then immunolabeled with Alexa647 (shown in gray), whereas the internalized receptors were immunolabeled with Alexa555 (red particles). Scale bar = 10 μm. *Bottom*, Histogram representing the mean ± SEM of the normalized ratio of the internalized particles and the surface GluN2A receptors (n = 31–115 cells from three independent experiments, $***p < 0.001$, ANOVA).

DISCUSSION

In post-synaptic neurons, the activity and the density of NMDARs at the membrane are factors that exert an important influence on neuronal function and synaptic plasticity. Thus, these parameters must be finely tuned, which is in part mediated by the (de)phosphorylation of NMDAR subunits. Much effort has been dedicated to identify and characterize the role of post-synaptic density (PSD)-enriched protein kinases that could potentially phosphorylate NMDARs (reviewed in Chen and Roche, 2007). Here, we show that DYRK1A interacts with and phosphorylates NMDAR GluN2A subunits at S^{1048}, a residue within the intracellular C-terminal domain. This phosphorylation provokes an increase in the surface density of GluN1/GluN2A, which may be, at least partially due to decreased internalization. Moreover, DYRK1A-mediated phosphorylation of GluN1/GluN2A modifies the electrophysiological properties of GluN1/GluN2A heteromers.

Despite their relevance in regulating the physiological activity of NMDARs, to date only a few protein kinases and phosphatases have been described that act on NMDARs (Chen and Roche, 2007; Van Dongen et al., 2009). For the GluN2 subunit in particular, tyrosine kinases (members of the Src family) and a small number of serine/threonine kinases (calmodulin kinase II, cyclin-dependent kinase 5, protein kinase A, B and C, and casein kinase 2) modulate the trafficking, stabilization at the cell surface, subunit composition or biophysical properties of NMDARs with a kinase-dependent subunit specificity (Omkumar et al., 1996; Nakazawa, 2000; Gardoni et al., 2001; Li et al., 2001; Wang et al., 2003; Chung et al., 2004; Jones and Leonard, 2005; Chen and Roche, 2009; Zhang et al., 2012; Murphy et al., 2014). These kinases phosphorylate GluN2 target residues within the large cytoplasmic tail, mostly the distal part of the primary amino acid sequence where GluN2 subunits interact with the scaffolding proteins PSD-95, PSD-97 and SAP-102. In terms of DYRK1A, and although we cannot completely rule out the existence of other DYRK1A phosphorylation sites, our results show that S^{1048}, located within the Cter proximal domain, is the main residue in GluN2A phosphorylated by DYRK1A. Structural prediction studies suggest that S^{1048} and its surrounding amino acids are unlikely to be located within secondary structures (alpha-helices and beta-sheets; Ryan et al., 2008), facilitating their easy recognition as a target site. This phosphorylation site, which has not been described previously, is conserved in different vertebrate species and it is not present in GluN2B, suggesting subunit specificity for this phosphorylation event at the S^{1048} position. Our results, however, do not allow to completely rule out the possibility of DYRK1A phosphorylating the GluN2B subunit. Further studies must be directed to unveil whether DYRK1A might regulate NDMAR activity by phosphorylating both GluN2 subunits.

The ability of DYRK1A to phosphorylate GluN2A would require close proximity of the two proteins, achieved either by their direct physical interaction or their presence in the same macromolecular protein complex. Our data do not allow us to distinguish between these possibilities, since the presence of GluN2A in DYRK1A immunocomplexes could reflect both a direct interaction with the GluN2A subunit and/or recruitment to the NMDARs through binding to GluN1 or any other scaffold protein associated with the heteromeric complexes. Although preliminary results from interaction studies in heterologous expression systems support a direct interaction between DYRK1A and NMDARs, further experiments will be necessary to assess the potential of scaffold protein(s) to mediate DYRK1A-GluN2A

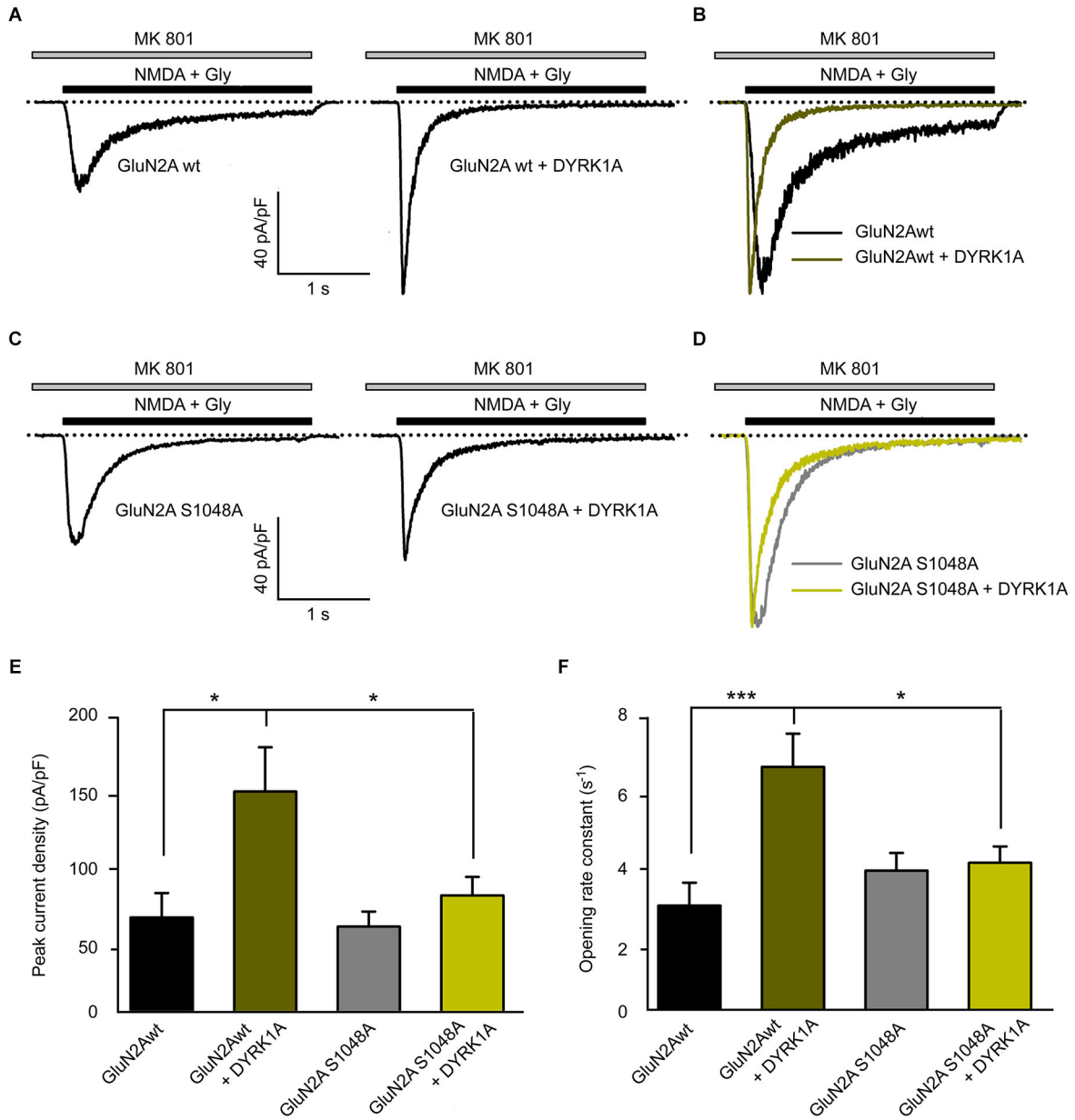

FIGURE 5 | Dual specificity tyrosine-phosphorylation-regulated kinase 1A increases the NMDA-elicited current amplitude and opening rate. **(A–D)** Representative NMDA-elicited currents recorded from HEK-293T cells expressing HA-GluN1/HA-GluN2A receptors (panels **A,B**, wild-type GluN2A; panels **C,D**, GluN2A^{S1048A} phospho-deficient mutant) in the presence (right) or absence (left) of GFP-DYRK1A. Whole-cell currents were elicited by perfusion of 1 mM NMDA with 50 μM glycine, in the continued presence of the open-channel blocker MK-801 (5 μM). **(B,D)** NMDA-evoked currents shown in panels **(A)** and **(C)** normalized to the same peak amplitude. **(E)** Average peak NMDA-evoked current density in cells transfected with NMDARs (GluN2A^{wt} or GluN2A^{S1048A}) in the presence or absence of DYRK1A (*$p < 0.05$, ANOVA followed by Bonferroni *post hoc* test). **(F)** Average opening rate of heterologously expressed NMDARs (GluN2A^{wt} or GluN2A^{S1048A}) in the presence or absence of DYRK1A (***$p < 0.001$ and *$p < 0.05$, ANOVA followed by a Bonferroni *post hoc* test).

interactions in neurons, as described for other Ser/Thr kinases regulating NMDARs.

In the mouse brain, the spatio-temporal expression pattern of DYRK1A partially overlaps with GluN2A-containing NMDARs (Monyer et al., 1994; Martí et al., 2003; Paoletti et al., 2013), suggesting a potential functional interaction in the hippocampus, cortex and/or cerebellum. At the subcellular level, DYRK1A exists as a nuclear and cytoplasmic protein, the latter composed of three pools: soluble, cytoskeletal-associated and membrane-bound proteins (Martí et al., 2003; Aranda et al., 2008; Kaczmarski et al., 2014). This distribution complicates the identification of the subcellular compartment(s) in which

DYRK1A interacts with GluN1/GluN2A receptors. Moreover, the mechanisms controlling DYRK1A intracellular trafficking remain still elusive. A recent study shed light on the neuronal activity-dependency of *Drosophila Minibrain* (*Mnb*), the fruitfly *DYRK1A* homologous gene (Chen et al., 2014). In their study, Chen and collaborators found that synaptic activity increases Mnb mobilization to endocytic zones and promoted efficient synaptic vesicle recycling by dynamically regulating synaptojanin function during periods of robust synaptic activity. In line with these findings, NMDARs activity could trigger the recruitment of DYRK1A to GluN2A subunits, resulting on their phosphorylation and the subsequent cellular and electrophysiological changes.

The DYRK1A-dependent reduction in receptor endocytosis probably underlies the increase of NMDARs at the cell surface. Several components of the endocytotic machinery (dynamin 1, amphiphysin 1 and synaptojanin 1), as well as clathrin-adaptor proteins (AP180), have previously been described as DYRK1A substrates (Chen-Hwang et al., 2002; Adayev et al., 2006; Murakami et al., 2012; Chen et al., 2014). DYRK1A-mediated phosphorylation of these proteins appears to regulate their protein-protein interactions. Moreover, overexpression of DYRK1A appears to inhibit endocytosis in transferrin internalization assays (Kim et al., 2010). Therefore, it might be argued that the effects of DYRK1A on GluN1/GluN2A internalization result from a direct effect on endocytotic proteins or the regulation of clathrin-coated vesicles formation. Although we do not exclude the possibility that these DYRK1A activities might contribute to the modulation of GluN1/GluN2A internalization, the absence of any additional effect on internalization of the phospho-deficient mutant GluN2A^{S1048A} in combination with DYRK1A, strongly suggests that GluN1/GluN2A reduced internalization depends specifically on S^{1048} phosphorylation of GluN2A by DYRK1A.

Functionally, DYRK1A phosphorylation of GluN2A at S^{1048} modifies the biophysical properties of GluN1/GluN2A receptors, increasing both the peak current density and channel gating. The DYRK1A-induced increase in the surface density of NMDARs could be responsible for the increase in peak current density. Further electrophysiological experiments will be important to determine whether DYRK1A might also affect GluN1/GluN2A channel conductance and potentially contribute to the observed increase of NMDA-elicited currents. In addition, phosphorylation of GluN2A by DYRK1A favors the opening of GluN1/GluN2A channels, a parameter that is independent of NMDARs density. This alteration, together with the increased peak current, might alter NMDA-induced Ca^{2+} transients when DYRK1A is overexpressed. Indeed, a prolonged decay of NMDA-elicited Ca^{2+} transient was previously observed in synaptosomes and primary neuronal cultures from TgDyrk1A transgenic mice overexpressing Dyrk1A. We interpreted this as a genomic effect resulting from the increased *Grin2a* transcription and the concomitantly higher levels of GluN2A in the brains of these animals (Altafaj et al., 2008). In the light of the current data, we propose that the alterations observed in TgDyrk1A mice may be strongly influenced by a direct effect of DYRK1A on NMDAR density and function.

It is noteworthy that DYRK1A has been proposed as a candidate gene for some of the neuropathological phenotypes in DS, due to its location within HSA21, the fact that its protein product is overexpressed in DS individuals, and given that murine models overexpressing DYRK1A exhibit DS-like phenotypic alterations (Guimerà et al., 1996; Altafaj et al., 2001, 2013; Ahn et al., 2006; Dowjat et al., 2007; Guedj et al., 2009; Laguna et al., 2013). Dual specificity tyrosine-phosphorylation-regulated kinase 1A gain-of-function models have hippocampal-dependent cognitive alterations similar to those observed in Ts65Dn mice, the best characterized trisomic murine model for DS in which *Dyrk1A* is overexpressed, among other genes (reviewed in Sérégaza et al., 2006). Trisomic Ts65Dn mice display synaptic plasticity alterations that have been attributed to an excitatory/inhibitory neurotransmitter imbalance. While there is evidence supporting the role of an excessive inhibition of GABAergic transmission (Kleschevnikov et al., 2004; Fernandez and Garner, 2007; Martínez-Cue et al., 2013), the participation of NMDA-mediated over-activation of hippocampal circuitry in this phenomenon has also been proposed (Costa et al., 2008; Siddiqui et al., 2008; Scott-McKean and Costa, 2011). Within a trisomic context, NMDAR dysregulation in DS murine models may be the pathological output of the multiple gene products altered in DS (Siddiqui et al., 2008). The results presented here suggest a molecular model in which DYRK1A would be one such protein product. Dual specificity tyrosine-phosphorylation-regulated kinase 1A overexpression would increase the phosphorylation of GluN2A-containing NMDARs, increasing their surface density. The elevation of membrane NMDAR levels, together with the modification of their biophysical properties by DYRK1A, would dysregulate Ca^{2+} signaling, contributing to the synaptic alterations observed in murine models of DS. In summary, we provide the molecular, cellular and functional evidence that DYRK1A directly affects NMDARs, supporting the contribution of NMDA-elicited glutamatergic dysfunction to the excitatory-inhibitory neurotransmitter imbalance proposed to drive the pathophysiology of DS.

AUTHORS' CONTRIBUTIONS

Cristina Grau, Krisztina Arató, José M. Fernández-Fernández, Aitana Valderrama and Xavier Altafaj performed experiments. Cristina Grau, Krisztina Arató, José M. Fernández-Fernández, Carlos Sindreu, Cristina Fillat, Isidre Ferrer, Susana de la Luna and Xavier Altafaj analyzed the data. Xavier Altafaj wrote the manuscript with revisions from José M. Fernández-Fernández, Carlos Sindreu and Susana de la Luna and input from co-authors. All authors read and approved the final manuscript. Cristina Grau and Krisztina Arató are co-first authors.

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Actin-dependent mechanisms in AMPA receptor trafficking

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The precise regulation of AMPA receptor (AMPAR) number and subtype at the synapse is crucial for the regulation of excitatory neurotransmission, synaptic plasticity and the consequent formation of appropriate neural circuits for learning and memory. AMPAR trafficking involves the dynamic processes of exocytosis, endocytosis and endosomal recycling, all of which involve the actin cytoskeleton. The actin cytoskeleton is highly dynamic and highly regulated by an abundance of actin-binding proteins and upstream signaling pathways that modulate actin polymerization and depolymerization. Actin dynamics generate forces that manipulate membranes in the process of vesicle biogenesis, and also for propelling vesicles through the cytoplasm to reach their destination. In addition, trafficking mechanisms exploit more stable aspects of the actin cytoskeleton by using actin-based motor proteins to traffic vesicular cargo along actin filaments. Numerous studies have shown that actin dynamics are critical for AMPAR localization and function. The identification of actin-binding proteins that physically interact with AMPAR subunits, and research into their mode of action is starting to shed light on the mechanisms involved. Such proteins either regulate actin dynamics to modulate mechanical forces exerted on AMPAR-containing membranes, or associate with actin filaments to target or transport AMPAR-containing vesicles to specific subcellular regions. In addition, actin-regulatory proteins that do not physically interact with AMPARs may influence AMPAR trafficking by regulating the local actin environment in the dendritic spine.

Keywords: synaptic plasticity (LTP/LTD), endocytosis, exocytosis, glutamate receptor, dendritic spine

INTRODUCTION

AMPA receptors (AMPARs) mediate the majority of fast synaptic excitation in the brain. Therefore, the precise regulation of AMPAR number and subtype at the synapse is crucial to excitatory neurotransmission, synaptic plasticity and the consequent formation of appropriate neural circuits during learning and memory. AMPAR trafficking involves the dynamic processes of exocytosis, endocytosis and endosomal recycling. In addition, receptors may be restricted in their movement to maintain their localization at the postsynaptic membrane, or at intracellular compartments. The molecular mechanisms that underlie AMPAR trafficking under basal conditions and during certain forms of synaptic plasticity are the topics of very active research and some excellent review articles (Shepherd and Huganir, 2007; Henley et al., 2011; Van Der Sluijs and Hoogenraad, 2011; Anggono and Huganir, 2012).

The actin cytoskeleton is highly dynamic and highly regulated. It is composed of monomeric globular (G)-actin, which polymerizes to form actin filaments (F-actin), and an abundance of actin-binding proteins and upstream signaling pathways regulate actin polymerization and depolymerization. Depending on the relative activity of these multiple regulatory mechanisms, adenosine triphosphate (ATP)-bound G-actin monomers are polymerized at the plus (or "barbed") end of an actin filament, and adenosine diphosphate (ADP)-bound monomers depolymerize from the minus (or "pointed") end (Lee and Dominguez, 2010). This process is known as actin "treadmilling" and can generate forces to bring about movement in the cell. The actin cytoskeleton plays critical roles in cell morphology and cell motility, ie defining the shape and movement of the entire cell (Pollard and Cooper, 2009). Actin dynamics are also used by the cell to generate forces that manipulate membranes in the process of vesicle biogenesis, and also for propelling vesicles and larger endosomal compartments through the cytoplasm to reach their destination. For example, the role of the dynamic actin cytoskeleton in endocytosis has been intensively studied in non-neuronal cells, and a highly complex mechanism involving numerous actin-regulatory molecules involved in this process is emerging (Galletta and Cooper, 2009; Mooren et al., 2012). SNARE-mediated membrane fusion events are required for the final insertion of receptors into the plasma membrane in the process of exocytosis. This is also an active process that has been shown to require actin dynamics (Porat-Shliom et al., 2013). In addition, trafficking mechanisms exploit more stable aspects of actin by using actin-based motor proteins to traffic vesicular cargo along actin filaments (Kneussel and Wagner, 2013).

Following a brief review of early work demonstrating the importance of the actin cytoskeleton in AMPAR trafficking, this paper will focus on proteins that either bind to or regulate the

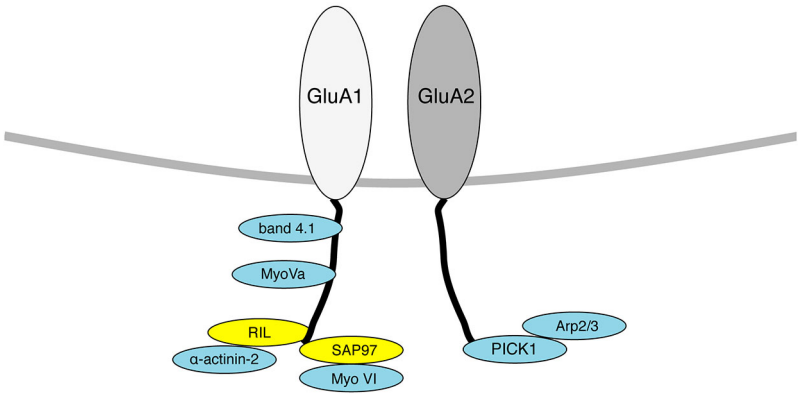

FIGURE 1 | Diagram indicating AMPAR interacting proteins that also associate with the actin cytoskeleton. Proteins that bind directly to F-actin are shown in blue, proteins that act as a scaffold to bring actin binding proteins to GluA1/2 are shown in yellow. Only proteins that have a known physical association with AMPAR subunits are shown here.

actin cytoskeleton to influence AMPAR trafficking. Many of these proteins physically associate with AMPAR subunits (**Figure 1**). In some cases, the evidence indicates that a specific protein plays a critical role in regulating AMPAR trafficking or localization, but the precise trafficking event that is affected, or the mechanistic details of the interaction with the actin cytoskeleton are unclear.

Dendritic spines are highly enriched in dynamic actin filaments, reflecting the highly plastic nature of this subcellular compartment. The spine actin cytoskeleton has an important structural role, since actin polymerization is associated with spine enlargement, and depolymerization with spine shrinkage. (Bosch and Hayashi, 2012; Fortin et al., 2012). The actin-dependent mechanisms that underlie spine structural plasticity are outside the scope of this paper, although some actin-based pathways play a role in regulating both AMPAR trafficking and structural plasticity (Fukazawa et al., 2003; Gu et al., 2010; Rocca et al., 2013; Bosch et al., 2014). The spine is a highly active trafficking compartment, so a high concentration of dynamic F-actin in the spine is well-placed to regulate various aspects of receptor trafficking, especially that of AMPARs. Early indications that the actin cytoskeleton is involved in AMPAR localization at the synapse came from studies using actin depolymerizing drugs such as latrunculin. Cultured neurons exposed to latrunculin showed reduced clustering of GluA1-containing AMPARs in dendritic spines (Allison et al., 1998), and reduced surface expression at synapses (Kim and Lisman, 1999; Zhou et al., 2001). Moreover, the F-actin stabilizing drug Jasplakinolide blocked ligand-stimulated AMPAR internalization (Zhou et al., 2001). Taken together, these studies showed that F-actin is involved in maintaining AMPARs at synapses, and also that actin depolymerization is required for the removal of AMPARs from the synaptic plasma membrane. Further studies showed that long term potentiation (LTP) was blocked in the presence of either latrunculin or phalloidin, which is another F-actin stabilizing agent, indicating that the dynamic actin cytoskeleton is required for the potentiation of AMPAR function (Kim and Lisman, 1999). A later report from the same authors suggested that although a pool of AMPARs is dynamically regulated by the actin cytoskeleton, a distinct pool remains stable in the presence of actin-disrupting agents, and therefore may not be regulated by actin (Kim and Lisman, 2001).

ACTIN-ASSOCIATED PROTEINS THAT REGULATE AMPAR LOCALIZATION OR TRAFFICKING

PROTEIN 4.1

One initial interpretation of these data was that AMPARs are somehow anchored to actin filaments at the synapse to cluster them at the postsynaptic density (PSD), restricting their lateral mobility and their endocytosis from the plasma membrane. This would implicate either a direct interaction between AMPAR subunits and actin filaments, or the involvement of linker proteins that would mediate such an association. Around the same time as these studies, a number of labs were characterizing novel AMPAR protein interactions that had been discovered by yeast-2-hybrid screens, and the discovery that AMPAR subunit GluA1 interacts directly with the F-actin-associated proteins 4.1N and 4.1G provided a molecular mechanism for this model (Shen et al., 2000). 4.1 proteins were originally identified in erythrocytes and are critical for the organization of the spectrin–actin cytoskeleton and for the association of the cytoskeleton with membranes via interaction with membrane proteins (Baines et al., 2014). A role for 4.1G/N in actin-dependent AMPAR localization was demonstrated in experiments with GluA1 lacking the 4.1G/N binding site, which showed reduced surface expression in biotinylation assays, and this mutation occluded the effects of latrunculin treatment (Shen et al., 2000). Although these experiments were carried out in heterologous cells and not in neurons, 4.1G/N appeared to perform the role of stabilizing surface AMPARs by providing a link with actin filaments. In contrast to the static anchoring function proposed by Shen et al., a dynamic role for 4.1 proteins was suggested by a more recent study using live imaging of super-ecliptic pHluorin (SEP)-tagged GluA1 in conjunction with TIRF microscopy. SEP is a pH-sensitive variant of GFP that fluoresces at neutral pH (cell surface), and is quenched at low pH (intracellular vesicles/endosomes) (Ashby et al., 2004).

This technique enables the anlaysis of GluA1 insertion events in real time, which are visualized as fluorescent puncta that rapidly appear and then dissipate gradually over time as the receptors diffuse away from the site of insertion. Knockdown of 4.1N expression using shRNA caused a reduction in the frequency of insertion events at extrasynaptic sites. While the actin cytoskeleton *per se* was not studied in this paper, the data suggest a role for 4.1N in AMPAR exocytosis rather than in surface stability (Lin et al., 2009). Based on this dynamic model, disrupting 4.1 protein function would still cause reduced AMPAR surface levels because of reduced receptor insertion into the plasma membrane. The precise molecular mechanism that underlies such a dynamic role for 4.1N is unclear. These observations were questioned by a report of a mutant mouse expressing only 22% of wild-type 4.1N levels and lacking 4.1G entirely. While synaptosomal levels of GluA1 were modestly reduced in the double "knockout", both basal synaptic transmission and LTP in CA1 region of hippocampal slices were unaffected, suggesting that the 4.1-mediated link with the actin cytoskeleton is dispensable for AMPAR localization at the synapse and for regulated trafficking (Wozny et al., 2009).

RIL/α-ACTININ-2

Another AMPAR interacting protein that associates with the actin cytoskeleton is RIL (reversion-induced LIM protein), which binds GluA1 C-terminus and also the F-actin cross-linking protein α-actinin-2. Although this interaction is not well-characterized, it is proposed to play a role in enhancing surface and synaptic expression of AMPARs by regulating endosomal recycling (Schulz et al., 2004). Exogenously expressed RIL colocalizes with transferrin receptors (TfR) in COS cells and enhances the localization of exogenous GluA1 to TfR positive compartments. In neurons, RIL overexpression causes increased enrichment of AMPARs in dendritic spines, and increased mEPSC amplitude. It is unclear whether this occurs via dynamic control of the actin cytoskeleton, or via the targeting of AMPAR-containing endosomes to actin filaments. A possible mechanism might be that RIL mediates an interaction between endosomal GluA1 and actin filaments, and myosin-based transport shuttles the recycling receptors to the plasma membrane (see following section). Indeed, actinin-4 has been shown to function in complex with MyoV to regulate transferrin receptor recycling (Yan et al., 2005). However, RIL has been reported to influence F-actin dynamics in non-neuronal cells (Vallenius et al., 2004), suggesting that alternative mechanisms could be involved.

MYOSIN MOTOR PROTEINS

An important aspect of AMPAR trafficking is the transport of AMPAR-containing vesicles or larger endosomal compartments to and from the sites of exo- and endocytosis respectively. Myosins are actin-based motor proteins that hydrolyze ATP to generate mechanical force, which is directed as movement along actin filaments (Soldati and Schliwa, 2006). Various myosin isoforms are involved in AMPAR trafficking, the best characterized being MyoV and MyoVI, which are plus end directed and minus end directed motors, respectively. Plus end directed motors move towards the barbed (plus) end of actin filaments, and hence tend to direct cargo to the cell periphery. In contrast, minus end directed motors move towards the pointed (minus) end of actin filaments, and a major role is in the movement of endocytic vesicles away from the plasma membrane (Hartman et al., 2011). MyoVI is a minus-end directed motor that associates with AMPARs via the scaffold protein SAP97, which in turn binds GluA1 via a PSD-95/discs large/zona occludens (PDZ) interaction (Leonard et al., 1998; Wu et al., 2002). These early studies used biochemical techniques to describe the protein interactions, and it was later shown that this complex also contains the endocytic adaptor protein AP2, and that AMPAR internalization stimulated by insulin or by AMPA is abolished in cultured neurons from MyoVI knockout mice (Osterweil et al., 2005). This suggests a role for MyoVI in AMPAR internalization, probably in the transport of endocytic vesicles from the sites of endocytosis at the plasma membrane to endosomal compartments. A more recent study used a C-terminal fragment of MyoVI as a dominant negative to disrupt endogenous MyoVI-SAP97 interactions. In contradiction to Wu et al. and Osterweil et al., neurons expressing this construct show reduced surface expression of endogenous AMPARs analyzed by immunocytochemistry in cultured hippocampal neurons (Nash et al., 2010). The same treatment causes a complete block of NMDAR-dependent AMPAR insertion at the plasma membrane stimulated by brief (3×1 s) transient depolarization.

In contradiction to Osterweil et al., these results suggest a role for MyoVI in trafficking AMPARs towards the plasma membrane, rather than in internalization. A possible explanation is that the C-terminal fragment of MyoVI used by Nash et al. binds to and hence blocks interactions with the N-terminus of SAP97, which includes the L27 domain (Wu et al., 2002). SAP97 is a multifunctional scaffold protein, and multimerization via the L27 domain has been suggested to be required for AMPAR targeting to the synapse (Nakagawa et al., 2004).

Two distinct myosin motor-dependent mechanisms have been suggested for the forward traffic of AMPARs to the synapse in response to LTP induction. MyoVa can bind directly to the GluA1 C-terminus, and is required for LTP in CA1 neurons of organotypic slice cultures, but not for constitutive AMPAR trafficking under basal conditions, which was also assayed electrophysiologically (Correia et al., 2008). In contrast, biochemical experiments showed that MyoVb interacts with the recycling endosome protein complex Rab11-FIP2 in a Ca^{2+} dependent manner (Wang et al., 2008). A direct interaction between MyoVb and AMPAR subunits was not tested in this study. Time-lapse imaging of fluorescently-tagged TfR expressed in cultured hippocampal neurons demonstrated that MyoVb promotes the entry of recycling endosomes into dendritic spines in response to chemical LTP induction. Experiments using SEP-GluA1 to report surface accumulation of exogenous AMPARs showed that this mechanism is required for the surface delivery of AMPARs in response to chemical LTP (Wang et al., 2008). The same authors also used electrophysiological techniques to demonstrate a requirement for MyoVb in LTP in hippocampal CA1 neurons. Another report suggested that MyoVb also affects surface AMPAR expression under basal conditions by expressing a mutant form of MyoVb lacking the region required for interacting with Rab11, and analyzing surface expression

of endogenous GluA1 (Lise et al., 2006). Interestingly, MyoVa is also regulated by Ca^{2+} (Wang et al., 2004), although specific Ca^{2+}-dependent interactions relevant to AMPAR trafficking have not been revealed. Furthermore, MyoVa dominant negative reduces Rab11 localization to spines, suggesting that it may too have general effects on recycling endosomal entry into spines (Correia et al., 2008). Whether both MyoVa and MyoVb do indeed play critical yet subtly different roles in AMPAR trafficking to synapses, or the different results reflect the use of different experimental approaches (dominant negative MyoVa vs. siRNA for MyoVb) that disrupt the same mechanism is unclear.

ADF/COFILIN

Once AMPAR-containing vesicles or endosomes reach the postsynaptic membrane, SNARE-mediated membrane fusion events are required for the incorporation of receptors into the plasma membrane. Although specific SNARE proteins and specific plasma membrane domains for AMPAR insertion have been identified (Kennedy et al., 2010; Jurado et al., 2013), little is known about the specific machinery that regulates the actin cytoskeleton during these events. However, a role for actin dynamics in AMPAR insertion into the spine plasma membrane has been demonstrated. Actin Depolymerizing Factor (ADF)/cofilin is a ubiquitous actin-binding protein, which is involved in the reorganization of actin filaments by causing depolymerization of F-actin at the minus end of filaments, and also by severing actin filaments (Sarmiere and Bamburg, 2004). Hence cofilin reduces the proportion of F-actin, but also increases the pool of G-actin available for subsequent polymerization, hence increasing F-actin turnover. Cofilin activity is tightly regulated by phosphorylation at Serine 3; phosphorylation by LIM kinase deactivates the protein, and dephosphorylation of the same site by Slingshot phosphatases activates cofilin (Mizuno, 2013). The expression of cofilin phosphorylation mutants that either constitutively activate or deactivate cofilin demonstrated that activated cofilin is required for the insertion of SEP-GluA1 into the spine plasma membrane following the chemical induction of LTP in live imaging experiments in cultured neurons (Gu et al., 2010). Consistent with a role for cofilin in enhancing AMPAR synaptic expression, Slingshot knockdown by RNAi causes a reduction in AMPAR EPSC frequency and amplitude in both dissociated cultures and organotypic slice cultures, and also blocks synaptic plasticity induced by infusion of active CaMKII (Yuen et al., 2010). A requirement for cofilin in LTP was also demonstrated by the generation of cofilin knockout mice, which showed a complete lack of LTP in CA1 neurons of hippocampal slices (Rust et al., 2010). In this paper, LTP-induced increases in surface-expressed AMPARs were not investigated, but instead cofilin was shown to play a role in AMPAR surface diffusion, which was studied using single-particle tracking of endogenous AMPARs labeled with quantum dot conjugated antibodies. It is well-established that synaptic AMPARs show markedly less diffusion at synaptic compared to extrasynaptic sites (Opazo and Choquet, 2011). While the lateral diffusion of AMPARs at synaptic sites was unaffected, extrasynaptic receptors were significantly less mobile in the absence of cofilin (Rust et al., 2010). Hence, cofilin appears to be involved in AMPAR trafficking to the synapse by regulating exocytosis and also by modulating the surface diffusion of extrasynaptic receptors, which affects the probability of a surface-expressed receptor being incorporated into the synapse (Opazo and Choquet, 2011). Further work will be needed to determine whether these observations reflect a general requirement for increased actin turnover during these dynamic trafficking events in the spine, or whether cofilin physically interacts with AMPARs or associated scaffold proteins to mediate temporally and locally precise changes in actin dynamics.

The role of actin dynamics in AMPAR lateral mobility was studied further by Kerr and Blanpied, who employed high-resolution FRAP (fluorescence recovery after photobleaching) of SEP-GluA1 and also of PSD scaffold proteins to determine their mobility within the PSD, and analyzed the effects of pharmacological manipulations of the actin cytoskeleton. These experiments suggested that while AMPARs show very little free diffusion within the PSD, they are restricted to spatial subdomains that are defined by subsynaptic scaffolds and the actin cytoskeleton. Actin dynamics causes remodeling of the underlying scaffold, which in turn causes continuous spatial readjustments of AMPAR subdomains and hence their positioning within the PSD (Kerr and Blanpied, 2012). The actin-regulatory machinery responsible for regulating the dynamics of scaffold proteins and AMPARs in the PSD were not investigated in this study. Another important finding from this paper was that loss of actin filaments had only a very small effect on AMPAR synaptic localization within the timescale of the experiment. A dramatic loss of F-actin was observed after just 5 min of latrunculin treatment, but only a small (yet still increasing) loss of SEP-tagged AMPAR subunits was recorded at 10 min (Kerr and Blanpied, 2012). This result does not support a role for actin filaments in simply anchoring AMPARs at the synapse, but instead may reflect a role for actin dynamics in regulating receptor trafficking events at extrasynaptic sites.

PICK1 AND THE ARP2/3 COMPLEX

As well as being involved in the maintenance or increase in AMPAR surface expression at the synapse, the dynamic regulation of the actin cytoskeleton is also involved in reducing the levels of surface-expressed AMPARs. The actin-nucleating Arp2/3 complex is the major catalyst for the formation of branched actin networks that mediate changes in membrane geometry (Campellone and Welch, 2010). Proteins such as N-WASP, WAVE and related proteins bind and activate the Arp2/3 complex, and are highly regulated so that changes in cell morphology or vesicle trafficking occur at appropriate times and subcellular locations (Takenawa and Suetsugu, 2007). PICK1 binds GluA2/3 subunits via its PDZ domain, and plays a critical role in reducing AMPAR surface expression during LTD (Kim et al., 2001; Terashima et al., 2008). PICK1 also binds directly to F-actin via the BAR domain, and to the Arp2/3 complex via a C-terminal portion of the protein including a critical tryptophan residue, W413. These interactions were defined by *in vitro* assays using purified protein components as well as co-immunoprecipitations from native tissue (Rocca et al., 2008). In *in vitro* pyrene-tagged actin polymerization assays, PICK1

inhibits Arp2/3-mediated actin nucleation and polymerization, and both F-actin and Arp2/3 interactions are required for this inhibitory activity (Rocca et al., 2008). PICK1 inhibits the Arp2/3 complex by competing with Arp2/3 activators such as N-WASP for binding to the complex, but also has a direct inhibitory effect that can be observed in the absence of other proteins in the in vitro pyrene assay. In antibody-feeding immunocytochemistry experiments, molecular replacement with a PICK1 W413A mutant blocks AMPAR internalization in response to chemical LTD induction in cultured neurons (Rocca et al., 2008). Furthermore, the same mutant blocks CA1 LTD in hippocampal slices (Nakamura et al., 2011). These results demonstrate that the inhibition of Arp2/3-mediated actin polymerization by PICK1 is required for AMPAR internalization. However, it is unclear whether PICK1 functions mainly at the level of the plasma membrane to promote AMPAR endocytosis, or at the recycling endosome to restrict AMPAR recycling, or both. Both trafficking processes involve BAR domain proteins that are involved in bending or tubulating membranes, and both also involve actin dynamics as regulators of mechanical force to control vesicle formation or tubulation (Galletta and Cooper, 2009; Van Der Sluijs and Hoogenraad, 2011; Mooren et al., 2012; Suetsugu and Gautreau, 2012).

The PICK1-F-actin and the PICK1-Arp2/3 complex interactions are modestly auto-inhibited by an intramolecular interaction between the PDZ domain and the BAR domain, and Arp2/3 inhibition is enhanced by the binding of a GluA2 C-terminal fragment to the PICK1 PDZ domain in *in vitro* actin polymerization assays (Rocca et al., 2008). This suggests a mechanism to ensure that maximal Arp2/3 inhibition is temporally and spatially focused to promote PICK1-mediated AMPAR trafficking. PICK1 is a Ca^{2+} sensor that responds to NMDAR-mediated Ca^{2+} influx to enhance its interaction with GluA2 (Hanley and Henley, 2005), hence actin dynamics in the vicinity of AMPARs are modulated by NMDAR stimulation in response to the induction of synaptic plasticity. A further level of regulation is provided by the small GTPase Arf1, which interacts directly with PICK1 to reduce Arp2/3 binding and consequent inhibition of Arp2/3 activity in *in vitro* assays. (Rocca et al., 2013). Arf1 appears to inhibit PICK1 under basal conditions, since molecular replacement with an Arf1 mutant that does not bind PICK1 causes the loss of surface AMPARs in cultured neurons, which occludes subsequent NMDA-induced internalization. Arf1 binds PICK1 preferentially in its active, guanosine triphosphate (GTP)-bound state, hence a signaling event to increase PICK1-mediated Arp2/3 inhibition to promote trafficking would require a switch from GTP-bound to GDP-bound Arf1. Indeed, a reduction in GTP bound Arf1 in response to NMDAR stimulation in cultured neurons is blocked by siRNA-mediated knockdown of the Arf GAP GIT1 (Rocca et al., 2013), which is a protein previously implicated in AMPAR trafficking (Ko et al., 2003). This model also suggests the involvement of an Arf1 GEF to maintain a basal level of GTP-Arf1 and hence a low basal level of Arp2/3 inhibition by PICK1 at appropriate subcellular locations, presumably close to synapses. Further work will determine the spatial organization of this process and the precise mechanism of NMDAR-stimulated Arf GAP activity by GIT1.

ARC/ARG3.1

Arc/Arg3.1 associates with the actin cytoskeleton, but does not bind actin directly, and the intermediate protein involved in this interaction is unknown. Arc also interacts with the endocytic proteins endophilin and dynamin (Chowdhury et al., 2006) to promote AMPAR internalization during homeostatic synaptic plasticity in cultured neurons and certain forms of memory (Shepherd et al., 2006; Liu et al., 2012). None of these proteins binds AMPAR directly, and the specific molecular interactions involved in AMPAR trafficking, including a potential role for the association of Arc with actin, are unclear. An interesting observation linking Arc to actin dynamics is that Arc synthesis causes cofilin phosphorylation (Messaoudi et al., 2007). Since phosphorylated cofilin is inactive, this suggests that Arc synthesis has a stabilizing influence on actin dynamics. Cofilin has not been implicated in AMPAR internalization *per se*, so this influence of Arc on actin dynamics may be independent of its role in AMPAR endocytosis.

CPG2

Another actin-associated protein involved in regulating AMPAR internalization is CPG2 (Candidate Plasticity Gene 2), which binds F-actin directly and colocalizes with clathrin at postsynaptic endocytic zones (Cottrell et al., 2004; Loebrich et al., 2013). CPG2 knockdown causes an increase in surface-expressed AMPARs, an increase in synaptic strength, and an accumulation of clathrin-coated vesicles close to synapses, suggesting that it may play a role in a late phase of endocytosis, such as vesicle movement away from the plasma membrane (Cottrell et al., 2004; Loebrich et al., 2013). Consistent with this hypothesis, F-actin dynamics are thought to play a critical role in the late stages of clathrin-coated pit invagination and subsequent vesicle mobilization (Merrifield, 2004). The CPG2-actin interaction is enhanced by PKA phosphorylation of CPG2, and expression of phospho-null mutant CPG2 reduces basal AMPAR internalization, suggesting that actin binding is required for trafficking. However, the specific function of the actin binding property of CPG2 with respect to AMPAR trafficking is currently unknown.

CONCLUDING REMARKS

It is clear that the actin cytoskeleton plays a critical role in controlling the dynamic localization of AMPARs, by regulating multiple points in the trafficking pathway (**Figure 2**). However, the mechanistic details are still far from clear. Fundamental aspects of receptor trafficking have been defined in non-neuronal mammalian cells, which will probably give further clues about the mechanisms at play in neurons to regulate AMPARs. However, the atypical environment of the dendritic spine, which is a small, confined compartment with a very high concentration of dynamic actin filaments, suggests that actin-dependent receptor trafficking mechanisms may involve characteristics that are specific to this environment. Actin-binding proteins or actin-regulatory proteins that associate with AMPAR subunits or with postsynaptic scaffolds have already been shown to be critical regulators of AMPAR trafficking, but a more complete understanding of the spatial and temporal regulation of actin dynamics in relation to AMPARs and to the PSD is necessary. In addition, further work is

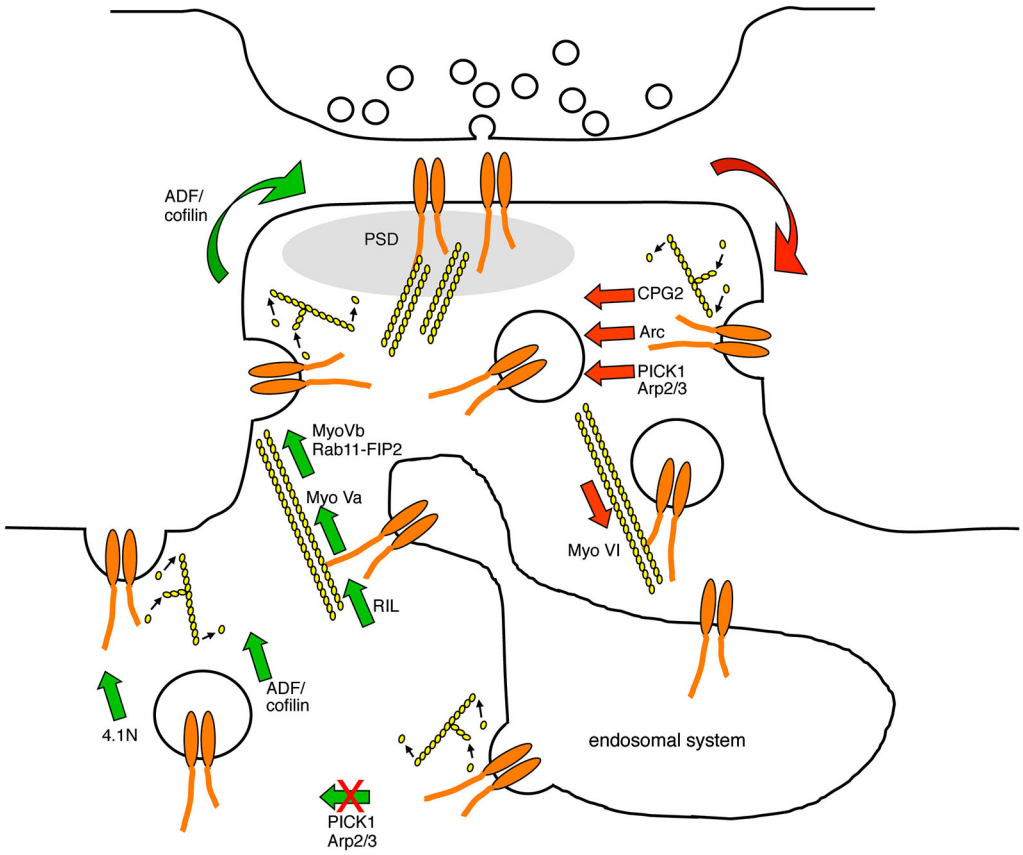

FIGURE 2 | Diagram indicating the points in the AMPAR trafficking pathway that are known to be regulated by actin-based protein machinery. AMPARs are endocytosed at endocytic zones adjacent to the PSD in a process that involves the modulation of F-actin turnover, and the actin-biding proteins CPG2, Arc, and the Arp2/3 inhibitor PICK1. AMPAR-containing endocytic vesicles are transported away from the plasma membrane along F-actin tracks by the minus-end directed actin motor protein myosin VI. In the recycling endosome, AMPARs associate with actin filaments via RIL and the plus-end directed motor protein myosin Va, which direct AMPAR traffic towards the plasma membrane. In addition, AMPAR-containing recycling endosomes associate with the plus-end directed motor protein myosin Vb via Rab11-FIP2, which pulls the endosome into the spine to increase the availability of AMPARs for subsequent plasma membrane insertion. PICK1 restricts AMPAR recycling back to the plasma membrane in a process that is likely to involve F-actin turnover. The insertion of AMPARs into the plasma membrane requires the activity of ADF/cofilin and the actin-binding protein 4.1N. Red arrows represent trafficking events that are involved in reducing AMPAR surface expression, and green arrows represent trafficking events that are involved in increasing AMPAR surface expression.

needed to unravel the upstream regulation of these mechanisms to drive AMPAR trafficking leading to changes in synaptic strength.

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The dendritic SNARE fusion machinery involved in AMPARs insertion during long-term potentiation

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Sorting endosomes carry α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPARs) from their maturation sites to their final destination at the dendritic plasma membrane through both constitutive and regulated exocytosis. Insertion of functional AMPARs into the postsynaptic membrane is essential for maintaining fast excitatory synaptic transmission and plasticity. Despite this crucial role in neuronal function, the machinery mediating the fusion of AMPAR-containing endosomes in dendrites has been largely understudied in comparison to presynaptic vesicle exocytosis. Increasing evidence suggests that similarly to neurotransmitter release, AMPARs insertion relies on the formation of a SNARE complex (soluble NSF-attachment protein receptor), whose composition in dendrites has just begun to be elucidated. This review analyzes recent findings of the fusion machinery involved in regulated AMPARs insertion and discusses how dendritic exocytosis and AMPARs lateral diffusion may work together to support synaptic plasticity.

Keywords: AMPARs, SNAREs, dendritic exocytosis, syntaxin-3

INTRODUCTION

As integral membrane proteins, synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) make use of the entire secretory pathway to reach their final destination at the postsynaptic density (PSD) of dendritic spines. In neurons, the endoplasmic reticulum (ER) can extend into dendrites where it serves as the site for protein biosynthesis as well as an internal calcium storage organelle (Torre and Steward, 1996; Spacek and Harris, 1997; Gardiol et al., 1999; Cui-Wang et al., 2012). These early trafficking steps through the secretory pathway greatly influence the number of available AMPARs since exit from the ER is a limiting step controlled by numerous signaling pathways (Standley et al., 2000; Scott et al., 2003; Hawkins et al., 2004; Horak et al., 2008). According to this notion, retention of AMPARs in the ER has been associated to impairments in synaptic potentiation elicited in CA3-CA1 synapses in the hippocampus (Broutman and Baudry, 2001). After departure from the ER, newly synthesized AMPARs reach the Golgi apparatus (GA) which in neurons is located both in the peri-nuclear region and in discrete Golgi outposts at dendritic branch points (Lowenstein et al., 1994; Horton and Ehlers, 2003; Horton et al., 2005; Ye et al., 2007). Following processing including glycosylation and peptide cleavage, mature AMPARs leave the GA in discrete membranous carriers, largely recycling endosomes (RE), which are then exocytosed at the dendritic plasma membrane. The fusion of these AMPAR-containing endosomes is believed to be highly regulated as it influences surface receptor composition and cell morphology. Two types of endosome exocytosis have been proposed: a constitutive recycling pathway that maintains an steady supply of lipids and membrane proteins and an activity-dependent fusion that underlies acute and long-term changes of molecular composition and synaptic function such as long-term synaptic potentiation (LTP) (reviewed in Shepherd and Huganir, 2007; Henley et al., 2011; Huganir and Nicoll, 2013).

The final step of intracellular membrane fusion is generally controlled by Sec1/Munc-18-like proteins (SM proteins) and the formation of a SNARE complex (Südhof, 2012). The assembly of the SNARE complex into a stable four-helix bundle occurs by the interaction of the SNARE motifs from syntaxin, synaptobrevin and SNAP proteins (**Figure 1**). SNARE complex formation is an exothermic process thought to provide the energy required for membrane fusion (Jahn and Scheller, 2006). According to their universal role in membrane fusion, previous work suggested that SNARE-dependent exocytosis mediates the fusion of AMPAR-containing endosomes with the postsynaptic membrane (Lledo et al., 1998; Lu et al., 2001; Kennedy et al., 2010; Jurado et al., 2013). However whereas the presynaptic SNARE fusion machinery has been identified, the composition of postsynaptic SNARE complexes has remained unclear until recently. Moreover, it is still uncertain whether the same pool of AMPARs-containing endosomes is capable of undergoing both constitutive and activity-dependent exocytosis via a similar SNARE fusion machinery. The identification of distinct SNARE molecules specifically involved in constitutive and/or regulated AMPARs insertion is particularly important since it may provide novel targets to selectively manipulate synaptic transmission and plasticity such as LTP which is thought to be implicated in learning and memory (Malenka and Bear, 2004; Neves et al., 2008). Recent efforts to elucidate the composition of postsynaptic SNAREs involved

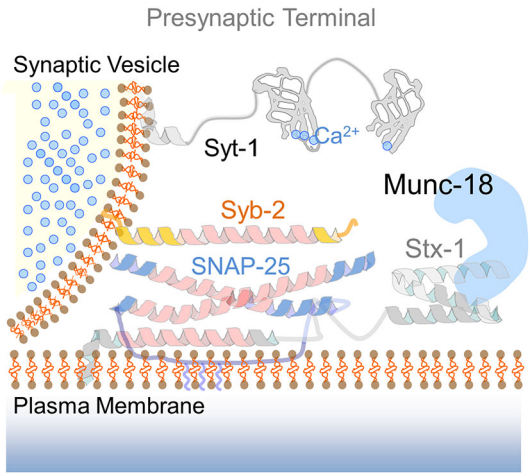

FIGURE 1 | The presynaptic SNARE complex. Drawing of the SNARE fusion machinery mediating calcium-dependent exocytosis of synaptic vesicles. The cartoon illustrates the functional elements of the presynaptic SNARE complex: syntaxin-1 (Stx-1) in an open conformation via interaction with the SM protein Munc-18, SNAP-25 and synaptobrevin-2 (Syb-2). The calcium sensor synaptotagmin-1 (Syt-1) with two calcium-binding C2 domains is located at the vesicle membrane. Upon calcium entry, Syt-1 interacts with complexin (not shown) to promote neurotransmitter release.

in activity-dependent exocytosis suggest that membrane fusion at the postsynaptic compartment is molecularly distinct from its presynaptic counterpart. Unfortunately, the fusion machinery underlying constitutive AMPARs insertion has received less attention despite its crucial role in maintaining basal synaptic strength. For this reason, here we primarily review data from experiments addressing the mechanism of AMPARs exocytosis during NMDAR-dependent LTP elicited in CA3-CA1 synapses in acute hippocampal slices or by activating N-methyl-D-aspartate (NMDA) receptors (NMDARs) in cultured neurons. NMDAR-dependent LTP is arguably the best studied form of long-term plasticity and whose deficit in different cell types and brain regions may contribute to several prominent neurological and neuropsychiatric disorders (Geschwind and Levitt, 2007; Kauer and Malenka, 2007; Clapp et al., 2012; Ehlers, 2012). In addition to discussing the fusion machinery of AMPARs-containing endosomes, we consider how regulated exocytosis may cooperate with other membrane processes such as receptors lateral diffusion to control the number of synaptic AMPARs, therein synaptic transmission and plasticity in the healthy brain.

AMPARs EXOCYTOSIS DURING LTP

In general, LTP can be elicited by brief repetitive stimulation of excitatory afferents (Bliss and Lomo, 1973; Malenka and Bear, 2004) which raises postsynaptic calcium levels mainly due to the activation of synaptic NMDARs (Collingridge et al., 1983; Kauer et al., 1988). Intensive research over the last three decades has demonstrated that postsynaptic calcium influx ultimately increases the number of synaptic AMPARs (Malenka and Bear, 2004; Huganir and Nicoll, 2013). However the role of calcium-dependent exocytosis during LTP has not been fully appreciated until more recently. Numerous signal transduction pathways were suggested to play a role in translating the calcium signal into LTP (Sanes and Lichtman, 1999; Malenka and Bear, 2004). Compelling evidence using genetic and pharmacological approaches indicated that calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) played a mandatory role in long-lasting increase of synaptic strength (Malenka et al., 1988; Malinow et al., 1989; Silva et al., 1992; Pettit et al., 1994; Lledo et al., 1995, 1998; Giese et al., 1998; Lisman et al., 2012). Due to the prominent role of CaMKII in LTP, it was initially assumed that AMPARs insertion was only indirectly regulated by calcium, in contrast to the calcium-regulated exocytosis observed in the presynaptic terminal. Electron microscopy studies revealed the presence of recycling endosomes in dendrites and dendritic spines (Cooney et al., 2002) suggesting that these dendritic vesicles may function as internal membrane stores of AMPARs. Furthermore, the presence of these dendritic endosomes occasionally observed as membrane-bound strongly suggested that they may interact and fuse with the plasma membrane to deliver their cargo in response to neuronal activity (Carroll et al., 1999; Lüscher et al., 1999; Beattie et al., 2000; Ehlers, 2000; Zhu et al., 2002; Park et al., 2004).

Early evidence that calcium-dependent synaptic potentiation requires a SNARE fusion machinery acting in dendrites came from LTP experiments in which botulinum neurotoxin B (BoNT/B), that cleaves synaptobrevins, and other inhibitor peptides of the SNARE complex were infused through the recording pipette (Lledo et al., 1998). Each of these inhibitors efficiently blocked the expression of LTP suggesting that exocytosis of AMPARs-containing endosomes is an essential step during synaptic potentiation. In parallalel to electrophysiological evidence for AMPARs exocytosis, the first optical demonstration of activity-triggered exocytosis in dendrites was reported (Maletic-Savatic and Malinow, 1998). In this pioneer study, neurons incubated with the lipophilic styryl dye FM1-43, a common reagent for the study of neurotransmitter release, incorporated the dye into postsynaptic compartments that destained within minutes upon neuronal stimulation (Maletic-Savatic and Malinow, 1998). These findings provided the first glimpse into postsynaptic exocytosis and suggested that dendritic vesicles may undergo activity-dependent fusion.

Over the years, advances on live cell imaging resulted in the appreciation of the morphological rearrangements that dendritic spines experience during synaptic potentiation. Structural plasticity of dendritic spines during LTP is often observed as a rapid increases of the spine head volume upon NMDAR activation, thus implying that membrane components are provided rapidly to support local growth (Murakoshi and Yasuda, 2012). Dendritic exocytosis of recycling endosomes containing surface receptors and other membrane proteins may provide an efficient way to support both synaptic and structural plasticity. According to this notion, live cell imaging studies using clostridial neurotoxins that disrupt SNARE complexes, or expression of dominant-negative SNARE proteins provided strong evidence for the role of activity-dependent exocytosis in supporting spine growth upon LTP induction (Park et al., 2004, 2006; Kopec et al., 2006, 2007; Yang et al., 2008).

Finally, recent work using shRNA-mediated knock-down of several SNARE proteins and high-resolution live cell imaging has confirmed the role of SNARE-dependent fusion during LTP (Kennedy et al., 2010; Jurado et al., 2013). Surprisingly despite the crucial role of CaMKII-dependent signaling in synaptic potentiation the exocytosis of AMPARs-containing endosomes may not be directly linked to CaMKII activity. Instead, postsynaptic exocytosis has been shown to require on small GTPases from the Ras and Rab families, which have been demonstrated to play a role in AMPAR mobilization upon NMDAR activation (Zhu et al., 2002). Numerous independent findings currently support the notion that AMPAR delivery to the plasma membrane is a calcium-regulated fusion event that involves activity-dependent exocytosis of AMPARs-containing endosomes (Lledo et al., 1998; Shi et al., 1999; Hayashi et al., 2000; Lu et al., 2001; Passafaro et al., 2001; Park et al., 2004; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Ahmad et al., 2012; Jurado et al., 2013). Altogether, these data strongly support the hypothesis that regulated exocytosis of AMPAR-carrying vesicles may underlie both functional and structural aspects of synaptic potentiation.

A POSTSYNAPTIC SNARE COMPLEX FOR LTP

Membrane fusion events in eukaryotic cells are carried out by SNARE proteins. In neurons, the presynaptic SNARE complex is formed by the interaction of the vesicle SNARE protein (v-SNARE), synaptobrevin-2/vesicle-associated membrane protein 2 (Syb-2/VAMP2), and plasma membrane target SNARE proteins (t-SNAREs), syntaxin-1 and SNAP25 (Jahn and Fasshauer, 2012; Rizo and Südhof, 2012; **Figure 1**). Among the t-SNAREs, syntaxins exist in either a "closed" or an "open" conformation. Syntaxin "open" conformation must be achieved in order to form a functional SNARE complex. The conformational change of syntaxin-1 is facilitated by the interaction between its C-terminus and SM proteins (Khvotchev et al., 2007; Shen et al., 2007; Südhof and Rothman, 2009). In addition to this interaction, syntaxin-1 binds to small regulatory proteins known as complexins, this binding has been proposed to arrest the SNARE complex in a "primed" state until calcium influx invades the axon terminal and vesicles are finally fused (McMahon et al., 1995; Giraudo et al., 2006; Tang et al., 2006; Xue et al., 2008; Maximov et al., 2009). The final coupling of synaptic vesicle exocytosis to calcium is mediated by neuronal synaptotagmins, a family of transmembrane proteins with at least one calcium-binding domain (C2 domain) (Geppert et al., 1994; Fernández-Chacón et al., 2001; Pang et al., 2006; Xu et al., 2007). Calcium binding to synaptotagmin-1 C2 domains removes the complexin brake and promotes the binding of synaptotagmin to both the plasma membrane and the SNARE complex thereby triggering fusion (Rizo and Südhof, 1998; Tang et al., 2006; Südhof, 2012). Analogous to presynaptic fusion, AMPAR exocytosis has been shown to rely on SNAREs, although the composition of postsynaptic SNARE complexes involved in both constitutive and activity dependent recycling remains a topic of active research (Kennedy et al., 2010; Ahmad et al., 2012; Jurado et al., 2013) (please note the different molecular composition of the postsynaptic SNARE complex illustrated in **Figure 2** to the canonical presynaptic SNARE complex in **Figure 1**). Consistent with this notion, complexins have emerged as important regulators of calcium-dependent exocytosis of AMPAR-carrying endosomes in LTP. Data from mice lacking complexin-2 provided early evidence to a potential role of complexins in LTP (Takahashi et al., 1999; Huang et al., 2000). More recently, the essential role of complexins in synaptic potentiation has been demonstrated using viral-mediated knock-down approaches *in vivo* (Ahmad et al., 2012). Ahmad et al. showed that complexins -1 and -2 control dendritic exocytosis of AMPARs during hippocampal LTP, although they may not be required for constitutive exocytosis. These findings further strengthen the involvement of a SNARE-dependent fusion during regulated AMPARs insertion.

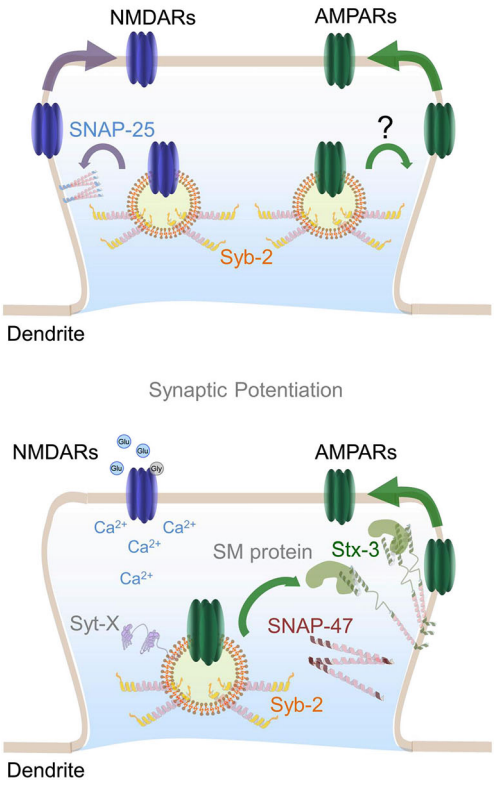

FIGURE 2 | Postsynaptic SNARE complex involved in AMPARs insertion during LTP. Top panel represents dendritic SNARE proteins involved in constitutive trafficking of NMDARs and AMPARs. SNAP-25 is depicted as membrane-bound regulating constitutive NMDARs exocytosis whereas the vesicle SNARE synaptobrevin-2 (Syb-2) may be an integral component of both AMPARs and NMDARs-containing endosomes. Bottom panel illustrates the formation of a specific postsynaptic SNARE complex involved in AMPARs exocytosis upon NMDAR activation. SNAP-47 is shown in close proximity to syntaxin-3 which is anchored to the plasma membrane in an open conformation by its interaction with an unknown postsynaptic SM protein. In a similar fashion to SNAP-25, Syb-2 is depicted regulating constitutive recycling of AMPARs. Plasma membrane-bound Syntaxin-3 molecules may constitute micro-domains or hot spots for exocytosis of AMPARs-containing endosomes during LTP. Calcium influx into the postsynaptic terminal promotes the assembly of a SNARE complex constituted by Stx-3, SNAP-47 and Syb-2, as well as complexin (not shown) and a postsynaptic synaptotagmin isoform (Syt-X) still to be identified.

COMPOSITION OF POSTSYNAPTIC SNARE COMPLEX DURING LTP

In contrast to presynaptic neurotransmitter release, the composition of postsynaptic SNARE complexes mediating calcium-dependent AMPAR exocytosis in dendrites has just recently begun to be elucidated. In a similar fashion to axonal exocytosis, SNARE complexes in dendrites are constituted by three families of SNARE proteins: syntaxins, SNAPs and synaptobrevins. SNARE complex assembly is mediated by the interaction of the SNARE motifs, present in all SNARE proteins (note that two SNARE motifs are contributed by SNAP-25 and one by syntaxin-1 and synaptobrevin-2) (Hayashi et al., 1994; Sutton et al., 1998). Intriguingly, despite their specificity *in vivo* (Südhof and Rothman, 2009), SNARE-dependent interactions exhibit promiscuity *in vitro* (Fasshauer et al., 1999; Yang et al., 1999) suggesting that different SNARE complexes constituted by distinct combinations of syntaxin, SNAP and synaptobrevin isoforms may coexist within the same cell to regulate fusion events at different subcellular compartments. Moreover, different SNARE isoforms of for example membrane-bound syntaxins may sort distinct SNARE complexes to discrete membrane compartments or regions within the plasma membrane (i.e., postsynaptic membrane vs. presynaptic membrane) indicating hot spots for exocytosis. Consisting with this, immunohistochemistry and electron microscopy studies have exposed the presence of several SNAREs proteins at the somato-dendritic region in different brain areas. For example, syntaxin-3, SNAP-25 and synaptobrevin-2 have been detected in dendrites of nigral dopaminergic neurons which is indirect evidence for their involvement in dendritic secretion of neuromodulators like dopamine or perhaps neuropeptides (Witkovsky et al., 2009). Additionally, distinct SNARE-dependent mechanisms seem to underlie calcium-dependent fusion of secretory vesicles from axons and dendrites in hypothalamic neurons (Landry et al., 2003). Below, we review our current knowledge of SNAREs involved in AMPARs insertion during LTP following a chronological order from the first evidence for the role of postsynaptic synaptobrevins to the more recent work in SNAP proteins and syntaxins.

POSTSYNAPTIC SYNAPTOBREVINS

Synaptobrevin -1 and -2 isoforms are small transmembrane proteins that belong to a larger VAMP family (Ernst and Brunger, 2003; Brunger et al., 2009). Interestingly, synaptobrevin-2 has long been known to undergo transcytosis, an early intracellular trafficking event that temporally drives axonal vesicles to the dendritic compartment (Sampo et al., 2003; Wisco et al., 2003; Yap et al., 2008; Ascaño et al., 2009). Identification of vesicles containing classical presynaptic molecules in dendrites, even if only temporarily during early development, raised the intriguing possibility that proteins critical for presynaptic function may also act at postsynaptic locations. According to this idea, some of the first evidence that AMPARs insertion during LTP requires postsynaptic exocytosis came from experiments where synaptobrevin-mediated fusion was disrupted using botulinum toxin B which cleaves VAMP family SNARE proteins infused into postsynaptic neurons via the recording pipette (Lledo et al., 1998). This early observation led to a model where AMPAR-containing endosomes fuse with the plasma membrane upon LTP induction (**Figure 2**).

In addition to functional plasticity, several studies have shown that postsynaptic exocytosis likely mediated by postsynaptic synaptobrevins is required for structural plasticity at glutamatergic synapses (Park et al., 2004, 2006; Kopec et al., 2006, 2007; Yang et al., 2008). Upon NMDARs activation dendritic spines have been shown to increase their volume (Murakoshi and Yasuda, 2012). This stimulus-induced spine growth is blocked by the infusion of botulinum toxin B or expression of dominant-negative SNARE proteins in postsynaptic neurons (Park et al., 2006; Kopec et al., 2007; Yang et al., 2008), indicating that SNARE complex-mediated membrane fusion is required for both structural and synaptic plasticity.

More recently, experiments in cultures prepared from synaptobrevin-2 KO mice indicated that synaptobrevin-2 contributes to maintaining both synaptic and extrasynaptic AMPARs (Jurado et al., 2013). This observation raises the question of which SNARE proteins control the constitutive and regulated delivery of AMPARs to the plasma membrane. Synaptobrevin-2 may be a component of the AMPAR-containing organelles involved in both pathways, although other R-SNAREs must contribute as well since surface levels of AMPARs were only partly reduced in cells lacking synaptobrevin-2.

POSTSYNAPTIC SNAPs

SNAP-25

A functional SNARE-complex requires at least one copy of the plasma membrane-associated SNAREs known as SNAPs (Synaptosomal-associated proteins), with SNAP-25 being the canonical protein at the presynapse (Jahn and Scheller, 2006; Rizo and Rosenmund, 2008; Südhof and Rothman, 2009). Immunohistochemistry in cultured hippocampal neurons have identified several SNAP isoforms in dendrites, including SNAP-25 in similar fashion to other SNAREs which exhibit ubiquitous expression patterns (Südhof, 2012). Moreover, SNAP-25 has even been found in PSD fractionations suggesting a role in dendritic membrane fusion (Jordan et al., 2004; Chen et al., 2006). According to this, *in vivo* knock-down of SNAP-25 impairs NMDAR-mediated transmission in slices and decreases synaptic NMDAR levels in cultured neurons without affecting basal transmission or AMPARs levels (Jurado et al., 2013). These findings are consistent with previous work that shows a role of SNAP-25 in NMDAR trafficking (Lau et al., 2010). Taken together, these results indicate a rather specific role of SNAP-25 in regulating NMDAR-containing endosomes and therefore in controlling the threshold of NMDAR-dependent LTP induction. Furthermore, these findings support the hypothesis that NMDARs and AMPARs are transported via distinct vesicles (Fong et al., 2002; Washbourne et al., 2002) and are sorted via different intracellular pathways to synaptic sites (Jeyifous et al., 2009). Whereas AMPARs are believed to undergo forward trafficking to the plasma membrane via the GA likely through dendritic Golgi outpost, NMDAR may traffic via nonconventional secretory pathway involving CASK and SAP97 (Jeyifous et al., 2009).

SNAP-23

A role for SNAP-23, a SNAP-25 homolog, in glutamate receptor trafficking has been recently suggested. Using immunohistochemistry, Suh et al., showed that endogenous SNAP-23 is highly enriched in dendrites and dendritic spines (Suh et al., 2010). Furthermore, postsynaptic knock-down of SNAP-23, but not SNAP-25, reduced the size of NMDA-evoked currents without affecting presynaptic glutamate release in cultured hippocampal slices. These findings suggest that SNAP-23 may influence AMPARs exocytosis indirectly by regulating surface NMDARs and thereby modulating the induction of synaptic potentiation. However a SNAP-23 shRNA introduced *in vivo* did not subsequently affect LTP in acute hippocampal slices (Jurado et al., 2013). This apparent contradiction may be explained by the use of robust induction protocols to elicit LTP in acute slices in comparison to the milder protocols required to induce potentiation in cultured neurons. Nevertheless, neither SNAP-23 nor SNAP-25 seem to play a direct role in regulated exocytosis of AMPAR-containing endosomes during LTP, although may affect plasticity by controlling NMDAR function.

SNAP-47

SNAP-47, a newly identified SNAP protein (Holt et al., 2006), has been showed to play a role in LTP using an *in vivo* knock-down strategy (Jurado et al., 2013). Immunocytochemistry and structured illumination microscopy have revealed a widespread distribution of endogenous SNAP-47 in both neuronal cell bodies and neuronal processes (Holt et al., 2006; Jurado et al., 2013). Importantly, SNAP-47 knock-down did not alter basal AMPAR- or NMDAR-mediated synaptic responses or basal AMPAR surface expression, providing evidence for a specific role of SNAP-47 in activity-dependent AMPAR exocytosis but not in constitutive trafficking. Moreover, as a genuine SNARE, SNAP-47 has been shown to assemble into stable SNARE complexes with syntaxin-1 and synaptobrevin-2 *in vitro* (Holt et al., 2006). According to this, mutagenesis of SNAP-47 confirmed that a SNARE-dependent interaction is critical for its role in LTP (Jurado et al., 2013).

Sequence comparison of SNAP-47 with other SNAP-25 homologs has revealed SNAP-47 unusual structure that may reflect its functional specialization at the postsynaptic site. SNAP-47 has a long N-terminal stretch and an extended loop between its two SNARE motifs. Also, in contrast to SNAP-23 and SNAP-25, which are predominantly bound to the plasma membrane, SNAP-47 lacks an immediately identifiable membrane anchor sequence which suggest it may be partly cytosolic (Holt et al., 2006). These structural differences of SNAP-47 may be advantageous for regulating membrane fusion at subcellular locations where exocytotic domains are not permanent but rather transiently defined (Yudowski et al., 2007; Yang et al., 2008; Petrini et al., 2009; Patterson et al., 2010).

POSTSYNAPTIC SYNTAXINS

More recent efforts to elucidate the identity of the postsynaptic SNARE complex have been dedicated to the characterization of postsynaptic syntaxins. Syntaxins are small transmembrane proteins that comprised a family of 15 members from which only four (syntaxin 1–4), localize to the plasma membrane where they cluster into microdomains that may support SNARE complex assembly (Lang et al., 2001; Ohara-Imaizumi et al., 2004; Low et al., 2006; Sieber et al., 2006, 2007; Kennedy et al., 2010). These features suggest that identification of syntaxin clusters in dendrites may provide clues to the exact location of AMPARs exocytosis.

Given the prominent role of complexins in calcium-dependent dendritic fusion (Takahashi et al., 1999; Huang et al., 2000; Ahmad et al., 2012), it is reasonable to assume that a syntaxin capable of interacting with complexin (Pabst et al., 2000) will be implicated in LTP. Consistent with this logic, syntaxin-3 has been recently proposed to control AMPARs insertion via a complexin-dependent mechanism (Jurado et al., 2013). Analysis of LTP elicited in acute hippocampal slices from mice expressing shRNAs against different syntaxins revealed that syntaxin-3, but not -1 or -4, plays a critical role in LTP but does not participate in constitutive or presynaptic exocytosis. Structured illumination microscopy showed a relatively ubiquitous distribution of endogenous syntaxin-3 including dendrites and cell bodies. Interestingly, the same syntaxin-3 shRNA in dissociated hippocampal neurons blocked the increase in surface expression of endogenous AMPARs upon NMDAR activation, a cell culture model of LTP (Lu et al., 2001; Passafaro et al., 2001; Park et al., 2004). More importantly, both the block of LTP and AMPARs insertion were rescued by reintroducing syntaxin-3 which rules out potential off-target effects of the shRNA being used. Further structure/function analysis replacing endogenous syntaxin-3 by a non complexin-binding mutant confirmed that syntaxin-3/complexin interaction is necessary for the function of postsynaptic SNARE complexes implicated in AMPARs exocytosis. These results suggest that postsynaptic syntaxin-3 via complexins may constitutively restrict AMPARs insertion until calcium influx reaches the postsynaptic compartment in a similar fashion to their function at presynaptic terminals (Giraudo et al., 2006; Tang et al., 2006; Huntwork and Littleton, 2007; Maximov et al., 2009; Xue et al., 2009; Yang et al., 2010). Furthermore, in a manner analogous to syntaxin-1 in presynaptic terminals, syntaxin-3 was shown to require the binding of SM proteins. This requirement of postsynaptic SM proteins was shown using a molecular replacement strategy in which a syntaxin-3 mutant with a deletion of the SM-binding sequence was ineffective to restore synaptic potentiation in the absence of endogenous syntaxin-3 (Jurado et al., 2013). These findings suggest that a postsynaptic Munc18-like protein still to be identified is likely to catalyze the assembly of the postsynaptic SNARE complex involved in LTP.

Surprisingly syntaxin-4 a syntaxin isoform that does not bind to complexin (Pabst et al., 2000), has also been suggested to mediate AMPARs exocytosis (Kennedy et al., 2010). This evidence is primarily supported by the block of recycling endosomes exocytosis marked with superecliptic pHluorin (SEP)-fused transferrin receptors (TfR-SEP) by a specific syntaxin-4 shRNA. This apparent discrepancy may in large part be explained by the differences in the methods used to assay the fusion of AMPARs-containing endosomes, as endogenous AMPARs like those assayed by electrophysiology may traffic differently from

overexpressed recombinant receptors. Nonetheless, these results raise the intriguing possibility that different syntaxin isoforms may coexist in postsynaptic compartments and sort different cargos via independent microdomains (Puthenveedu et al., 2010; Temkin et al., 2011).

LOCATION AND TIMING OF AMPARs EXOCYTOSIS

Although the role for postsynaptic exocytosis in synaptic plasticity is now clear, the specific locations and timing of AMPARs exocytosis continue to be an active matter of debate. Most studies exploring this issue have yielded inconsistent results (Gerges et al., 2006; Kopec et al., 2006, 2007; Park et al., 2006; Yudowski et al., 2007; Yang et al., 2008; Lin et al., 2009; Makino and Malinow, 2009; Petrini et al., 2009; Kennedy et al., 2010; Opazo et al., 2010; Patterson et al., 2010; Tanaka and Hirano, 2012). While some have suggested that activity stimulates exocytosis in the soma and dendritic shafts (Yudowski et al., 2007; Yang et al., 2008; Lin et al., 2009; Makino and Malinow, 2009; Petrini et al., 2009; Opazo et al., 2010; Opazo and Choquet, 2011; Tanaka and Hirano, 2012), others support insertion directly into stimulated dendritic spines (Gerges et al., 2006; Kopec et al., 2006; Park et al., 2006; Kennedy et al., 2010; Patterson et al., 2010).

Early work to determine the timing of AMPARs exocytosis used an irreversible photoactivable AMPAR inhibitor to analyze the exchange rate of synaptic or extrasynaptic AMPARs upon electrical stimulation or glutamate uncaging (Adesnik et al., 2005). Surprisingly, exchange of synaptic AMPARS took place only after several hours, a timescale much slower than previously thought. In contrast, AMPAR currents measured at the cell body by glutamate uncaging recovered within minutes, suggesting more rapid cycling of receptors at the neuronal soma under basal conditions (Adesnik et al., 2005). Unfortunately, no direct measurements of endogenous AMPAR exocytosis exist, and its time course in living synapses remains unknown. Although electrophysiology experiments are useful to assay the timing and functional relevance of dendritic exocytosis, determining the location of AMPARs insertion requires imaging technologies. Efforts to visualize the location of AMPARs exocytosis have largely relied on optical probes based on SEP, a pH-sensitive GFP variant, which is fluorescent at neutral pH but is quenched when inside acidic vesicles (Miesenböck et al., 1998). SEP-labeled AMPARs, particularly GluA1 subunit-containing receptors, have been used in a number of studies to directly identify AMPARs exocytosis in dendrites (Kopec et al., 2006, 2007; Yudowski et al., 2007; Jaskolski et al., 2009; Lin et al., 2009; Makino and Malinow, 2009; Araki et al., 2010; Kennedy et al., 2010; Patterson et al., 2010). Two-photon glutamate uncaging at individual dendritic spines has revealed that SEP-GluA1 is inserted in the dendritic shaft in neighboring areas of activated spines (Makino and Malinow, 2009). Conversely, a recent study demonstrated that exocytosis of AMPARs-containing endosomes occurs within spines (Kennedy et al., 2010). This last study used transferrin, a marker for recycling endosomes, to demonstrate that endosomes already present in dendritic spines undergo fusion similarly to those in the dendritic shaft. Differences in experimental and imaging conditions most likely underlie the disparity of results obtained using these visualization approaches. Nonetheless, these collective data have been incorporated into a prominent hypothesis in the field that postulates that AMPARs are first inserted into the extra/peri-synaptic surface, then diffuse laterally to the PSD (Borgdorff and Choquet, 2002; Ehlers et al., 2007; Yudowski et al., 2007; Heine et al., 2008; Makino and Malinow, 2009), where they are retained by interactions with scaffold proteins (Henley et al., 2011; MacGillavry et al., 2011; Opazo and Choquet, 2011). In this scenario AMPARs exocytosis is required to replenish the peri-synaptic pool of freely moving surface receptors that will be sequestered by PSD scaffolds during potentiation.

Related to the issue of the location of AMPARs insertion is the question whether AMPARs exocytosis is required for LTP induction or just for LTP maintenance. First experiments using postsynaptic loading of SNARE inhibitors showed that membrane fusion inhibition was effective in shortening the duration of LTP without affecting induction (Lledo et al., 1998). These results support the notion that exocytosis may be critical for LTP maintenance by supplying the pool of surface AMPARs that can then freely diffuse to synaptic locations. However, recent evidence from *in vivo* molecular manipulations of several SNARE proteins and complexins has shown an almost complete block of LTP right after stimulation (Ahmad et al., 2012; Jurado et al., 2013) suggesting that early insertion of AMPARs may be necessary for eliciting synaptic plasticity. A potential explanation for this apparent conflict may be the different methods used for blocking exocytosis. Detection of synaptic effects using acute infusions in the cell body may be delayed by the necessity of the infused molecule to reach the specific synapses that are being stimulated. Future work in this topic is guaranteed which will provide answers to these questions likely by employing novel cutting-edge visualization techniques such as super-resolution microscopy.

CONCLUDING REMARKS

Despite the fact that SNARE-dependent fusion machinery is involved in both pre and postsynaptic exocytosis, there are important differences in the properties of fast neurotransmitter release and activity-dependent AMPARs insertion during LTP. In presynaptic terminals, small synaptic vesicles are docked at the plasma membrane in specialized active zones and primed such that fusion occurs rapidly, within milliseconds following a rise in calcium. In the other side of the synapse, AMPARs-containing endosomes are not tightly coupled to the dendritic plasma membrane but instead may require myosin-dependent trafficking into dendritic spines (Correia et al., 2008; Wang et al., 2008) which would explain the slow exocytosis kinetics in the range of seconds or minutes (Yudowski et al., 2007; Yang et al., 2008; Petrini et al., 2009; Patterson et al., 2010). The reported differences in the composition of the postsynaptic SNARE complex vs. its presynaptic counterpart could account for these significant functional differences. Moreover, all known membrane fusion reactions that require complexin also require a synaptotagmin isoform (Xu et al., 2007; Cai et al., 2008; Schonn et al., 2008) which suggests that a postsynaptic synaptotagmin may control calcium-dependent synaptic plasticity. Interestingly, synaptotagmin-1, the major trigger of fast neurotransmitter release, is not required for LTP (Ahmad et al., 2012) implying that a different synaptotagmin still to be identified could be involved.

In summary, multiple SNAREs have been found in dendrites where they seem to play an essential role in controlling the constitutive and regulated exocytosis of glutamate receptors. Particularly, we have reviewed convincing evidence suggesting that the t-SNARE proteins Stx-3 and SNAP-47 and the v-SNARE protein synaptobrevin-2 are essential components of the postsynaptic vesicle fusion machinery that is required for LTP. Furthermore, postsynaptic synaptobrevin-2 may also contribute to constitutive postsynaptic AMPAR trafficking, and a postsynaptic SNARE complex constituted by SNAP-25 and/or SNAP-23 may control constitutive trafficking of NMDARs (**Figure 2**). Future efforts to elucidate the detailed molecular mechanisms including postsynaptic synaptotagmins and SM proteins involved in both synaptic transmission and plasticity will be critical for understanding the neural basis of many aspects of normal and pathological brain function.

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The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity

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In the mammalian central nervous system, excitatory glutamatergic synapses harness neurotransmission that is mediated by ion flow through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). AMPARs, which are enriched in the postsynaptic membrane on dendritic spines, are highly dynamic, and shuttle in and out of synapses in an activity-dependent manner. Changes in their number, subunit composition, phosphorylation state, and accessory proteins can all regulate AMPARs and thus modify synaptic strength and support cellular forms of learning. Furthermore, dysregulation of AMPAR plasticity has been implicated in various pathological states and has important consequences for mental health. Here we focus on the mechanisms that control AMPAR plasticity, drawing particularly from the extensive studies on hippocampal synapses, and highlight recent advances in the field along with considerations for future directions.

Keywords: AMPAR, homeostatic plasticity, Hebbian plasticity, synaptic plasticity, synaptic transmission, trafficking

INTRODUCTION

The birth of modern neuroscience arguably started with the seminal work of Cajal (1852–1934, Doyle, 1939) who identified neurons as individual units embedded within the vastly complex network of brain tissue. However, little was known about how these intricate and beautiful cells communicated with each other until the advent of more sophisticated techniques that allowed probing of the communication across the synaptic cleft. Studies at the neuromuscular junction, an experimental preparation that was more accessible than the brain, demonstrated that postsynaptic receptors were largely stable and were generally unresponsive to changes in activity level (Fambrough and Hartzell, 1972; Sanes and Lichtman, 1999). Whether this applied to the central nervous system was begun to be answered in the 1970s and 80s, when Bliss and Lømo, working in rabbit hippocampus, first demonstrated that a stimulus could cause an increase in synaptic strength that was long lasting, termed long-term potentiation (LTP: Bliss and Lømo, 1973). The discovery of LTP set in motion the background for the flurry of studies aimed to test if memories are stored at subsets of synapses distributed throughout neuronal networks, and if changes in these tiny structures underlie the ability to learn new behaviors. A particular class of glutamatergic receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs: Beneyto and Meador-Woodruff, 2004), is a key determinant of synaptic strength, and the plasticity of AMPARs is the focus of this review. This is a large field that has spanned over three decades now, and its progress has relied on diverse experimental approaches using *in vitro* and *in vivo* preparations, from biochemistry, cell biology, electrophysiology, to state-of-the-art imaging combined with increasingly sophisticated genetic manipulation.

After a brief introduction to the discovery and history of AMPARs, this review focuses on their role in postsynaptic plasticity in the hippocampus and the recent advances over the last few years. How do AMPARs initially get to the cell surface, and once there, how are they targeted to and retained at synapses? Neighboring synapses sharing the same dendrite may experience significantly different activity levels, and this impacts AMPAR mobility and synaptic retention. Furthermore, AMPAR subunits are differentially regulated by neuronal activity, especially with respect to enzyme-mediated phosphorylation/dephosphorylation cycles that drive their insertion or removal from the synapse. The incorporation of calcium-permeable AMPARs into synapses in response to stimuli is also an important modulation. Neurons are capable of a variety of plastic changes, and synapse strength is both regulated locally and across thousands of synapses cell-wide. How are AMPARs differentially regulated by these separate forms of plasticity? Finally we will discuss changes in AMPAR plasticity in age-related cognitive decline and brain pathologies, and the implications for normal neuronal function.

WHAT ARE AMPARs?

AMPARs are tetrameric, cation-permeable ionotropic glutamate receptors, and are expressed throughout the brain (Beneyto and Meador-Woodruff, 2004). The four AMPAR subunits (GluA1–GluA4) are encoded by the genes GRIA1-GRIA4, and are assembled as dimers-of-dimers to form the hetero-tetrameric receptors (Hollmann and Heinemann, 1994; Traynelis et al., 2010), although homo-tetrameric receptors have been reported (Wenthold et al., 1996; Lu et al., 2009). Upon binding of glutamate, the pore opening allows the influx of Na^+ ions (along with K^+ efflux) to depolarize the postsynaptic compartment; however, depending on the subunit composition and the RNA

editing, AMPARs also permit Ca^{2+}-influx, which has important consequences for plasticity by engaging Ca^{2+}-dependent signaling events.

The four AMPAR subunits are highly homologous (around 70% amino acid residue identity) with conserved transmembrane and extracellular domains (Collingridge et al., 2004). The C-terminal intracellular tails are diverse amongst the subunits, and alternative splicing and RNA editing contribute to additional variants. Alternative splicing at the so-called flip/flop exon produces subunit variants with distinct receptor desensitization properties (Lambolez et al., 1996). Moreover, in the adult brain, most GluA2 subunits undergo RNA editing that replaces a glutamine with a positively charged arginine in the pore-forming region of the assembled channel; this Q/R editing prevents Ca^{2+}-influx. Therefore, in the adult brain, the majority of GluA2-containing AMPARs are largely Ca^{2+}-impermeable (99%, Greger et al., 2003) and they also show a lower single channel conductance (Cull-Candy et al., 2006; Traynelis et al., 2010) along with a slightly increased decay time. In contrast, GluA2-lacking AMPARs are Ca^{2+}-permeable (CP-AMPARs), and have a higher single channel conductance (Swanson et al., 1997) and faster rise and decay kinetics. GluA2-lacking AMPARs also display an intracellular block by polyamines, which can be displaced by stimuli delivered close to one another; this phenomenon manifests as a postsynaptic form of paired-pulse facilitation of synaptic responses (Rozov and Burnashev, 1999). A precise role for CP-AMPARs in synaptic plasticity is hotly debated (see below).

WHERE ARE AMPARs LOCATED?

AMPARs are enriched at excitatory glutamatergic synapses, where they sit in the postsynaptic membrane opposite the presynaptic active zone where glutamate-filled vesicles fuse with the plasma membrane and release their contents into the synaptic cleft. The number of AMPARs at a particular synapse ranges from tens to hundreds, and at mature synapses, it correlates well with spine size and synaptic strength (Matsuzaki et al., 2001). AMPARs are highly dynamic, showing lateral mobility along the cell surface between synaptic and extrasynaptic regions and also undergo constitutive trafficking to and from the cell surface with a surface half-life measured in tens of minutes (Nishimune et al., 1998). Changes in AMPAR number at the synapse is one of the major ways by which the efficacy of synaptic transmission can be altered. Following patterned neuronal activity, AMPARs shuttle into or out of synapses, resulting in long lasting changes in synaptic strength (Lüscher et al., 1999). LTP and long-term depression (LTD) are the most actively studied forms of synaptic plasticity that are thought to represent cellular correlates of particular types of learning and memory.

Prior to reaching synapses, AMPAR trafficking from the endoplasmic reticulum is regulated by various accessory proteins (for example TARPs and cornichons, see Haering et al., 2014) and deficits in these proteins lead to dysregulation in AMPAR trafficking and their expression at synapses. Along dendrites, AMPARs are trafficked through interactions with kinesin (Perestenko and Henley, 2003; Shin et al., 2003) and GRIP1 (Setou et al., 2002), although dynein may also play a role (Kapitein et al., 2010). Some AMPARs may be inserted into the plasma membrane at the soma and then laterally diffuse along the cell surface to synapses (Adesnik et al., 2005). Importantly, the mRNA coding for GluA1 and GluA2 AMPAR subunits can be detected in dendrites together with protein translation machinery (Grooms et al., 2006). Accordingly, many studies have demonstrated the occurrence of local dendritic translation of GluA1 and GluA2, and that such events can supply AMPARs in these cellular compartments under basal conditions and in response to changes in neuronal network activity (Steward and Levy, 1982; Tang and Schuman, 2002; Ju et al., 2004; Grooms et al., 2006). As we will see below, synapses and their complement of glutamate receptors are able to be regulated at every level, from a single synapse, to a dendritic branch, and in some cases, across the entire neuronal arbor. How the control mechanisms operating at different subcellular domains interact with each other and are synergistically integrated within a single neuron is an exciting topic of research.

HOW DO AMPARs ARRIVE AT THE SYNAPSE?—AMPAR INSERTION AT THE PLASMA MEMBRANE

The site of exocytosis of AMPARs is not completely clear. Various studies have suggested the insertion site as the soma (Adesnik et al., 2005), dendrite (Yang et al., 2008; Makino and Malinow, 2009; Patterson et al., 2010), or the spine, directly (Wang et al., 2008; Kennedy et al., 2010, see **Figure 1**). The consensus is that AMPARs are first delivered to extrasynaptic regions, and then diffuse into synapses where they are retained, and both steps are regulated by neuronal activity. AMPAR exocytosis is mediated by SNARE proteins (soluble NSF attachment protein receptors; Lüscher et al., 1999) and synaptic receptors are removed by dynamin-dependent endocytosis (Carroll et al., 1999), although they may be first trafficked laterally along the cell surface away from the synapse. Different AMPAR subunits display distinct exocytosis properties. In general, short-tailed heterodimers (GluA2/3) cycle continuously in and out of the membrane, and maintain the surface pool of synaptic receptors (Passafaro et al., 2001; Shi et al., 2001), whilst AMPARs containing long-tailed subunits (GluA1/2 and GluA2/4) are inserted into synapses in an activity-dependent manner (Hayashi et al., 2000; Shi et al., 2001). Simply increasing the number of extrasynaptic AMPARs is not sufficient to potentiate synapses (for example by overexpression of stargazin, see Schnell et al., 2002), implying that other additional steps are required to stabilize the receptors at the synapse. Postsynaptic density (PSD)-95 appears to fulfill this role, as PSD-95 overexpression selectively promotes synaptic accumulation of AMPAR without altering surface AMPAR number (Bats et al., 2007).

That exocytosis of AMPARs mediates the increase in synaptic strength during LTP in hippocampal CA1 neurons is supported by findings in which blocking dendritic membrane fusion events with botulinum toxins or by infusing peptides that interfere with NSF binding to SNAP, impairs the magnitude of synaptic potentiation (Lledo et al., 1998). Conversely, inhibiting endocytosis or interfering with the interaction between NSF and GluA2 prevents LTD expression (Lüscher et al., 1999), highlighting the importance of AMPAR trafficking in the expression of synaptic plasticity.

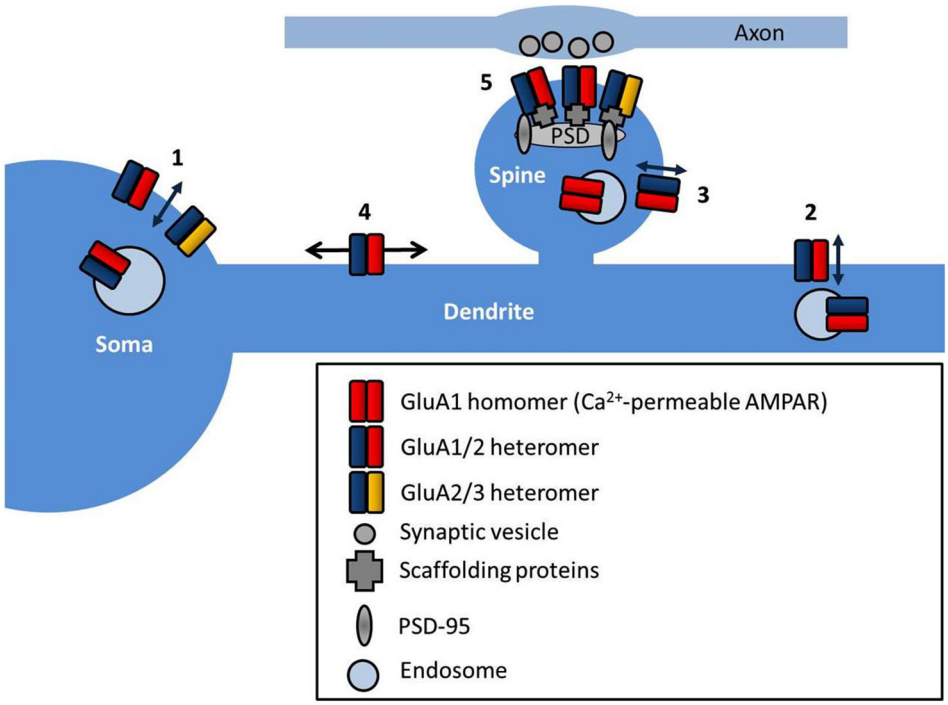

FIGURE 1 | AMPAR subcellular localization and sites of trafficking. AMPARs are exocytosed at multiple locations in neurons such as the soma (1), dendrites (2) and directly into the spine (3). AMPARs freely diffuse at the cell surface extrasynaptically (4), and are trapped at synapses by interactions with scaffold proteins at the postsynaptic density (PSD) (5).

Tagging AMPAR subunits extracellularly with the pH-sensitive GFP mutant, super ecliptic pHluorin (SEP, Miesenböck et al., 1998), which is quenched in acidic endosomes but fluoresces brightly at the surface, has facilitated direct monitoring of cell surface AMPARs. Imaging studies using these SEP-tagged AMPAR subunits have provided insights into the temporal relationship between spine structural changes and the delivery of receptors to the synaptic plasma membrane as well as the order of accumulation of different receptor subunits at synapses. Using a chemical LTP (chemLTP) induction protocol in hippocampal organotypic slices, Kopec et al. (2006) have shown that SEP-GluA1 (and to a lesser extent SEP-GluA2) enter spines upon stimulation, and this is preceded by a structural enlargement of the spine head. The timing of subunit insertion that follows the spine enlargement is also supported by electrophysiology experiments using pairing-induced LTP (Hayashi et al., 2000) and by *in vivo* experience-driven forms of plasticity at the barrel cortex (Takahashi et al., 2003) and associative fear conditioning in the lateral amygdala (Rumpel et al., 2005). Another study in cultured hippocampal neurons used total internal reflection microscopy (TIRF) to limit the SEP-AMPAR signals to those very close to the membrane (Tanaka and Hirano, 2012). Careful monitoring of the temporal order of GluA1, GluA2 and GluA3 insertion following LTP-type stimuli has revealed a fast insertion of GluA1 (within 5 min) followed by GluA2 (5–10 min) and finally GluA3 (20–30 min).

Other imaging studies have suggested the existence of multiple types of AMPAR insertion events that are reminiscent of the different modes of synaptic vesicle exocytosis at the presynaptic terminal. Similarly to full collapse vesicle fusion and kiss-and-stay or kiss-and-run fusion events that have been reported for neurotransmitter release, on the postsynaptic side, some AMPAR insertion events involve receptor delivery to the plasma membrane followed by a quick diffusion of the receptors away from the insertion site that is compatible with full collapse, whilst others show retention of the AMPAR clusters at the cell surface for tens of seconds that is similar to the kiss-and-stay mode (Yudowski et al., 2007; Jullié et al., 2014). Whether these different classes of events indicate a difference in cargo function or content is not yet clear, nor whether neuronal activity can bias the delivery mode towards one or the other. However, it seems logical that these variations in the mode of AMPAR insertion are mechanistically linked to the cellular demand for synaptic components. As discussed below, the extrasynaptic pool of AMPARs acts as the source of receptors for synapses to capture. Petrini et al. (2009) showed that after potentiation synaptic AMPAR number increased due to increased receptor exocytosis and stabilization at the synapse. Curiously, disrupting peri-synaptic sites of receptor endocytosis also impaired potentiation, suggesting that constitutively cycling of AMPARs to and from the surface is required for the correct expression of plasticity. Several studies have suggested that the spine neck provides a mechanical intracellular diffusion barrier (Kusters et al., 2013), and that recruitment of AMPARs to the spine can be modified by endocytosis of membrane within the spine (Jaskolski et al., 2009). Clarifying

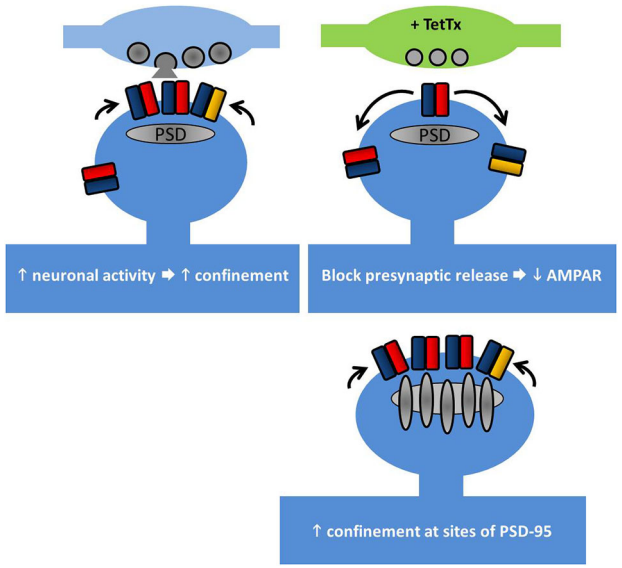

FIGURE 2 | Lateral diffusion and synaptic retention of AMPARs depends on neuronal activity. AMPAR retention at synapses is regulated by multiple factors. Top left, increasing neuronal activity reduces the surface diffusion of AMPARs at synapses and increases their trapping. Top right, at individual synapses where the presynaptic release has been chronically blocked, GluA1 retention is reduced. Bottom right, PSD-95 acts to retain AMPARs at synapses. Overexpression of PSD-95 increases synaptic AMPAR accumulation, but not overexpression of stargazin (see main text).

the sites of exo-endocytosis of AMPARs is therefore crucial for understanding the regulation of synaptic strength under basal conditions and in response to synaptic activity.

"AND YET IT MOVES"—AMPAR SURFACE DIFFUSION AND PLASTICITY

Once at the cell surface, AMPARs are highly mobile and they laterally diffuse along the cell surface. AMPAR diffusion in the plane of the plasma membrane has been mapped using single-particle tracking, showing the contributions of their location, the level of neuronal activity, and the receptor subtype in affecting the type of movement. Whereas extrasynaptic AMPARs diffuse freely, within synapses they exhibit slowing and can become immobilized. In particular, GluA2 subunits diffuse slower in general as neurons mature, and exhibit trapping at synapses. The level of neuronal activity also affects the speed of diffusion, with increased activity slowing the movement of the subunits (Borgdorff and Choquet, 2002; Groc et al., 2004). That slowing of receptor diffusion within synapses could be mediated in part by the interaction with the synaptic scaffold proteins is suggested by the observations in which GluA1 diffusion is slowed at sites of exogenously overexpressed PSD-95, and that GluA1 diffusion is increased upon expressing a stargazin mutant lacking the PDZ-binding motif, which also reduces the immobile fraction of GluA1 (Bats et al., 2007, illustrated in **Figure 2**).

The role of input activity in controlling receptor diffusion has been elegantly addressed using tetanus toxin (TetTx) to silence individual presynaptic inputs (Ehlers et al., 2007). Postsynapses apposed to TetTx-positive presynaptic boutons tend not to capture GluA1 subunits as they pass through the synapse, despite the slowing of their diffusion (**Figure 2**). Notably, short-term activity blockade (1–4 h of TTX/APV/CNQX) does not produce the same effect, suggesting that the change in GluA1 diffusion involves a chronic form of structural reorganization at postsynapses lacking presynaptic input activity. Interestingly this study by Ehlers et al. (2007) hints at the existence of nanodomains within the postsynapse (see below) by showing that the confinement radius of AMPARs at active synapses is smaller than at inactive synapses.

The diffusional exchange of AMPARs between synaptic and peri-synaptic regions allows neurons to fine-tune extremely short-term forms of plasticity. AMPARs have a relatively low affinity for glutamate (Lisman and Raghavachari, 2006), and for effective activation they need to be positioned close to or directly opposite presynaptic sites of glutamate release. Cross-linking of surface AMPARs with an antibody to retard their diffusion increases paired-pulse depression (PPD) and decreases the variability of excitatory postsynaptic current (EPSC) amplitude (Heine et al., 2008). This suggests that the rapid diffusional exchange of AMPARs to and from synapses contribute to the recovery from desensitization.

Several other factors can change the synaptic trapping and diffusional properties of AMPARs including corticosteroids (Groc et al., 2008), n-cofillin (Rust et al., 2010), extracellular matrix components (Frischknecht et al., 2009; Szepesi et al., 2014), CaMKII (Opazo et al., 2010) and the endocytosis and recycling of AMPARs (Petrini et al., 2009). It has also been demonstrated that loss of synaptic AMPARs is preceded by transient extrasynaptic endocytosis (Ashby et al., 2004), indicating that the pool of extrasynaptic AMPARs is co-regulated with the synaptic pool. In addition, blocking dynamin to interfere with AMPAR endocytosis can increase AMPAR lateral diffusion (Jaskolski et al., 2009). These observations further support the link between events related to synaptic strength regulation and AMPAR surface motility.

Recently even finer measurements of AMPAR surface diffusion have been made possible with the advent of light-based super-resolution microscopy. Using three different super-resolution approaches (uPAINT, sptPALM, STED), Nair et al. (2013) have revealed the existence of nanodomains (between 60 and 130 nm in diameter) within spine heads where GluA1 and GluA2 subunits are concentrated. Reducing PSD-95 protein levels in neurons decreases the number of receptors per cluster and also reduces miniature EPSC (mEPSC) amplitude, suggesting that these clusters correspond to the postsynaptic target of the presynaptically released glutamate. In a parallel study, a detailed examination of the fine structure of PSD-95 (MacGillavry et al., 2013) has similarly revealed small enriched nanodomains of PSD-95 within the PSD and that these structures can concentrate AMPARs (depicted in **Figure 3**). The precise functionality of these nanodomains remains to be elucidated, but modeling data suggests that the concentration of AMPARs (and associated scaffold proteins) into nanodomains can strongly affect basal transmission, EPSC variability, and recovery from desensitization (MacGillavry et al., 2013; Nair et al., 2013).

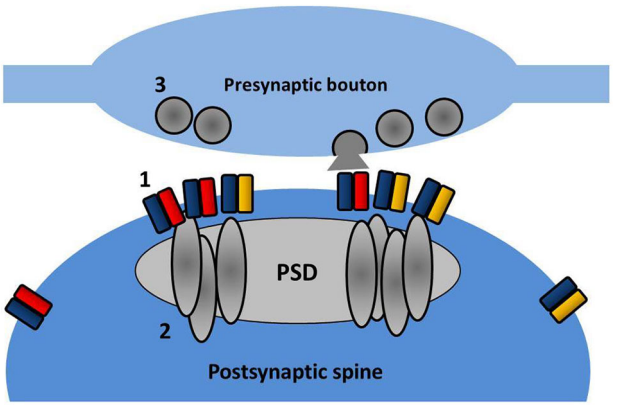

FIGURE 3 | Nanodomain organization of AMPARs and PSD partners. Within the spine, AMPARs (1) and PSD-95 (2) are concentrated into sub-diffraction sized clusters. These may reflect the effective positioning of the postsynaptic receptor population opposite presynaptic sites of vesicle fusion (3).

Collectively, these data demonstrate that the level of neuronal activity and modulation of neuronal signaling can control subunit-specific behavior of AMPARs, particularly their incorporation and retention at synaptic sites, and in turn, affect synaptic plasticity. In a simplified model, at synapses, PSD proteins trap and anchor surface AMPARs in response to increases in neuronal or synaptic signaling and release the receptors when activity levels are low. How different forms of activity-dependent synaptic plasticity affect the distribution and composition of synaptic nanodomains is an extremely exciting and promising topic for future research. The postsynaptic nanodomain might be equally matched by the heterogeneous presynaptic organization, for example, representing hotspots of synaptic vesicle priming and fusion.

LTP—MAKING A MEMORY

LTP of synaptic strength can be induced by a variety of electrical, pharmacological and behavioral paradigms. Classical LTP, as originally described by Bliss and Lømo (1973) can be stable for months, and presumably mechanisms such as these underlie our own memories, which in humans can span several decades. The key change during LTP is an increase in the number of AMPARs at a subset of synapses (see **Figure 4**). Presynaptic changes can also contribute to LTP (for reviews, see Kullmann, 2012; Padamsey and Emptage, 2013), but here we focus exclusively on postsynaptic mechanisms.

LTP is typically induced by high frequency tetanic stimulation, which leads to Na^+-influx through AMPARs, depolarization of the postsynaptic compartment, and activation of NMDARs to permit Ca^{2+}-influx; this sets off a cascade of phosphorylation events to potentiate synaptic transmission. The primary change following tetanic stimulation is the gross increase in AMPAR number at the synapse, but hidden within this is a series of subtle temporal and subunit-specific effects. The primary signaling effector (and the most studied molecule) is CaMKII in the postsynaptic neuron. This kinase is transiently activated following LTP induction (Lee et al., 2009), translocates to the synapse (Shen and Meyer, 1999) and phosphorylates target proteins, including GluA1 (Barria et al., 1997; Mammen et al., 1997), whose phosphorylation at S831 enhances single channel conductance (Derkach et al., 1999) and open probability (Banke et al., 2001). Therefore, CaMKII signaling alone can potentiate synaptic transmission, although more recent work suggests that formation of GluA1/2 heterotetramers occludes the S831-mediated increases in channel conductance/open probability (Oh and Derkach, 2005), and places the GluA2 subunit in the dominant role for the secondary modulation of AMPAR function associated with LTP.

In addition to S831, S845 on the GluA1 subunit, which is targeted by PKA, is also found to be phosphorylated after LTP in the hippocampal CA1 region (Barria et al., 1997; Lee et al., 2000). The degree of phosphorylation however depends on the activity history of the synapse (Lee et al., 2000). Knock-in mice that carry at these sites either phosphomimic or phosphonull residues display a lower threshold for spike-timing dependent plasticity and either deficits in LTP or LTD (Lee et al., 2003, 2010; see below for LTD).

PKC is also capable of phosphorylating GluA1, and phosphorylation at S818, which is increased during LTP, is required for LTP induction (Boehm et al., 2006). PKC can also phosphorylate T840, and mutating this site results in deficient LTP in slices prepared from older animals (over 3 months of age) but not from juvenile animals (3–4 weeks old); this suggests an age-dependent component to this form of phosphorylation-dependent modulation of plasticity (Lee et al., 2007).

LTP AND SILENT SYNAPSES

Some synapses have no AMPARs at their resting state and instead just contain NMDARs. Following LTP induction, AMPARs are rapidly trafficked into these "silent synapses" and contribute to the depolarization of the postsynaptic neuron (Isaac et al., 1995; Liao et al., 1995). The existence of silent synapses has been supported by immunolabeling studies in cultured neurons where some synapses only label for NMDARs and not AMPARs (Gomperts et al., 1998; Liao et al., 1999, 2001). The fast "unsilencing" of these synapses during LTP may enable the network to quickly and strongly encode new memories, although more work is needed to clarify how such a form of potentiation could be advantageous over inserting additional AMPARs into existing synapses. Moreover, the detailed molecular basis by which particular silent synapses switch to active ones remains to be established. Presumably alterations in the PSD traps AMPARs at the target synapse, which is paralleled by increased extrasynaptic trafficking of AMPARs to maintain the surface pool.

CALCIUM-PERMEABLE AMPARs IN PLASTICITY

As discussed above, CP-AMPARs, which lack a GluA2 subunit or contain an unedited GluA2 subunit, have a capacity to augment or even replace Ca^{2+}-entry through NMDARs to play a role in synaptic plasticity. Exactly how CP-AMPARs contribute to plasticity is unclear, with conflicting evidence in the literature. In one study, LTP induction has been shown to trigger a rapid but transient synaptic insertion of CP-AMPARs that are replaced by GluA2-containing AMPARs within 30 min, and where blocking

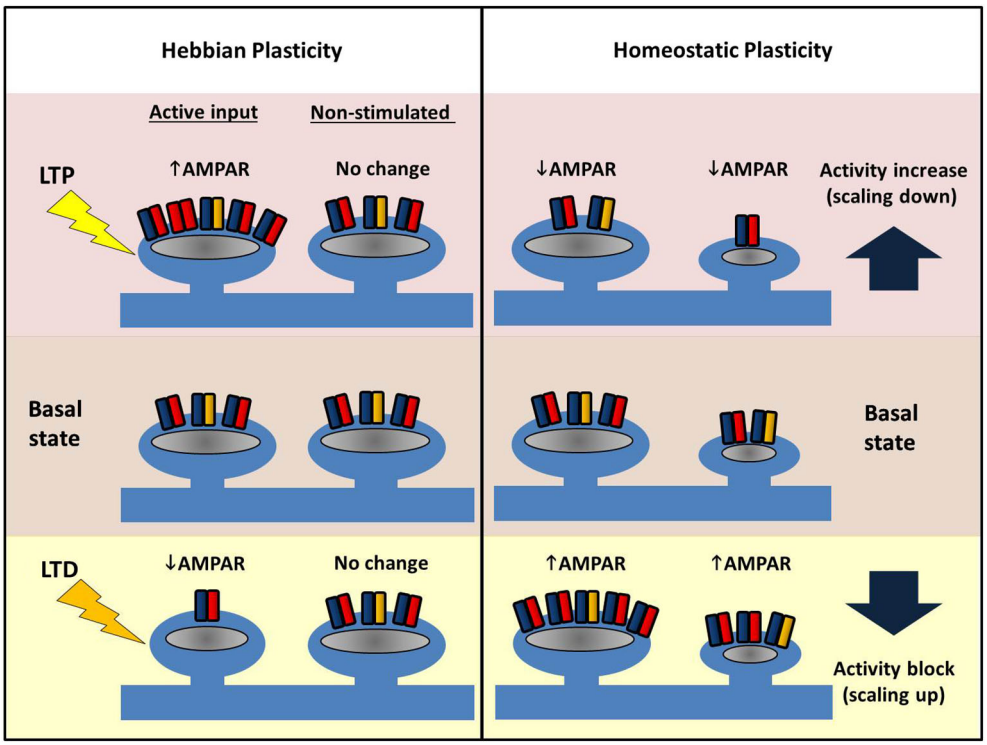

FIGURE 4 | Comparing Hebbian and homeostatic plasticity. During Hebbian forms of plasticity synapses change their number of AMPARs in an input-specific fashion. Different patterns of activity can either cause strengthening (LTP, top left) or weakening of synapses (LTD, bottom left) via AMPAR trafficking. Potentiation or depression is limited to stimulated synapses, and neighbors are unaffected. In contrast, during homeostatic plasticity altered levels of neuronal activity drives changes in synaptic AMPAR number across the entire dendritic arbor. Blocking pre- and postsynaptic spiking with TTX causes AMPARs to accumulate at excitatory synapses (bottom right). Conversely increasing network activity (for example with a $GABA_AR$ antagonist) causes a reduction in synaptic AMPAR (top right). Crucially this form of plasticity conserves the relative strength difference between synapses.

CP-AMPARs reduces the magnitude of potentiation and CP-AMPARs (Plant et al., 2006). Others have presented data suggesting that CP-AMPARs are delivered to peri-synaptic sites prior to LTP expression (Yang et al., 2008), and that CP-AMPARs maintain the ability of synapses to undergo LTP and spine size expansion (Yang et al., 2010).

Insertion of CP-AMPARs involves phosphorylation events. Guire et al. (2008) showed that CP-AMPAR insertion depends upon CaMKI activity, which in turn requires actin polymerization to recruit synaptic CP-AMPARs, and others have demonstrated a role for PKC phosphorylation (Yang et al., 2010). Another study has linked CP-AMPARs to mEPSC amplitude increases and spine head enlargement following chemLTP in cultured neurons (Fortin et al., 2010), and suggested that downstream of CP-AMPARs, the Rac/PAK/LIM kinase pathway can control spine actin turnover. Phosphorylation of GluA1 at S845 has been reported to play a role in stabilizing GluA1 homomers and retaining CP-AMPARs at peri-synaptic sites (He et al., 2009). The same study has also demonstrated that LTD is accompanied by a reduction of these receptors, and that in mice expressing a GluA1-S845A mutant, peri-synaptic CP-AMPARs are lost. In contrast, no involvement of CP-AMPARs has been seen in hippocampal CA1 LTP in other studies (Adesnik and Nicoll, 2007; Gray et al., 2007).

Multiple studies using GluA2 KO mouse models have demonstrated enhanced LTP in these animals LTP (Jia et al., 1996; Meng et al., 2003; Asrar et al., 2009). Consistently, conditional loss of GluA2 in mice results in increased LTP with no requirement for NMDARs and with no effect on LTD (Wiltgen et al., 2010). These studies also highlight non-overlapping roles of proteins involved in LTP, in that CP-AMPAR-dependent LTP is independent of CaMKII (Asrar et al., 2009) and animals lacking both GluA2 and GluA3 are still able to undergo potentiation (although they show deficits in basal synaptic transmission; Meng et al., 2003).

As discussed below, CP-AMPARs appear to have a role in compensatory, homeostatic forms of plasticity. Perhaps the reported differences in the requirement for CP-AMPARs in LTP and LTD reflect differences in the experimental set up including synapse type, their history of activity, the experimental protocol used to elicit plasticity, and the developmental state of the tissue. The ionic properties of these receptors make them potentially very powerful plasticity players at the synapse. Notably, the mechanism that orchestrates the transient synaptic incorporation of CP-AMPARs is a fascinating one to study. How might some synapses be able to selectively trap GluA2-lacking AMPARs for a short period, only to replace them with GluA2-containing receptors? Does it require a specific set of scaffold proteins with

a high binding affinity for GluA2-lacking AMPARs that become unmasked in the PSD?

LTD—WEAKENING OF SYNAPSES

Hippocampal synapses are typically bidirectionally plastic, and while LTP may be the cellular correlate of learning and memory, a mechanism to weaken synapses is necessary too. LTD is one such process, and it may underlie forgetting (Nabavi et al., 2014; see **Figure 4**). Classical hippocampal LTD is dependent on NMDARs (Dudek and Bear, 1992), and its induction engages high affinity Ca^{2+}-sensing molecules downstream of the NMDAR activation (Mulkey and Malenka, 1992) such as calcineurin (Mulkey et al., 1994; Jurado et al., 2010). This in turn triggers dephosphorylation events on targets such as GluA1 (Lee et al., 1998, 2000, 2003), leading to depression of synaptic strength via removal of AMPARs (Beattie et al., 2000; Carroll et al., 2001). Although both LTP and LTD are dependent on NMDAR activation and culminate in changes in the number of synaptic AMPARs, the spatio-temporal nature of the intracellular Ca^{2+} rise dramatically impacts the direction of plasticity. GluA1 S845 on the C-terminal tail appears to be required for LTD, as mice carrying an alanine replacement display perturbed LTD (Lee et al., 2010). In contrast, GluA1 S831A mutants show no LTD (or LTP) deficits, whilst the double phosphomutants show impaired LTD as well as a faster decay of LTP (Lee et al., 2003). Interactions between GluA2 and AP2 also contribute to LTD (Lee et al., 2002), and the same region on GluA2 overlaps with the site for NSF interaction, which is required to maintain synaptic AMPAR (Nishimune et al., 1998), but the domain itself is not directly involved in LTD.

A kinase anchoring protein 150 (AKAP150) plays a key role in LTD. AKAP150 can interact with calcineurin and drives NMDAR-dependent removal of AMPARs from the synapse (Jurado et al., 2010). The interplay between AKAP150, PKA and PSD-95 seems particularly important. AKAP150 targets both PKA and PKC to synapses, and the loss of AKAP150 perturbs synaptic transmission (Tunquist et al., 2008). Additionally, preventing PSD-95 interaction with AKAP150 blocks NMDAR-dependent LTD but leaves metabotropic glutamate receptor (mGluR)-LTD intact in cultured neurons (Bhattacharyya et al., 2009). PSD-95 itself undergoes dephosphorylation at S295 following chemLTD induction (by bath applied NMDA) in cultured neurons, and overexpressing a PSD-95 S295A mutant prevents LTD (Kim et al., 2007).

Another key protein regulating LTD and AMPAR endocytosis is Protein Interacting with C Kinase 1 (PICK1). GluA2 is endocytosed upon phosphorylation at S880 by interacting with PICK1, which also involves PICK1-mediated inhibition of actin polymerization via the Arp2/3 complex (Rocca et al., 2008). This mechanism of AMPAR endocytosis is further regulated by the small GTPase Arf1, and overexpressing a mutant Arf1 that cannot bind PICK1 blocks NMDAR-dependent LTD (Rocca et al., 2013). Additional evidence for the importance of S880 phosphorylation on GluA2 in LTD is provided by the demonstration of increased phosphorylation of this residue following LTD induction (Kim et al., 2001) and of inhibition of LTD upon blocking GluA2/PICK1 interaction (Steinberg et al., 2006). However other groups have shown that S880 phosphorylation of GluA2 can reverse LTD and drive AMPARs to the cell surface by competing with PICK1 binding for GluA2 with GRIP/ABP (Daw et al., 2000). Furthermore, PICK1 knock-down does not prevent NMDA-driven AMPAR removal (Lin and Huganir, 2007). Altogether, these observations point to a role of PICK1 in regulating the intracellular pool of AMPARs after endocytosis, which in turn, can indirectly impact AMPAR internalization.

In the cerebellum there are different forms of LTD; one of the best studied is expressed at synapses between presynaptic parallel fibers and postsynaptic Purkinje cells. This cerebellar parallel fiber LTD shows several key differences compared to hippocampal LTD, including the requirement for GluA2 (Chung et al., 2003), NMDAR-independence (De Zeeuw et al., 1998) and mGluR1 activation (Linden and Connor, 1991). Knocking out GluA2 blocks cerebellar parallel fiber LTD (Chung et al., 2003) as does removing other AMPAR interactors, including PICK1 (Steinberg et al., 2006), and GRIP1 and GRIP2 (Takamiya et al., 2008). Tellingly, reducing endocytosis with inhibitors can block parallel fiber LTD (Wang and Linden, 2000) pointing at a general mechanistic requirement for the removal of AMPARs in LTD regardless of the synapse. Elsewhere in the cerebellum, a form of LTD has been identified at the synapses between mossy fibers and deep cerebellar nuclei. This too is NMDAR-independent, but requires postsynaptic calcium (Zhang and Linden, 2006).

Another well-studied form of LTD crucially involves mGluR activation. Activation of group 1 mGluRs (for example by (R,S)-3,5-dihydroxyphenylglycine, DHPG) induces a rapid removal of synaptic AMPARs. Whilst not covered here, we direct the reader to several excellent review articles on the subject (Gladding et al., 2009; Lüscher and Huber, 2010).

Many other proteins have been shown to modulate LTD to varying extents. Small GTPases Rap1 and Rab5 have both been implicated in hippocampal LTD (Zhu et al., 2002; Brown et al., 2005), along with PI3γ (Kim et al., 2011) and the JAK/STAT signaling pathway (Nicolas et al., 2012). The immediate early gene Arc/Arg3.1 also appears to play a role, as mice lacking this gene have impaired LTD and memory deficits (Plath et al., 2006).

The above notwithstanding, exactly how the behavior of AMPARs determines the outcome of LTD is still unclear, as mice lacking GluA1 (Selcher et al., 2012) or mice lacking both GluA2 and GluA3 (Meng et al., 2003) all show normal hippocampal LTD. In fact, even deleting all four AMPAR subunits and replacing them with kainate receptors can support LTD (Granger and Nicoll, 2014). That LTD generally requires a loss of AMPARs from the synapse seems to be a consistent result. Nevertheless, the exact series of events that drive this loss, and similarly to some aspects of LTP (Granger et al., 2013), the basis for the apparent redundancy of AMPAR subunits remains to be clarified.

HOMEOSTATIC PLASTICITY—NON-LOCAL AND ALL-ENCOMPASSING SYNAPTIC STRENGTH CHANGE

In addition to input-specific forms of plasticity, neurons respond to changes in the overall level of network activity, in a cell-autonomous fashion (Maffei and Fontanini, 2009; Vitureira et al., 2012). Individual cells must monitor the level of activity they experience (for example by the state of somatic Ca^{2+}-flux following action potentials) and compare it to some pre-set value, and then be able to adjust their synaptic protein complement to

offset changes in external activity. In most mammals large changes in network activity happen on a diurnal basis with the onset of sleep (Tononi and Cirelli, 2014), and in pathological states neuronal populations may lose their inputs due to tumorigenesis, focal brain damage or general degenerative syndromes (Small, 2004; Santos et al., 2010). Chronic disease can cripple specific populations of neurons in the brain (for example dopaminergic neurons in Parkinson's disease) leading to long-term changes in circuit function. This may develop in two stages, with a primary gradual reduction in drive of the affected population, followed by an eventual complete cessation of activity. Other disease states may selectively alter excitatory or inhibitory synapses across the neocortex. These synaptopathies will lead to imbalances across the central nervous system that neurons will attempt to correct as far as their internal mechanisms allow them. Dysregulation of AMPARs at the synapse is the vanguard for many of these diseases, and understanding the mechanisms that counterbalance these perturbations is critical for our understanding of the brain.

Investigations of homeostatic plasticity have often relied on simple, neuronal culture preparations. Experimentally, activity manipulation is achieved in a variety of ways: global pharmacological blockade of synaptic AMPARs and NMDARs heavily suppresses network activity, as does the addition of TTX that prevents action potentials, whereas $GABA_A$ receptor blockers increase the overall network activity through disinhibition (**Figure 4**). In a first demonstration of homeostatic synaptic response monitored by mEPSCs, visual cortical cultures were treated with various channel blockers for 2 days (Turrigiano et al., 1998). Both TTX and AMPAR inhibitor treatment were found to increase the amplitude of mEPSCs whereas bicuculline (a $GABA_A$ blocker) decreased the mEPSC amplitude, with the overall effect of maintaining the firing rate of the neuron despite the activity manipulation. Neurons thus adjust their synaptic AMPAR number in a manner that opposes the external changes in activity. Moreover, this is cell-wide and multiplicative such that the differences in individual synaptic weights are conserved. Consequently, this phenomenon—the activity-dependent bidirectional change in mEPSC amplitude—has been termed "synaptic scaling" as all of the individual postsynaptic strengths across the entire neuron are apparently scaled up or down by a uniform amount. Crucially, such a scaling process retains the information encoded in the relative original strengths of the connections, and thus a strong synapse will still be stronger than its weak neighbor after scaling (thus all the work described above on input-specific LTP and LTD is not in vain!). Further studies have indicated that GluA1 and GluA2 increase in a coordinated fashion during scaling up, and AMPA/NMDA ratios are also conserved (Watt et al., 2000). The latter point is interesting to consider with respect to LTP, where in the short-term, the number of AMPARs increases first, and later, NMDARs also increase to restore the ratio (Watt et al., 2004). Similar to LTP and LTD, both scaling up and down of synaptic AMPARs requires Ca^{2+}-dependent signaling pathways, some of which are shared (e.g., somatic Ca^{2+}-entry), but unlike LTP/LTD, synaptic scaling appears to rely strongly on signaling linked to GluA2 (see below).

Studies have also used local perfusion of drugs to selectively perturb synapses. Interestingly, global action potential firing and local spontaneous mEPSC events appear to play different but overlapping roles in regulating AMPARs. Sutton et al. (2006) has demonstrated that local blockade of NMDARs relieves a brake on local translation to promote the insertion of GluA1. This intriguing finding suggests that individual synapses sense alterations in presynaptic behavior, and are able to respond accordingly. Other strategies to induce local changes in activity have used presynaptic silencing using Kir2.1 (a hyperpolarizing K^+ channel, which when overexpressed, reduces AP firing, see Burrone et al., 2002) or expressing TetTx to prevent SNARE-dependent neurotransmitter release (Harms et al., 2005), and in both cases synapse-specific responses to the loss of input activity are observed. In addition, local application of TTX onto neuronal somata increases dendritic GluA2 fluorescence within 4 h (Ibata et al., 2008), suggesting that neurons are monitoring their activity level as a function of somatic activity. That this might be somatic Ca^{2+}-flux is supported by the finding that blocking all Ca^{2+} channels with $NiCl_2$, or L-type Ca^{2+} channels with nifedipine, have the same outcome.

At the level of AMPAR subunits, GluA2 is critical for homeostatic scaling up. Overexpressing a dominant-negative GluA2 C-terminal tail (but not GluA1 C-terminus) blocks this form of plasticity both in cultures and *in vivo* (Gainey et al., 2009). GluA2 KD via siRNA has no effect on basal mESPCs, suggesting that other subunits (largely GluA1) can compensate for the reduced GluA2. GluA2 KD however occluded synaptic scaling but not chemical LTP. As mentioned above, GluA2 KO animals can still express LTP (Jia et al., 1996; Meng et al., 2003; Asrar et al., 2009), and thus altogether these observations hint at non-overlapping functions for GluA2 in different forms of plasticity. A recent study in organotypic hippocampal slices by Arendt et al. (2013) first induced synaptic scaling with TTX and then induced LTP by electrical stimulation. They find that previous activity blockade enhances the subsequent LTP, which appears to be due to the formation of more silent synapses during the activity blockade that are then unsilenced during LTP induction. This suggests that larger structural changes that are not readily discernable may be associated with synaptic scaling.

As of now, multiple proteins have been implicated in synaptic scaling, including Arc (Shepherd et al., 2006), CaMKIV (Ibata et al., 2008), eIF4AIII (Giorgi et al., 2007), retinoic acid (Aoto et al., 2008), Plk2 (Seeburg et al., 2008; Evers et al., 2010), MeCP2 (Blackman et al., 2012), TNF alpha (Stellwagen and Malenka, 2006; Steinmetz and Turrigiano, 2010), beta3 integrins (Cingolani and Goda, 2008; Cingolani et al., 2008), and both PSD-93 and PSD-95 (Sun and Turrigiano, 2011). Beta-catenin KD occludes both scaling up and scaling down and also alters spine shape and decreases mEPSC amplitude without affecting mEPSC frequency. Interestingly, overexpression of a dominant-negative form of N-cadherin mimics the effects of beta-catenin knock down (Okuda et al., 2007; see also Vitureira et al., 2011), supporting the requirement for the N-cadherin/catenin adhesion complex in regulating synaptic AMPARs.

AMPAR PLASTICITY IN DISEASE—WHERE IS MY MIND?

Deficits in synaptic proteins are increasingly implicated in a variety of neurological disorders and neurodegenerative diseases. Any

pathological processes affecting the brain will impact synaptic function, although some more directly than others. For example, in Alzheimer's disease, dysregulated endocytosis of synaptic AMPARs and NMDARs may contribute to progressive memory loss (Tang, 2009). Moreover, amyloid-beta peptide, which is closely linked to Alzheimer's disease pathology, has been shown to impair synaptic plasticity (Shankar et al., 2008), facilitate hippocampal LTD (Li et al., 2009), and interfere with CaMKII activity and disrupt activity-dependent AMPAR trafficking (Gu et al., 2009). Animal models of Alzheimer's disease also highlight defects in synaptic AMPAR trafficking and abnormalities in LTP and LTD (Walsh and Selkoe, 2007).

Other disease states or brain syndromes involve alterations in AMPAR subunit composition. Epilepsy causes a loss of GluA1-containing AMPARs across the brain (Grigorenko et al., 1997), whilst exposure to cocaine drives increased levels of CP-AMPARs in dopaminergic neurons in the ventral tegmental area (VTA: Argilli et al., 2008; Bowers et al., 2010; Mameli et al., 2011). In particular, for the latter effect with cocaine exposure, a single dose delivered to a naïve animal produces changes in the VTA that mimics LTP (Ungless et al., 2001; Argilli et al., 2008). Within 3 h, CP-AMPAR expression increases, and renders such synapses unable to undergo a spike-timing-dependent form of LTP. The same VTA response follows injections of morphine, nicotine, ethanol or amphetamine (Saal et al., 2003). Whilst these drug effects are alarming, they at least indicate a potential target for treatment of addiction. Fascinatingly, voluntary administration of cocaine produces a potentiation of these synapses that lasts up to 3 months without further drug use (Chen et al., 2008), as opposed to less than 10 days following a single injection (Ungless et al., 2001).

WHERE DO WE GO FROM HERE? WHAT ARE THE OPEN QUESTIONS IN AMPAR PLASTICITY?

Undoubtedly, the list of proteins able to regulate synaptic AMPAR levels and their activity remains incomplete. A recent study on AMPAR auxiliary subunits in hippocampal dentate granule cells (DG-GCs) underscores the subtleties still being elucidated (Khodosevich et al., 2014). TARP-γ8 and CKAMP44 are both highly expressed in DG-GCs where they promote AMPAR surface expression and decrease the rate of receptor deactivation. However these two auxiliary subunits have opposite effects on AMPAR desensitization, leading to distinct short-term plasticity; furthermore, only TARP-γ8 is required for LTP expression. This study not only highlights how AMPAR behavior can be uniquely shaped by the cell-type specific expression of modulators with which they complex, but also emphasizes the diversity and flexible control of AMPAR function across the brain.

Whereas AMPAR auxiliary proteins undeniably expand the variety of AMPAR function, in what way do AMPARs with distinct subunit composition contribute to their functional diversity? As discussed above, GluA2-lacking CP-AMPARs participate in different forms of plasticity. Intriguingly, however, a recent report has raised questions about the subunit-specific requirements for recruiting AMPARs to synapses during LTP (Granger et al., 2013). By taking advantage of conditional mice mutants carrying floxed alleles of genes encoding for GluA1, GluA2 and GluA3, Granger et al. tested the effects of genetically ablating any combination of these three subunits on LTP. Surprisingly, they find that any one of the GluA subunits is sufficient for maintaining the enhanced synaptic strength, and even overexpressed kainate receptors can restore LTP in these animals. Using the same approach they have shown that LTD expression is also independent of glutamate receptor subtype (Granger and Nicoll, 2014). Altogether this data suggests that the extra-synaptic surface population of AMPARs is the key factor for providing synaptic receptors for LTP and LTD, although this may depend on the type of stimulus delivered. The unexpected degree of subunit redundancy is remarkable, and even more so given the presence of different accessory proteins that interact with specific glutamatergic receptors to confer the differences in receptor properties. Perhaps experimentally induced LTP is an extreme case of plasticity with reduced discrimination, and under physiological conditions, various aspects of cognitive functions could be driven by controlling and deciphering the subtle variations in synaptic AMPARs. Moreover, changes in AMPAR number, amongst other processes, across a widely distributed set of synapses contribute to network function that ultimately guides behavior, memories, and consciousness. Indeed, how close can we come to physiological stimuli that are sufficient to encode behaviors, whilst we watch the formation of necessary neuronal traces or engrams? This lofty goal may require more than simply changes in AMPARs in synapse remodeling, or the formation of new synaptic and neuronal connections, but would be a genuine high point in our scientific endeavors.

Live super-resolution light microscopy has only just begun to reveal the intricacies of molecular movement at the synapse. Whilst not quite reaching electron microscopy levels of resolution, the advantage of being able to image live tissues at resolutions well below 100 nm makes the technique highly attractive for studying the behavior of synaptic proteins in response to activity. Where exactly do AMPARs undergo exo-endocytic recycling and is this dependent on the subunit composition? Are the nanodomains described for AMPARs and PSD components mirrored by the organization of presynaptic structures? At the active zone, precisely where does presynaptic vesicle fusion take place and in what manner does the released glutamate affect the diffusion properties of synaptic and extra-synaptic AMPARs? Furthermore, targeting super-resolution imaging to *in vivo* synapses in their native milieu, especially in behaving animals, will likely uncover new aspects of AMPAR plasticity that may have been lost in *in vitro* preparations.

In another direction, different disease states are now being unraveled, with the causative genes and protein products being identified in humans and reassembled in animal models. Many pathological states of the brain feature deficits in synaptic transmission at their core, which have been termed "synaptopathies", and the aging global population has created a serious social and medical issue that neuroscientists must play their part in solving.

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AMPAR interacting protein CPT1C enhances surface expression of GluA1-containing receptors

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AMPARs mediate the vast majority of fast excitatory synaptic transmission in the brain and their biophysical and trafficking properties depend on their subunit composition and on several posttranscriptional and posttranslational modifications. Additionally, in the brain AMPARs associate with auxiliary subunits, which modify the properties of the receptors. Despite the abundance of AMPAR partners, recent proteomic studies have revealed even more interacting proteins that could potentially be involved in AMPAR regulation. Amongst these, carnitine palmitoyltransferase 1C (CPT1C) has been demonstrated to form an integral part of native AMPAR complexes in brain tissue extracts. Thus, we aimed to investigate whether CPT1C might be able to modulate AMPAR function. Firstly, we confirmed that CPT1C is an interacting protein of AMPARs in heterologous expression systems. Secondly, CPT1C enhanced whole-cell currents of GluA1 homomeric and GluA1/GluA2 heteromeric receptors. However, CPT1C does not alter the biophysical properties of AMPARs and co-localization experiments revealed that AMPARs and CPT1C are not associated at the plasma membrane despite a strong level of co-localization at the intracellular level. We established that increased surface GluA1 receptor number was responsible for the enhanced AMPAR mediated currents in the presence of CPT1C. Additionally, we revealed that the palmitoylable residue C585 of GluA1 is important in the enhancement of AMPAR trafficking to the cell surface by CPT1C. Nevertheless, despite its potential as a depalmitoylating enzyme, CPT1C does not affect the palmitoylation state of GluA1. To sum up, this work suggests that CPT1C plays a role as a novel regulator of AMPAR surface expression in neurons. Fine modulation of AMPAR membrane trafficking is fundamental in normal synaptic activity and in plasticity processes and CPT1C is therefore a putative candidate to regulate neuronal AMPAR physiology.

Keywords: glutamate receptors, GluA1, CPT1C, AMPAR trafficking, surface expression, electrophysiological recordings, cortical neurons, palmitoylation

INTRODUCTION

Glutamate is the neurotransmitter involved in the majority of excitatory synaptic processes in the brain. This amino acid activates primarily ionotropic glutamate receptors (iGluRs): NMDA, AMPA, and Kainate receptors. Amongst iGluRs, the AMPA receptors (AMPARs) are essential as they mediate 90% of the fast excitatory neurotransmission in the central nervous system (CNS). Although their main role relates to synaptic transmission, AMPARs are also responsible for some forms of activity-dependent synaptic plasticity, the process thought to underlie higher order cognitive functions such as learning and memory (Barry and Ziff, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003).

AMPARs are tetrameric structures formed by four different subunits: GluA1–A4 and can be found as homo- or heterotetrameric structures (Traynelis et al., 2010), heteromeric receptors being the most common combinations found in neurons, amongst different brain regions (Gallo et al., 1992; Kondo et al., 1997; Lu et al., 2009; Reimers et al., 2011). Their subunit composition is crucial for AMPAR properties and their roles in neurons largely rely on the different intracellular carboxyl-terminal (C-terminal) domains, which vary between subunits. AMPAR subunits can be found with long (GluA1, GluA2-long, and GluA4) or short (GluA2, GluA3, and GluA4-short) intracellular C-terminal domains (Köhler et al., 1994). The different C-termini of AMPAR subunits permit a great variability in protein-to-protein interactions dependant on both the distinct AMPAR subunits (Palmer et al., 2005) and the class of PDZ binding domain (Sheng and Sala, 2001; Cai et al., 2002). The C-terminal domain of GluA subunits also contains most of the well-characterized phosphorylation and palmitoylation sites. These posttranslational modifications allow a fine and complex regulation of AMPARs through the specific interaction of the receptor with multiple intracellular proteins, which play crucial roles in AMPAR trafficking and function (Anggono and Huganir, 2012; Lu and Roche, 2012).

Of the multiple proteins that transiently interact with AMPARs and that determine their trafficking, synaptic targeting and

recycling in neurons, some special attention must be given to transmembrane proteins that form integral part of the functional receptor. In addition to trafficking, these proteins modulate channel gating properties hence acting as genuine auxiliary subunits of the AMPARs. Amongst these, the most important are the *Transmembrane AMPA receptor Regulatory Proteins* (TARPs; Kato et al., 2010a; Straub and Tomita, 2012). Indeed, the vast majority of AMPARs in the CNS are associated with TARPs (Menuz et al., 2008; but see Schwenk et al., 2009) and they appear to be crucial for correct trafficking and synaptic targeting (Tomita et al., 2005). Depending on the TARP subtype, AMPAR trafficking properties are differentially modulated resulting in a differential synaptic integration (Jackson and Nicoll, 2011).

A recent proteomic study confirming the interaction of AMPARs with transient and integral partners of AMPARs has also identified a number of proteins capable of interacting with AMPAR subunits. One of them is Carnitine palmitoyltransferase 1C (CPT1C), which forms part of some macromolecular complexes of AMPARs in the brain (Schwenk et al., 2012). This protein is a member of Carnitine palmitoyltransferases, a family of enzymes that catalyzes the exchange of acyl groups between carnitine and CoA to facilitate the transport of long chain fatty acids from the cytoplasm to the mitochondria for β-oxidation (McGarry and Brown, 1997). CPT1C is a specific CPT1 brain isoform strongly expressed in the hypothalamus, the hippocampus, cortex, and cerebellum (Price et al., 2002). CPT1C is highly homologous to the other CPT1s: it has the ability to bind palmitoyl-CoA and maintains the same binding affinity as CPT1A for malonyl-CoA (the endogenous allosteric CPT1 inhibitor). However, CPT1C has a 100-fold lower catalytic activity than the other isoforms (Sierra et al., 2008). Moreover, it is located in the endoplasmic reticulum (ER) instead of the mitochondria (Sierra et al., 2008; Carrasco et al., 2012). The molecular mechanism of CPT1C action has not been unraveled yet, but some clues about its importance in mammalian brain function derive from CPT1C knockout mice studies. These KO mice show an impairment of motor functions, muscle strength, hypoactivity (Carrasco et al., 2013), behavioral learning deficits (Carrasco et al., 2012) and altered maturation of dendritic spines in hippocampal neurons indicating an important role of CPT1C in the CNS. Additionally, some results indicate that CPT1C is also involved in the control of food intake and energy expenditure (Wolfgang et al., 2006). It has also been described that a gain-of-function of CPT1C in the brain of transgenic mice results in severe growth retardation and in a reduction of brain weight (Reamy and Wolfgang, 2011).

In the present study we investigate whether CPT1C might affect AMPAR function. Our results confirm that GluA subunits are able to interact with CPT1C and this interaction modulates AMPAR surface expression in a subunit-dependent manner, without altering the gating properties of the receptor. Moreover we find that the palmitoylable cysteine residue located in the 585 position of GluA1 is crucial for CPT1C modulation of AMPAR surface level. Since it is clear that regulation of AMPAR membrane trafficking is critical for normal synaptic activity and for several forms of synaptic plasticity in the brain, the involvement of CPT1C in these processes is relevant for understanding AMPAR physiology.

MATERIALS AND METHODS

EXPRESSION CONSTRUCTS

AMPAR subunit cDNAs were a gift from Prof. Dr. Stephen Heinemann (Salk Institute, La Jolla, CA) and Prof. Dr. Peter Seeburg (Max Planck Institute, Heidelberg, Germany). pDs-Red-ER-KDEL was a generous gift of Juan Pablo Muñoz (IRB, Barcelona). GluA1-pIRES-mCherry: a pIRES vector expressing GluA1 and mCherry translated from a single bicistronic mRNA (used for heteromeric GluA1/GluA2 electrophysiological experiments). CPT1C plasmid vectors were a generous gift from Dr. Núria Casals (Universitat Internacional de Catalunya) and CPT1A-GFP was a gift from Dr. Dolors Serra (Universitat de Barcelona). Characteristics of CPT1C and CPT1A plasmid vectors were: (1) CPT1C-GFP: a plasmid containing CPT1C cDNA sequence C-terminally tagged with EGFP; this construct produces a protein of approximately 100 kDa (all experiments involving CPT1C have been performed with this plasmid unless otherwise stated); (2) CPT1C-pIRES: a pIRES vector expressing CPT1C and EGFP translated from a single bicistronic mRNA and (3) CPT1A-GFP: contains CPT1A cDNA sequence C-terminally tagged with EGFP. All cDNAs are from rat and plasmid vectors are all under the control of the same promoter (CMV promoter).

To obtain GluA1 cDNAs with mutations in the palmitoylation sites we used site-directed mutagenesis to change specific base pairs. Primers containing the mutation/s were designed and then synthetized by Integrated DNA Technologies (IDT). GluA1(C585S) and GluA1(C811S) mutant cDNAs resulted from changing the codon TGT to TCT and TGC to TCC, respectively. Both changes produce a cysteine to serine switch making these palmitoylation targets disappear. For the double mutant GluA1(C585,811S) we used GluA1(C585S) cDNA as a template and introduced the C811S mutation to create the GluA1(C585,811S) product, in which both palmitoylation sites from GluA1 were eliminated. The primers used for introducing the mutations were the following:

C585S: AAGGAT**C**TGACATTTCCCCCAGGTCCC
C811S: CCTTAATCGAGTTCT**C**CTACAAATCCCGTAGCG

All constructs were fully sequenced to verify sequence integrity.

CELL CULTURE AND TRANSFECTION

tsA201 cells – derived from HEK293, which do not express CPT1C protein (Sierra et al., 2008)—were maintained in DMEM:F12 containing 10% fetal bovine serum and 1% penicillin–streptomycin solution in 5% CO_2/95% air at 37°C. 24 h before transfection, 1.5×10^6 cells were plated into T25 flasks for coimmunoprecipitation (Co-IP) and Acyl Biotin Exchange assays (ABE) or 0.5×10^5 cells onto poly-D-lysine-coated coverslips for immunofluorescence (IF) and electrophysiological (EP) experiments. Cells were transiently co-transfected with 5.4 μg total cDNA (for Co-IP and ABE) and 0.6 μg total cDNA (for IF and EP) using X-tremeGENE transfection reagent (Roche) according to the manufacturers' directions. In all transfections the ratio used was 1:2 (GluA:CPT1C). Media was replaced 24 h after transfection

with fresh media containing 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide 50 μM (NBQX; Tocris-ABCam, Abcam) to prevent AMPAR-mediated toxicity. For EP experiments, cells were re-plated on glass coverslips to allow optimal density. All experiments were performed 48 h later.

NEURONAL CULTURES AND TRANSFECTION

Cortical neuron cultures were prepared from mouse embryos (E18). The cerebral cortex was isolated and maintained in cold Hank's Balanced Salt Solution (HBSS, Gibco) supplemented with 0.45 % glucose. After removal of the meninges, the cortical tissue was digested mildly with trypsin for 17 min at 37°C and mechanically dissociated. Cells were washed three times in HBSS and resuspended in Neurobasal medium supplemented with 2 mM Glutamax (Gibco) before filtering in 70 μm mesh filters (BD Falcon). Cells were plated onto glass coverslips (5×10^4 cells/cm^2) coated with 0.1 mg/ml poly-L-lysine (Sigma). 2 h after seeding, the plating medium was replaced by complete growth medium (Neurobasal medium supplemented with 2% B27 (Invitrogen) and 2 mM Glutamax) and the coverslips were incubated at 37°C in a humidified 5 % CO_2 atmosphere. Every 3–4 days, half of the conditioned medium was removed and replaced by fresh growth medium. Primary cultures were transfected with Lipofectamine 2000 on day 7 *in vitro* (7 DIV), according to the manufacturer's, instructions and the cells were fixed 72 h after transfection. All the experimental procedures were carried out according to European Union guidelines (Directive 2010/63/EU) and following protocols that were approved by the Ethics Committee of the Bellvitge Biomedical Research Institute (IDIBELL).

COIMMUNOPRECIPITATION

48 h after transfection, tsA201 cells were washed twice with room temperature PBS and collected in 1 ml 50 mM Tris-HCl (pH 7.4) with Protease Inhibitor cocktail (Sigma) on ice. All subsequent steps were performed at 4°C. Cells were lysed in a Polytron (VDI 12; VWR) at force 5, for 20 s, twice. Lysates were centrifuged at 1000 ×g for 10 min to pellet nuclei and unlysed cells. The supernatant was further centrifuged at 20,000 ×g for 30 min, and the membrane fraction (pellet) was resuspended in solubilisation buffer (1 % Triton X-100, 150 mM NaCl and 50 mM Tris-HCl pH 8, containing protease inhibitors) and homogenized with a Polytron for 20 s. After 20 min on ice, insoluble material was pelleted with a 30 min centrifugation at 20,000 ×g and the supernatant was quantified using the BCA method (Thermo Scientific). 200–400 μg of total protein were incubated with 4 μg of antibody overnight at 4°C with orbital agitation (antibodies: mouse anti-GluA1-NT (N-terminus), rabbit anti-GluA2 (cytoplasmic domain) both from Merck Millipore, rabbit serum anti-GFP from Invitrogen). Antibody-protein complexes were pulled down by incubating with 80 μl of Protein-A sepharose beads (Sigma) pre-equilibrated with solubilisation buffer, for 2 h. Precipitated complexes were washed in solubilisation buffer three times and eluted with 2 × SDS/DTT sample buffer, heated 10 min at 76°C and separated on SDS/PAGE. Before adding the antibodies, 10% of total protein (100 μl) was removed as input samples. 500 μl of pre-cooled acetone was added to the input samples, the mixture was vortexed and incubated overnight at −20°C. The precipitated proteins were cleared by centrifugation at 20,000 ×g for 20 min, supernatant was removed, and pellets were air-dried for 15–30 min and resuspended with appropriate volume of 2 × SDS/DTT buffer.

IMMUNOBLOTTING

Samples were separated by SDS/PAGE in 4-15% mini-protean TGX precast gels, transferred using Trans-Blot Turbo transfer system on nitrocellulose membranes (all from BioRad). Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% (wt/vol) BSA or non-fat skim milk. Peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibodies (Dako) diluted in blocking solution at 1:1000 dilution, were detected by using ECL reagent (Amersham) and a LAS3000 Intelligent Dark Box (Fujifilm) was used to report western-blots. Quantification of the western blots was performed with Image J (NIH).

IMMUNOCYTOCHEMISTRY

Immunofluorescence was performed in tsA201 cells grown on lysine treated coverslips, 48 h after transfections. Washes were always performed by immersion of the coverslips in PBS or PBS-G (20 mM Glycine in PBS). Composition of different solutions: Fixation solution (2% PFA in PBS), permeabilization solution (0.1% Triton X-100 in PBS-G), blocking solution (10% NGS, 2% BSA, 0.1% Triton X-100 in PBS-G), antibody incubation solution (4% normal goat serum and 0.1% BSA in PBS-G) and triton-antibody solution (antibody incubation solution containing 0.1% Triton X-100). Antibodies incubations were performed in a humid chamber at 37°C for 1 h.

For co-localization of CPT1C with GluA1 or GluA2 and for determining the level of surface expression of GluA1 in tsA201, the following method was used: staining surface AMPARs was achieved by labeling live cells with the mouse anti-GluA1-NT or mouse anti-GluA2 antibodies (both from Merck Millipore) in a 1:200 solution in DMEM:F12, for 7–10 min at 37°C. In neuronal cultures, the surface staining step was performed for 1 h at 37°C on fixed neurons (4% PFA + 4% sucrose). After washes in room temperature PBS, tsA201 cells were fixed for 15 min at room temperature and incubated with goat anti-mouse Alexafluor 555 (Molecular Probes) at 1:250 in antibody incubation solution. After several washes in PBS-G, cells were fixed again to preserve the binding of the first secondary antibody. Cells were subsequently permeabilized for 5–10 min and blocked for 30 min. Next, and in order to determine the total expression of AMPARs in each cell, GluA1 or GluA2 were labeled incubating the coverslips with the same mouse anti-GluA1-NT or mouse anti-GluA2 antibodies at 1:200 (in triton-antibody incubation solution). Following washes in PBS-G, cells were incubated with goat anti-mouse Alexafluor 647 (Molecular Probes) at 1:500 (in triton-antibody incubation solution). Coverslips were then washed and mounted with Fluoromount (Invitrogen).

For co-localization of CPT1C with GM-130 (Golgi marker), cells were transfected with CPT1C-GFP and Golgi staining was performed on fixed, permeabilized and blocked tsA201 cells by incubating cells with mouse anti-GM-130 (BD-Biosciences) at 1:50 in triton-antibody incubation solution and subsequently

with goat anti-mouse Alexafluor 555 (Molecular Probes) at 1:500.

For co-localization of CPT1C with an ER marker we co-transfected tsA201 cells with 600 ng of total DNA at a ratio of 1:2 (ER-KDEL: CPT1C). 48h after transfection cells were fixed for 15 min in 4% PFA, washed and mounted in Fluoromount.

CONFOCAL IMAGING AND IMMUNOFLUORESCENCE QUANTIFICATION

Confocal images were acquired on a spectral confocal microscope (Leica TCS-SL, CCiTUB), using 40× or 63× oil objective lenses, in multitracking mode to minimize channel crosstalk. Each image was taken through laser excitation lines 488, 543, and 633 and Differential Interference Contrast (DIC). For immunofluorescence quantification experiments the same settings for each condition and throughout experiments were used. For tsA201 cells, stacks of 0.7 μm were taken from different areas and for cortical neurons stacks were of 0.3 μm.

Quantification of GluA1 surface expression was performed using Image J (NIH). Each stack was Z-projected to the maximum intensity. With the freehand selection tool individual cells or dendrites co-expressing the receptor and CPT1C-GFP or GFP (expression verified by tracking fluorescence intensity in the green channel) were traced and fluorescence in each channel was measured. The fluorescence values of each cell/dendrite were then analyzed, red integrated density (INTDEN) being the value corresponding to surface expression of the receptor in that cell/dendrite, and blue INTDEN being the value for total expression of GluA1 in the same cell/dendrite (tsA201 cells with low levels of blue fluorescence were not quantified). The mean background intensity was obtained from three different areas of each image and subtracted from each measurement using the following formula:

$$\text{CTCF} = \text{INTDEN} - (\text{AREA}^{*}\ \text{MEAN FLUORESC BKGD}) \quad (1)$$

where CTCF stands for corrected total cell fluorescence. Then, the ratio surface to total was obtained from the CTCF value from red fluorescence (surface) divided by the CTCF value from blue fluorescence (total), and normalized to the reference condition (GluA1+GFP or GFP transfected neurons). Finally, column graphics including the mean ratio of each condition were plotted and the error bars (SEM) were obtained. A set of at least 3 different immunofluorescences for each condition was performed and 10–50 cells of each condition were analyzed for each immunofluorescence. For neuronal experiments, three separate independent cultures were performed and 70 and 80 dendrites from 21 and 23 neurons from control and test condition were analyzed.

Quantification of co-localization was performed using the Manders Overlap Coefficient (MOC) calculated in Image J via the JACoP plugin from images of single cells. This coefficient ranges between 1 and zero with values close to 1 being high co-localization, and values close to zero being low.

ELECTROPHYSIOLOGY: GENERAL PROCEDURES

Cells were visualized with an inverted microscope (IX50; Olympus). Electrodes were fabricated from borosilicate glass (1.5 mm o.d., 0.86 mm i.d., Harvard Apparatus) pulled with a PC-10 vertical puller (Narishige). Electrode resistance varied between configurations (see below). Macroscopic currents were recorded at room temperature (22-25°C) in the whole-cell configuration (wc) or from outside-out patches (o) excised from GFP-positive cells. Currents were recorded with Axopatch 200B amplifier, filtered at 2 kHz (wc) or 10 kHz (o) and digitized at 5 kHz (wc) or 50 kHz (o) using Digidata 1440A interface with pClamp 10 software (Molecular Devices Corporation). For all configurations the "extracellular" solution contained (in mM): 145 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH to 7.42 with NaOH). For fast agonist application, 10 mM glutamate was added to the "extracellular" solution. The "intracellular" solution contained (in mM): 145 CsCl, 2.5 NaCl, 1 Cs-EGTA, 4 MgATP, and 10 HEPES (pH to 7.2 with CsOH). Spermine tetrahydrochloride (Sigma Aldrich) was added to intracellular solution at 100 μM in all cases.

WHOLE-CELL RECORDINGS

Whole-cell recordings were made from isolated cells using thick-walled electrodes with a resistance of 3–5 MΩ, giving a final series resistance of 5–15 MΩ. A voltage ramp protocol was used to change the holding potential (0 to –80 mV then to +80 mV at a rate of 160 mV/s; with the voltage held at –80 mV for 200 ms previous to the ramp). Receptors were activated by a bath application of 1 mM glutamate plus 25 μM cyclothiazide (CTZ) to prevent receptor desensitization. Control traces were subtracted from stable agonist-activated responses and the average current recorded at −80 mV was measured. In all recordings, to control for differences in cell surface area, the response to glutamate was expressed as current density (–pA/pF; maximum current divided by input capacitance as measured from the amplifier settings).

The rectification index (RI) was defined as the absolute value of glutamate-evoked current at +60 mV divided by that at −60 mV:

$$\text{RI} + 60\text{mV}/-60\text{mV} = |\text{I}_{+60\text{mV}}|/|\text{I}_{-60\text{mV}}| \quad (2)$$

AGONIST APPLICATION TO EXCISED PATCHES

For out-side out patches we used electrodes with a final resistance of 8–12 MΩ. Rapid agonist application was achieved by switching between a continuously flowing control solution (extracellular solution diluted by 4%) and a glutamate-containing solution (extracellular solution plus 2.5 μg/ml sucrose and 10 mM glutamate). Solution switching was achieved by piezoelectric translation of a theta-barrel application tool made from borosilicate glass (1.5 mm o.d.; Sutter Instruments) mounted on a piezoelectric translator (P-601.30; Physik Instrumente). 100 ms jumps were applied to outside-out patches at a holding potential of −60 mV. At the end of each experiment, the adequacy of the solution exchange was tested by destroying the patch and measuring the liquid-junction current at the open pipette (10–90% rise time normally 200–300 μs).

The kinetics of desensitization of the glutamate-activated currents were determined by fitting the glutamate-evoked responses at V_m −60 mV to a double-exponential function in order to determine the weighted time constant ($\tau_{w,des}$):

$$\tau_{w,\text{des}} = \tau_f\left(\frac{A_f}{A_f + A_s}\right) + \tau_s\left(\frac{A_s}{A_f + A_s}\right) \quad (3)$$

where A_f and τ_f are the amplitude and time constant of the fast component of desensitization and A_s and τ_s are the amplitude and time constant of the slow component of desensitization.

NON-STATIONARY FLUCTUATION ANALYSIS (NSFA)

To deduce channel properties from macroscopic responses, glutamate (10 mM) was applied to outside-out patches (100 ms duration, 1 Hz, V_{hold} −60 mV) and the ensemble variance of all successive pairs of current responses were calculated. The single channel current (i) and the total number of channels in the patch (N) were calculated by plotting this ensemble variance against mean current ($\bar{I}$) and fitting with a parabolic function:

$$\sigma^2 = \sigma_B^2 + \left(i\bar{I} - \left(\frac{\bar{I}^2}{N}\right)\right) \quad (4)$$

where σ_B^2 is the background variance. Along with normal peak-to-peak variation in the currents due to stochastic channel gating, some patches presented a gradual decline in peak amplitude. The mean response was calculated from the periods of the recordings showing stable responses that were identified using a Spearman rank-order correlation test (Igor Pro with Neuromatic). The weighted-mean single-channel conductance was determined from the single-channel current and the holding potential corrected for the calculated liquid-junction potential (+4.9 mV; pClamp 10).

ACYL-BIOTIN EXCHANGE (ABE) ASSAY

Detection of palmitoylation levels of GluA1 subunits was performed as described in Brigidi and Bamji (2013). 48 h after transfection, tsA201 cells co-expressing GluA1 and GFP or GluA1 and CPT1C-GFP, were washed with ice-cold PBS and lysed with a 30 G syringe 6 times in ice-cold lysis buffer (1% IGEPAL, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, Protease Inhibitor Cocktail (Roche) and PMSF) containing 50 mM N-ethylmaleimide (NEM) (Sigma). All steps where performed at 4°C. Lysates were cleared by centrifugation at 16,000 xg for 30 min and the amount of protein in the supernatant was determined using the BCA method (Thermo Scientific). 750 μg – 1.5 mg of protein were used for overnight immunoprecipitation of GluA1 [4 μg of anti-GluA1-NT antibody (Merck Millipore)]. Then, protein-antibody complexes were pulled-down with Protein-A sepharose beads (Sigma) for 1–2 h. The total immunoprecipitate was then resuspended in lysis buffer with 10 mM NEM and it was split into two equivalent samples: one sample for specific cleavage and unmasking of the palmitoylated cysteine's thiol group by 1 M hydroxylamine treatment (+HAM sample) and a second equivalent sample but without the presence of HAM to control non-specific incorporation of biotin (-HAM sample). Before performing HAM treatment, samples were totally washed to avoid any presence of unbound NEM. 1 M HAM solution was prepared in pH 7.2 lysis buffer and ± HAM treatment was performed for 1 h at room temperature. After washes, selective labeling of the palmitoylated cysteine using a thiol-reactive biotinylation reagent, biotin-BMCC (1 μM) (Thermo Scientific) in pH 6.2 lysis buffer was performed for 1 h at 4°C in ± HAM samples. Then, the thiol-biotinylated proteins following the ABE steps were resolved by SDS-PAGE and Western Blotting was performed. Membranes were blocked with 3% BSA in TBS-T and incubated with Streptavidin-HRP (Invitrogen) (1:5000 from a 1 mg/ml stock in 0.3 % BSA). After stripping, the same membrane was incubated with an anti-GluA1-NT antibody (1:1000) to normalize palmitoylation levels to the amount of immunoprecipitated protein.

ANALYSIS AND STATISTICS

Electrophysiological recordings were analyzed using IGOR Pro (Wavemetrics Inc.) with NeuroMatic (Jason Rothman, UCL). Data are presented in the text as the mean ± SEM from n patches and in the figures as bar plots of the group mean, with error bars denoting the SEM. Comparisons between two groups were performed using the non-parametric Mann-Whitney U test. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism version 5.0d for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

AMPAR SUBUNITS COIMMUNOPRECIPITATE WITH CPT1C IN HETEROLOGOUS EXPRESSION SYSTEM

CPT1C has been shown to interact with native AMPAR subunits in rat brain tissue (Schwenk et al., 2012). Thus we first sought to determine if in our expression system CPT1C also interacted with AMPAR subunits. To analyse the interaction of GluA1 with CPT1C we performed coimmunoprecipitation assays from tsA201 cells transiently transfected with GluA1 and the construct CPT1C tagged with the green fluorescence protein GFP (CPT1C-GFP; see methods) (**Figure 1**). Membranes of these cells were immunoprecipitated either with anti-GluA1 or anti-GFP and subsequently detected using Western blot. Antibodies recognizing GFP were able to pull down GluA1 when co-expressed with CPT1C-GFP (**Figure 1A**; upper middle panel). The ∼100 KDa band was not detected with GluA1 antibody in anti-GFP immunoprecipitates from cells transfected only with GluA1 or CPT1C-GFP. Additionally, coimmunoprecipitation was observed by pulling down CPT1C-GFP with an anti-GluA1 antibody (**Figure 1A**; lower right panel). Correct expression of the different constructs was confirmed by Western blot of the input samples (**Figure 1A**; upper and lower left panels). Our results confirm previous observations and indicate that in tsA201 cells CPT1C physically associates with GluA1 subunit.

Equivalent conditions were used for detecting interaction of GluA2 with CPT1C and the same negative controls were performed. **Figure 1B** shows that GluA2 subunit coimmunoprecipitated with CPT1C-GFP when the latter was pulled-down with an anti-GFP antibody (upper middle panel). Additionally, the anti-GluA2 antibody was able to pull-down CPT1C (**Figure 1B**; lower right panel). The presence of both proteins was detected in the input samples (**Figure 1B**; upper and lower left panels). These results confirm that both GluA1 and GluA2 interact with CPT1C in tsA201 cells when both proteins are co-expressed.

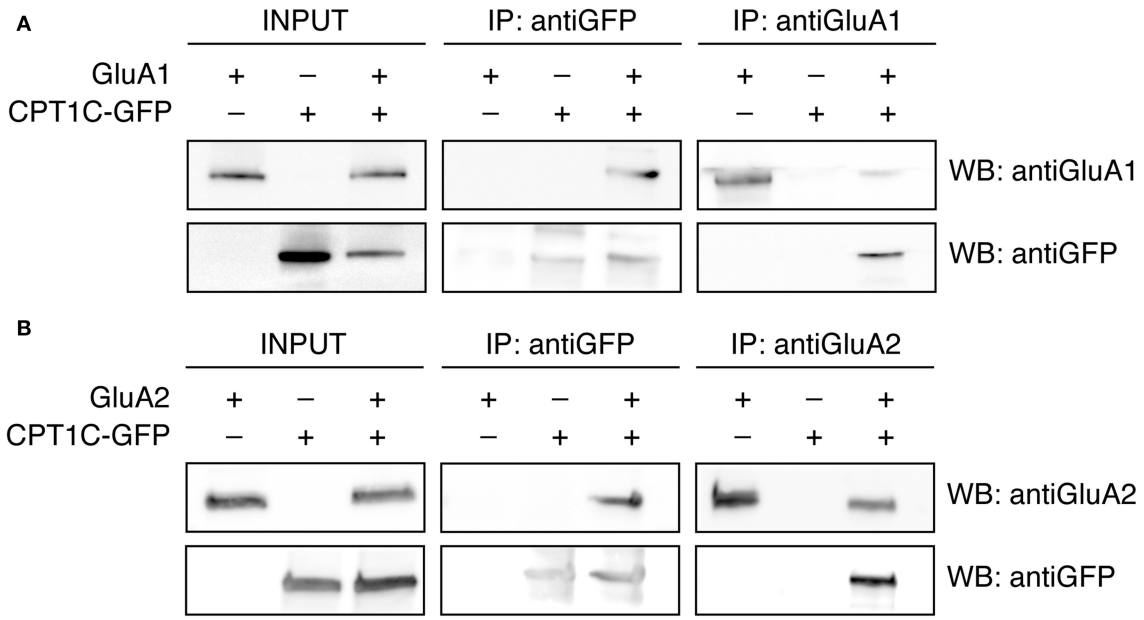

FIGURE 1 | GluA1 and GluA2 coimmunoprecipitate with CPT1C in expression systems. (A) Co-IP of the membranous fraction of tsA201 cells co-expressing either GluA1 alone or together with CPT1C-GFP confirming the interaction between CPT1C and GluA1. As a negative control GluA1 was co-expressed with an empty plasmid expressing GFP alone (pEGFP) and CPT1C-GFP was co-expressed with an empty plasmid (pcDNA3.0). Transfected cells were lysed and membranes were solubilized. 200-400 μg of solubilized membranes was immunoprecipitated with an anti-GFP antibody (IP: antiGFP) or with anti-GluA1-NT antibody (IP: antiGluA1). An input sample collected prior to immunoprecipitation of these extracts is shown as "INPUT." Inputs and immunoprecipitated samples were separated using SDS-PAGE and Western Blot was performed using anti-GluA1-NT (WB: antiGluA1) or anti-GFP (WB: antiGFP) antibodies. Immunoprecipitations were performed three times. **(B)** Same as in **(A)** but for tsA201 cells expressing GluA2 or GluA2 plus CPT1C-GFP. Membrane lysates were immunoprecipitated with an anti-GFP antibody (IP: antiGFP) or a rabbit polyclonal anti-GluA2 (cytoplasmic domain) (IP: antiGluA2). Western Blots were performed using mouse anti-GluA2 (WB: antiGluA2) or anti-GFP (WB: antiGFP) antibodies. Immunoprecipitations were replicated three times.

CPT1C INCREASES WHOLE-CELL CURRENTS OF GluA1-CONTAINING AMPARs

Previous results (Schwenk et al., 2012) and our coimmunoprecipitation experiments suggest that CPT1C and AMPARs form part of the same complex at some stage either during the biosynthetic pathway or as an integral part of the receptor at the cell surface. Given that, we wondered if the interaction between AMPAR subunits and CPT1C could have any functional consequences on AMPARs. To explore that possibility we transiently transfected tsA201 cells with GluA1 subunit in the absence and presence of CPT1C-GFP and we measured glutamate-evoked whole-cell currents at different holding potentials by applying a voltage ramp in the presence of 1 mM glutamate and 25 μM CTZ. **Figures 2A,B** illustrate two examples of currents recorded between −80 and +80 mV in cells transfected with GluA1 and GluA1+CPT1C, respectively. Our results show that whole-cell current density measured at −80 mV from cells expressing GluA1 together with CPT1C was higher than those currents recorded for GluA1 alone (**Figure 2C**; 66.97 ± 18.77 pA/pF for GluA1 vs. 159.4 ± 39.43 pA/pF for GluA1+CPT1C; $p = 0.0431$; Mann–Whitney *U*-test; $n = 11$ and 8 respectively).

For the coimmunoprecipitation experiments performed in **Figure 1** we used a GFP-tagged form of CPT1C due to the lack of a good commercially available anti-CPT1C antibody. To be consistent between experiments we carried out the electrophysiological experiments with the GFP-tagged form of CPT1C. However, we could not rule out that the fused GFP could somehow account for the whole-cell current increase seen in our recordings. To exclude that possibility we repeated our experiments with a non-tagged form of CPT1C protein (CPT1C-pIRES; see methods). We found a similar increase in the current density to that obtained with the GFP-tagged form (data not shown). Further, the effect of CPT1C on GluA1 could be due to a general CPT1 activity or feature and not due to the specific interaction of the isoform CPT1C with GluA1. To test this possibility we overexpressed the most ubiquitous form of CPT1, CPT1A, together with GluA1. We found similar current density values to those obtained with GluA1 alone (**Figure 2C**; 66.97 ± 18.77 pA/pF for GluA1 vs. 78.69 ± 37.10 pA/pF for GluA1+CPT1A; $p = 1.0000$; Mann–Whitney *U*-test; $n = 11$ and 5 respectively). This demonstrates the specificity of the CPT1C effect on AMPAR-mediated currents.

CPT1C is also present in AMPAR complexes isolated with GluA2 antibody (Schwenk et al., 2012 and **Figure 1B**). Therefore we investigated whether the effect of CPT1C on GluA2 subunit was similar to the one seen for GluA1 subunits by doing whole-cell density current experiments in GluA2 homomeric AMPARs. Unlike GluA1, **Figure 2D** shows that there was no increase in current density when CPT1C was co-expressed with GluA2 in tsA201 cells (61.85 ± 16.48 pA/pF for GluA2 vs. 52.28 ± 20.27 pA/pF for

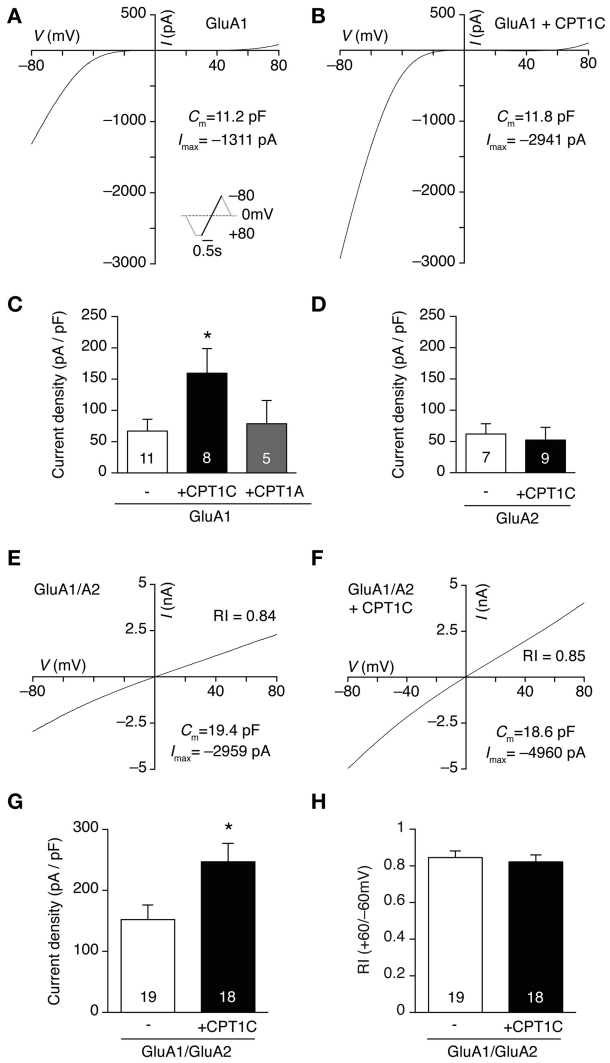

FIGURE 2 | CPT1C specifically increases glutamate-evoked currents of GluA1-containing AMPARs. (A) Whole-cell current-voltage (IV) relationship for a tsA201 cell expressing GluA1 homomeric receptors. The IV plot was obtained by ramping membrane potential from −80 to +80 mV at a rate of 160 mV/ s in the presence of 1 mM glutamate plus 25 μM CTZ to avoid receptor desensitization. 100 μM spermine was added to the pipette solution. Inset represents the voltage protocol used. **(B)** Same as **(A)** but for a cell expressing GluA1 plus CPT1C-GFP (CPT1C). **(C)** Average normalized currents at -80 mV for GluA1 alone or together with CPT1C or CPT1A. GluA1 current density (-pA/pF) was increased by co-expression with CPT1C (*$p < 0.05$; Mann–Whitney *U*-test) but was unaffected by CPT1A co-expression ($p > 0.05$; Mann–Whitney *U*-test). Numbers in bars denote the number of recordings. **(D)** Average current density for GluA2 alone or with CPT1C ($p > 0.05$; Mann–Whitney *U*-test). Numbers in bars denote the number of recordings. **(E)** Whole-cell IV in the same conditions as **(A)** but for a cell expressing GluA1/GluA2 heteromeric AMPARs. Rectification index (RI; I_{+60mV}/I_{-60mV}) gives a read-out of GluA2 incorporation. **(F)**. Same as **(E)** but for a cell expressing GluA1/GluA2 plus CPT1C. **(G)**. Average normalized currents at −80 mV for GluA1/GluA2 AMPARs with or without CPT1C. GluA1/A2 current density (-pA/pF) was increased by co-expression with CPT1C (*$p < 0.05$; Mann–Whitney *U*-test). Numbers in bars denote the number of recordings. **(H)**. Average RI for the cells recorded in both conditions showing no differences between groups.

GluA2+CPT1C; $p = 0.2991$; Mann–Whitney *U*-test; $n = 7$ and 9 respectively).

Most of glutamatergic neurons in CNS express GluA2-containing AMPARs either with GluA1, GluA3 or GluA4 (Gallo et al., 1992; Kondo et al., 1997; Lu et al., 2009; Reimers et al., 2011). Thus we decided to evaluate whether GluA1/GluA2 heteromeric receptors currents were similarly affected by CPT1C. **Figures 2E–G** shows that CPT1C was able to increase current density from heteromeric receptors (152.1 ± 23.94 pA/pF for GluA1/GluA2 vs. 246.9 ± 30.26 pA/pF for GluA1/GluA2+CPT1C; $p = 0.0236$; Mann–Whitney *U*-test; $n =$ 19 and 18 respectively). In these experiments, in order to favor GluA2 presence in the receptor we transfected in a 1:2 ratio (GluA1:GluA2) and the GluA1 plasmid expressed the mCherry protein (see methods) thus allowing the recording of GluA1-containing receptors. In fact, for both conditions, the red fluorescence patched cells displayed linear responses, which were not different between both groups confirming the presence of GluA2 (**Figure 2H**; $RI_{+60/-60}$= 0.84 ± 0.04 without CPT1C vs. 0.82 ± 0.04 with CPT1C; $p = 0.8910$; Mann–Whitney *U*-test; $n = 19$ and 18 respectively).

CPT1C DOES NOT ALTER GATING PROPERTIES OF AMPARs

In the current density experiments described above we found a significant increase in the glutamate-evoked GluA1-mediated currents when CPT1C was present. The total amount of current carried by a given population of receptors depends on several factors, which include the single channel conductance, the kinetics, the open probability of the receptor and the number of receptors contributing to the current. Any alteration in these parameters might result in changes in the current magnitude. So, one possibility would be that CPT1C could modulate the single channel conductance or open probability of AMPARs either by a direct interaction (Soto et al., 2007, 2014; Suzuki et al., 2008; Coombs et al., 2012; Shelley et al., 2012) or indirectly by phosphorylation (Derkach et al., 1999; Banke et al., 2000; Kristensen et al., 2011). So, we decided to investigate the mechanisms contributing to the current increase observed in AMPARs together with CPT1C. To determine whether AMPAR single channel conductance was altered by CPT1C we transfected GluA1 either alone or together with CPT1C in tsA201 cells and applied fast applications of glutamate (10 mM; 100 ms duration; 1 kHz) onto out-side out patches followed by non-stationary fluctuation analysis of the glutamate-evoked responses.

Figures 3A,B show typical responses for GluA1 homomers alone (**Figure 3A**) or together with CPT1C (**Figure 3B**). The single channel conductance of GluA1 homomers was not altered when co-expressed with CPT1C (**Figure 3C**; 16.53 ± 1.07 pS for GluA1 alone vs. 17.07 ± 1.31 pS for GluA1+CPT1C; $p = 0.8095$, Mann–Whitney *U*-test; $n = 17$ for both). CPT1C did not alter peak open probability ($P_{o,peak}$) of AMPARs (**Figure 3D**; 0.43 ± 0.04 for GluA1 vs. 0.40 ± 0.05 for GluA1+CPT1C; $p = 0.6052$, Mann–Whitney *U*-test; $n = 17$ for both). Similarly, the AMPARs kinetics measured as the desensitization decay time constant (see methods) were not changed (**Figure 3E**; 2.32 ± 0.18 ms for cells expressing GluA1 alone vs. 2.53 ± 0.17 ms for cells expressing

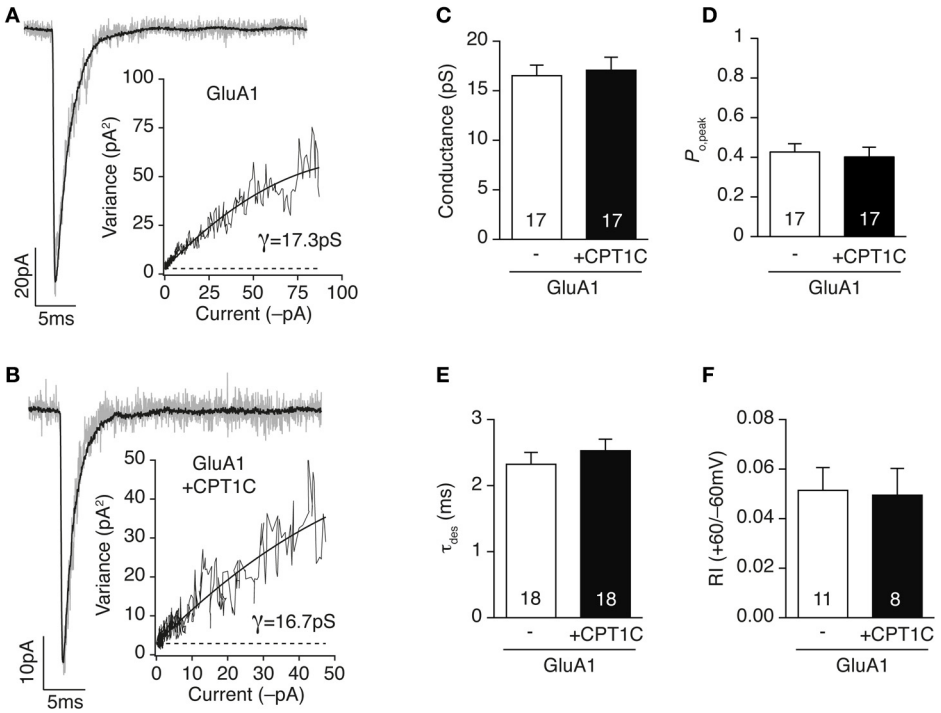

FIGURE 3 | CPT1C does not alter AMPAR gating properties. (A) Current activated by rapid application of 10 mM glutamate (100 ms, −60 mV) to an outside-out patch from a tsA201 cell expressing GluA1 alone. Gray trace represents a single response and black line the average of 37 responses. Inset shows the current variance vs. mean current plot for this patch. The weighted mean single-channel conductance estimate for this record was 17.3 pS. **(B)** Same as **(A)** but for a cell expressing GluA1 plus CPT1C-GFP (CPT1C). Black line is the average of 38 responses. Inset: current variance vs. mean current plot for this patch giving a weighted mean single-channel conductance of 16.7 pS. **(C–F)** CPT1C has no influence on either weighted single channel conductance (**C**; pS), peak open probability (**D**; $P_{o,peak}$), desensitization kinetics (**E**; τ_{des}) or rectification index (**F**; I_{+60mV}/I_{-60mV}) of GluA1 homomeric AMPARs. $P > 0.05$, Mann–Whitney *U*-test for all groups. Numbers in bars denote the number of recordings.

GluA1+CPT1C; $p = 0.3112$, Mann–Whitney *U*-test; $n = 18$ for both). Since AMPAR auxiliary subunits attenuate the strong block by polyamine of calcium permeable AMPARs (CP-AMPARs) (Soto et al., 2007, 2009), we evaluated if CPT1C was able to have a similar effect. We therefore measured the rectification index (RI) at +60/–60 mV (see methods) from the IV ramps experiments (**Figures 2A–C**), however we did not see any alteration in the strong inwardly rectifying IV relationship of CP-AMPARs (**Figure 3F**; 0.051 ± 0.009 for cells expressing GluA1 alone vs. 0.049 ± 0.011 for cells expressing GluA1+CPT1C; $p = 0.8554$, Mann–Whitney *U*-test; $n = 11$ and 8 respectively).

Taken together, data from **Figures 2**, **3** show that glutamate-evoked current density is increased by co-expression of CPT1C in GluA1 expressing cells but not those expressing only GluA2 subunit. Nonetheless, GluA1 channel properties (single channel conductance, peak open probability and desensitization kinetics) are unaffected suggesting an increase in receptor number at the cell surface. These results could also be indicating that both proteins do not associate at the plasma membrane despite a larger amount of current when CPT1C is co-expressed with GluA1.

AMPARs CO-LOCALIZE WITH CPT1C AT THE ER BUT NOT AT THE PLASMA MEMBRANE

Our results show a functional effect of CPT1C on GluA1. This effect though, seems not to be similar to that of "bona fide" auxiliary subunits, which exert several modulatory effects on AMPAR at the plasma membrane level (Jackson and Nicoll, 2011; Shanks et al., 2012). Therefore, to study the presence or absence of CPT1C at the cell surface, we visualized the location of the interaction between CPT1C and AMPAR subunits. With this aim, we co-transfected cells with CPT1C-GFP and GluA1 or GluA2 and performed immunofluorescence to differentially visualize surface AMPARs (red in **Figures 4A,B**) and the total pool of AMPARs subunits (blue in **Figures 4A,B**) by using confocal microscopy (for details, see methods).

Results in **Figures 4A,B** using cell lines indicate that CPT1C co-localize with both intracellular GluA1 and GluA2 (**Figures 4A,B**; light blue in left panels). Quantification of these co-localizations using Manders Overlap coefficient (MOC; see methods) is shown in **Figure 4D** (0.67 ± 0.14 for GluA1 and 0.59 ± 0.12 for GluA2; blue columns; $n = 10$). In fact, CPT1C expression seems to be restricted to areas close to the nucleus and with a reticular pattern (**Figures 4A,B**; green signal). Interestingly, there is no co-localization of CPT1C with surface receptors confirming that the interaction does not take place at the cell-surface (**Figures 4A,B**; right panels). **Figure 4D** (red columns) shows MOC for CPT1C and surface GluA1 (0.13 ± 0.09; $n = 10$) or surface GluA2 (0.11 ± 0.08; $n = 10$).

CPT1C has been described to co-localize with ER markers in transfected cell lines and in neurons (Sierra et al., 2008;

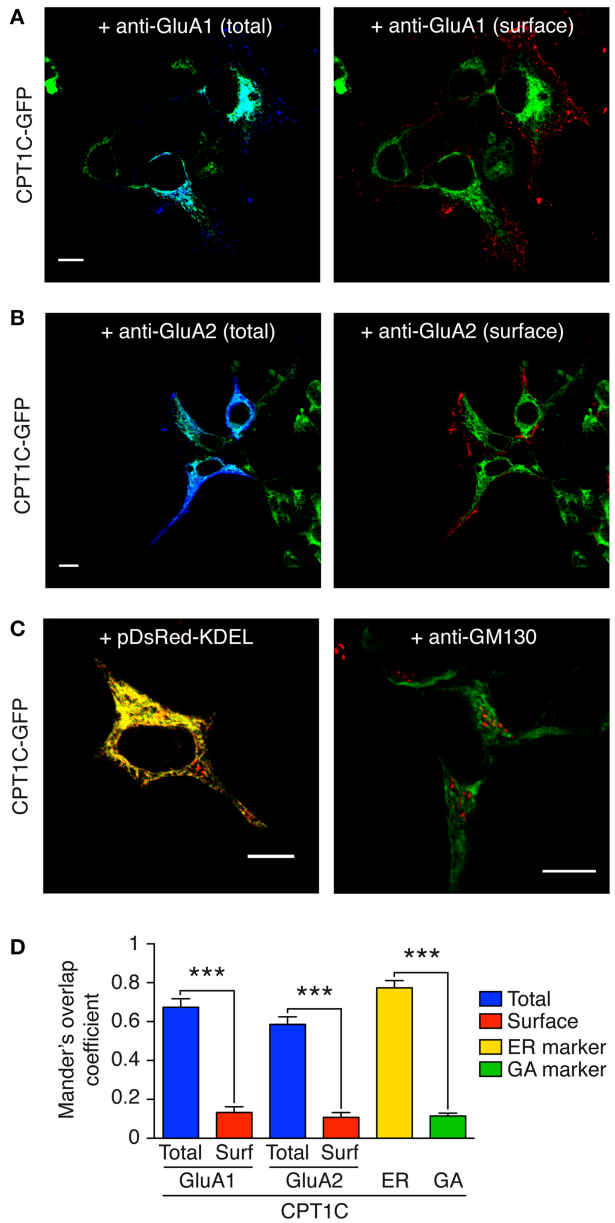

FIGURE 4 | GluA1 and GluA2 co-localize with CPT1C at intracellular compartments but not at the plasma membrane. CPT1C does not co-localize with the Golgi Apparatus marker GM-130. **(A)** Confocal image showing co-localization of CPT1C-GFP (green) with GluA1 (dark blue signal in left panel) in transfected tsA201 cells. Co-localization signal is displayed as light blue (left panel). There is no co-localization between CPT1C (green) and cell-surface GluA1 (red signal in right panel). Scale bar: 10 μm. **(B)** Confocal image showing co-localization of CPT1C (green) with GluA2 (dark blue signal in left panel) in transfected tsA201 cells. Co-localization signal is apparent as light blue color (left panel). There is no co-localization between CPT1C (green) and cell-surface GluA2 (red signal in right panel). Scale bar: 10 μm. **(C)** Co-localization (yellow signal in left panel) of CPT1C-GFP with ER marker (pDsRed-KDEL). Lack of co-localization (absence of yellow signal in right panel) of CPT1C (green) with the Golgi Apparatus marker GM-130 (red) in transfected tsA201 cells. Scale bars: 10 μm. **(D)** Representation of co-localization values quantified by Manders Overlap Coefficient (MOC; see Methods) expressed as mean ± SEM. MOC values for total GluAs and CPT1C (blue columns) were statistically different *(Continued)*

FIGURE 4 | Continued
from surface GluAs and CPT1C (red columns; $^{***}p < 0.001$ for both comparisons; Mann–Whitney *U*-test; $n = 10$ for all conditions). MOC values for CPT1C-ER marker (pDsRed-KDEL) show strong co-localization (yellow bar). MOC values for CPT1C-GM130 (GA marker; green bar) were similar to those of surface GluAs-CPT1C.

Carrasco et al., 2012). We confirmed this previous localization of CPT1C in the ER in our expression system (**Figure 4C**; left panel and **Figure 4D**; yellow column; MOC of 0.77 ± 0.04). Since GluA subunits dwell in the Golgi Apparatus during posttranscriptional modifications (Greger et al., 2002) we wondered if CPT1C and GluAs might interact at this level. However, we found that CPT1C shows poor co-localization with a Golgi Apparatus marker (GM-130) as shown in **Figure 4C** (right panel) and **Figure 4D** (green column; MOC of 0.11 ± 0.05; $n = 10$).

This data clearly demonstrates that GluA subunits are together with CPT1C at the ER level but not at the plasma membrane.

SURFACE EXPRESSION OF GluA1 IS INCREASED IN THE PRESENCE OF CPT1C

Our results showing that AMPAR gating at the plasma membrane level is not altered by CPT1C suggests that the increase in whole-cell currents might be explained by an increased number of receptors present at the cell surface.

To test this hypothesis we determined GluA1 surface expression using immunofluorescence quantification. We immunostained surface GluA1 in live transfected tsA201 cells (**Figures 5A,B**; red signal). We then permeabilized the cells and stained the total GluA1 pool (**Figures 5A,B**; blue signal). Given the variability in expression levels we calculated the ratio of the surface expression of GluA1 vs. the total expression level of GluA1 for the same cell.

As shown in **Figure 5C** the normalized ratio surface to total GluA1 was increased in cells co-expressing GluA1 and CPT1C-GFP (100 ± 5.53% for cells expressing GluA1 alone vs. 160.6 ± 6.24% for cells expressing GluA1+CPT1C; $p < 0.0001$, Mann–Whitney *U*-test; $n = 84$ and 90 cells respectively from 4 immunocytochemistry experiments for each condition). This result indicates a possible role of CPT1C in increasing GluA1 trafficking to the cell surface.

We wanted to extend these findings by studying CPT1C influence on surface expression of native AMPARs from neuronal cultures. Hence, we carried out immunofluorescence experiments in primary cortical neurons cultures at 10 DIV (**Figures 5D–F**). By using an equivalent methodology performed with tsA201 cells, we measured the GluA1 surface to total ratio in dendrites from GFP transfected neurons (**Figure 5D**) compared with CPT1C-GFP overexpressed neurons (**Figure 5E**). **Figures 5D2–D4, E2–E4** show examples of the analyzed dendrites. Neurons transfected with CPT1C increased the GluA1 surface to total ratio (100 ± 3.58 % for control GFP transfected neurons vs. 118.8 ± 5.65 % for CPT1C-GFP transfected neurons; $p = 0.0226$; $n = 70$ and 80 dendrites respectively from 3 different cultures each; **Figure 5F**).

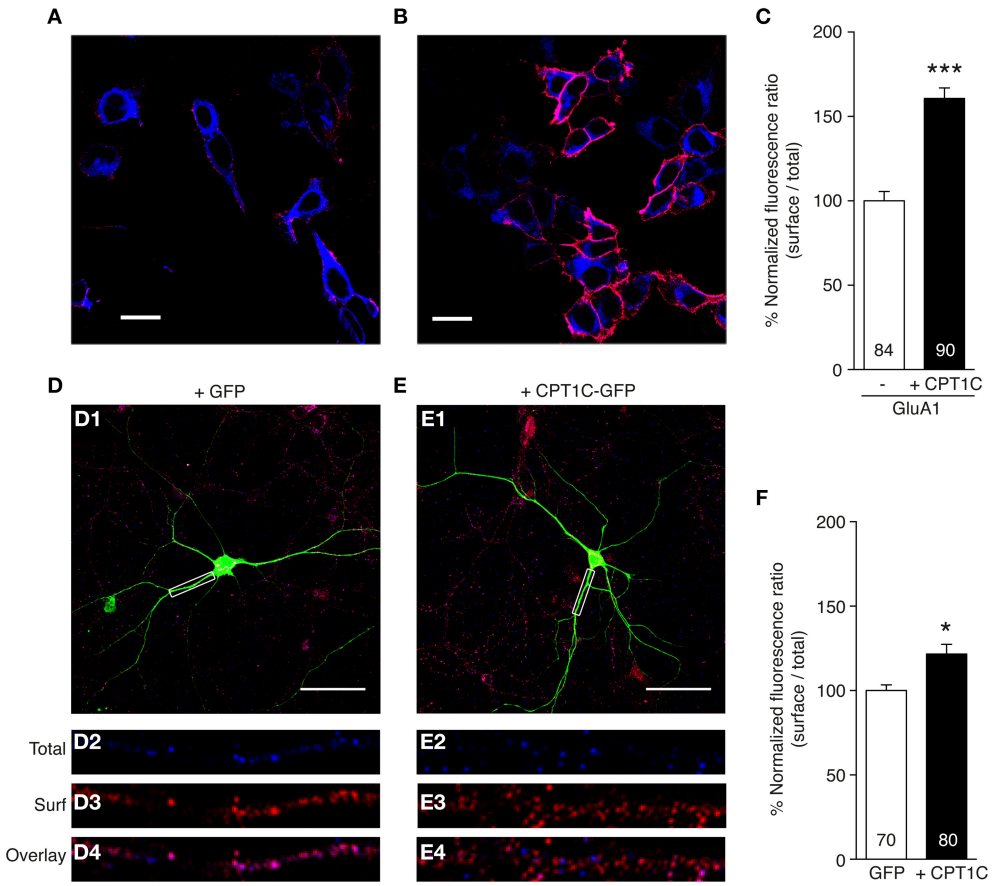

FIGURE 5 | CPT1C enhances surface expression of GluA1. (A,B) Representative single confocal images of tsA201 cells co-expressing GluA1 and GFP **(A)** or GluA1 and CPT1C-GFP **(B)**. Surface GluA1 was labeled in live cells with anti-GluA1-NT and Alexafluor 555 (red signal in the images). Subsequently cells were permeabilized and total GluA1 expression level was labeled with the same primary antibody but with Alexafluor 647 (blue signal in the images). Scale bars: 20 μm. **(C)** Quantification of the GluA1 surface to total ratio normalized to GluA1, expressed as a percentage. GluA1 surface expression was increased by co-expression with CPT1C (***$p < 0.001$; Mann–Whitney *U*-test). Data are means ± SEM. Numbers in bars denote the number of cells quantified from 4 different immunofluorescence experiments. **(D)** Example of a DIV 10 cortical neuron transfected with GFP. Surface GluA1 was labeled in fixed cells with anti-GluA1-NT and Alexafluor 555 (red signal in the images). Subsequently cells were permeabilized and total GluA1 expression level was labeled with the same primary antibody but with Alexafluor 647 (blue signal in the images). D1: field image showing the GFP in green, the surface GluA1 in red and the total GluA1 in blue. Scale bar: 50 μm. D2: magnification of the dendritic boxed area in D1 for total GluA1. D3: same as D2 but for surface GluA1. D4: overlay of intracellular and surface GluA1 for box in D1. **(E)** Example of a DIV 10 cortical neuron transfected with CPT1C-GFP in the same conditions as D. E1: field image showing the CPT1C-GFP in green, the surface GluA1 in red and the total GluA1 in blue. Scale bar: 50 μm. E2: magnification of the dendritic boxed area in E1 for total GluA1. E3: same as E2 but for surface GluA1. E4: overlay of total and surface GluA1 for box in E1. **(F)** Quantification of endogenous GluA1 surface to total ratio normalized to GFP transfected neurons, expressed as a percentage. GluA1 surface to total ratio was increased by overexpression of CPT1C-GFP (*$p = 0.0226$; Mann–Whitney *U*-test). Data represent means ± SEM. Numbers in bars denote the number of dendrites quantified from 3 different cultures.

These results show that CPT1C favors the trafficking of GluA1-containing AMPARs in both heterologous cells and neurons.

CPT1C ENHANCING EFFECT ON SURFACE EXPRESSION AND CURRENT DENSITY IS MEDIATED BY GluA1 C585

Our data suggest an effect of CPT1C on the trafficking of GluA1. This effect could be performed by a chaperone-like activity of CPT1C or by some posttranslational modification mediated by this protein directly on GluA1. It has been demonstrated that the posttranslational modification palmitoylation affects AMPAR trafficking (Hayashi et al., 2005; Lin et al., 2009; Yang et al., 2009). This modification consists in the reversible introduction of a lipid palmitate in some specific cysteine residues present in proteins. Given that it has been described that CPT1C can bind palmitoyl-CoA (Sierra et al., 2008) we addressed the question whether the observed increase in surface expression of GluA1 could be mediated by changes in the palmitoylation state of GluA1 due to CPT1C.

To check this possibility we first obtained two mutant forms of GluA1 that cannot be palmitoylated at previously described palmitoylable cysteine residues 585 and 811 (Hayashi et al., 2005) by changing the cysteine for a serine (C585S or C811S; see methods). We also tested the double mutant form GluA1(C585,811S).

Hence, we studied the effect of these mutations and their co-expression with CPT1C-GFP in cell lines firstly using the immunofluorescence quantification of surface receptors and also studying the current density.

Figures 6A–C show confocal images of surface GluA1 quantification experiments for GluA1 constructs alone (left panels) and together with CPT1C (right images). Quantification of 3 immunocytochemistry experiments for each condition is presented in **Figure 6D** and **Table 1**. In parallel we carried out whole-cell current density experiments as described in **Figure 2** to directly assess the effect of CPT1C on the glutamate-evoked currents of GluA1 mutants (**Figure 6E** and **Table 2**).

We replicated the immunofluorescence and electrophysiology results obtained in **Figures 2**, **5** in parallel with the mutant forms of GluA1. As previously found, CPT1C increased the surface/total ratio of GluA1 (100.0 ± 4.86% for GluA1 vs. 182.6 ± 10.25% for GluA1+CPT1C; $p < 0.001$; **Figure 6D** and **Table 1**) and the current density of homomeric GluA1 (85.86 ± 21.95 pA/pF for GluA1 vs. 155.5 ± 24.22 pA/pF for GluA1+CPT1C; $p = 0.0334$; **Figure 6E** and **Table 2**).

We observed that in the absence of CPT1C, GluA1(C585S) expression was enhanced at the cell surface by 1.97-fold compared to native GluA1 ($p < 0.001$; **Figure 6D** and **Table 1**) and that the glutamate-evoked current carried by GluA1(C585S) was

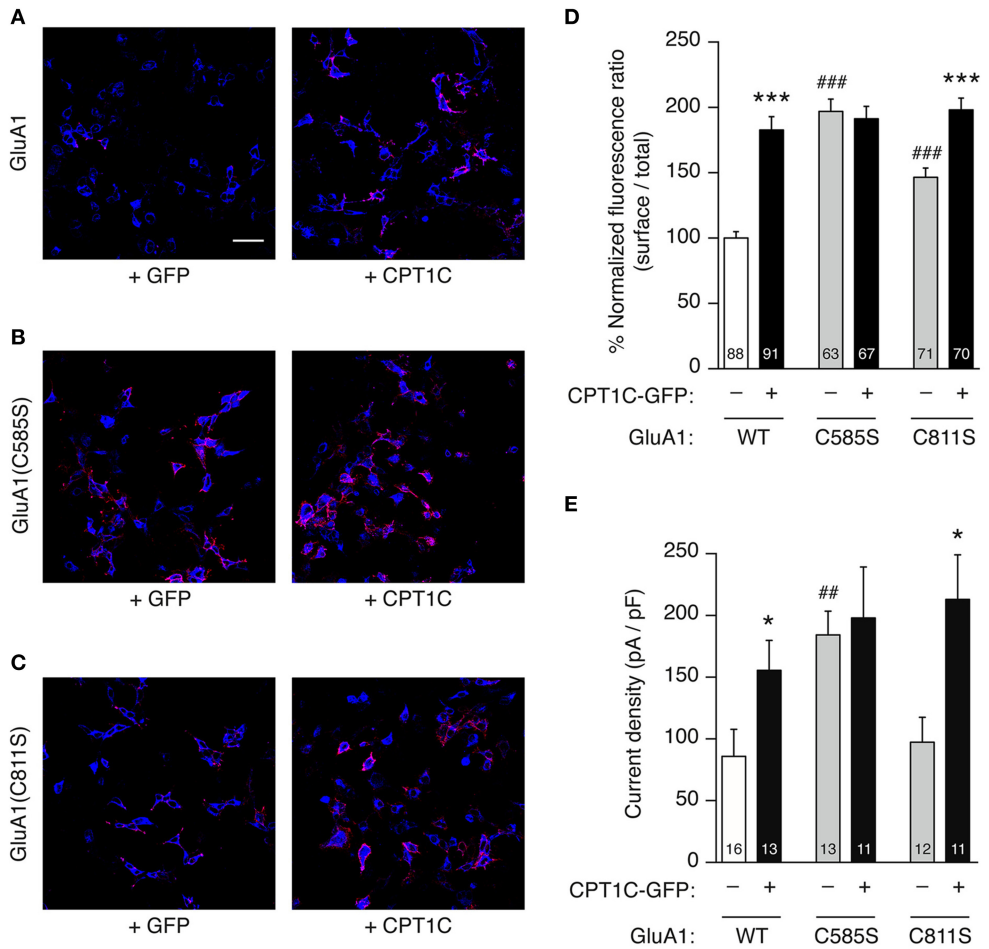

FIGURE 6 | GluA1 C585 is critical for the enhancement of current density and surface expression by CPT1C. (A–C). Representative single confocal images of tsA201 cells co-expressing different versions of GluA1 with (right panels) or without (left panels) CPT1C-GFP. In the images, surface GluA1 is shown in red and total GluA1 in blue. Scale bar: 50 μm. **(A)** Native GluA1 co-expressing GFP (+GFP) as the control condition, or CPT1C-GFP (+CPT1C). **(B)**. Same as **(A)** but for cells expressing GluA1 containing the point mutation C585S that abolishes palmitoylation at this residue. **(C)** Same as **(A,B)** but for GluA1 with the point mutation C811S. **(D)** Quantification of the GluA1 surface to total ratio normalized to GluA1, expressed as a percentage. GluA1 surface expression was increased by co-expression of CPT1C for both GluA1 and GluA1(C811S) (***$p < 0.001$; Mann–Whitney *U*-test) but not for GluA1(C585S). Non-palmitoylable forms of GluA1 increased surface expression of the receptors when compared to wild-type GluA1 (WT) (###$p < 0.001$; Mann–Whitney *U*-test). Numbers in bars denote the number of cells quantified from 3 different immunofluorescence experiments. **(E)** Averaged normalized currents at −80 mV for different versions of GluA1 alone or together with CPT1C. Current density (-pA/pF) was increased by co-expression of CPT1C with native GluA1 (WT) or mutant GluA1(C811S) (*$p < 0.05$; Mann–Whitney *U*-test) but not for GluA1(C585S). GluA1(C585S) increased the current density when compared to native GluA1 (##$p < 0.01$; Mann–Whitney *U*-test). Numbers in bars denote the number of recordings.

increased to the same degree (2.15-fold; $p = 0.0041$, **Figure 6E** and **Table 2**). These results are in keeping with previous findings (Hayashi et al., 2005). Interestingly, the effects of CPT1C co-expression and the GluA1(C585S) mutation were not additive.

Table 1 | Normalized fluorescence ratio (% of surface vs. total) values.

Receptor		Normalized fluorescence ratio (Surface/ Total) (%)	*n*	*p*-value (vs. no CPT1C)	*p*-value (vs. GluA1)
GluA1	–	100.0 ± 4.86	88		
	+CPT1C	182.6 ± 10.25	91	<0.001	
GluA1(C585S)	–	196.9 ± 9.41	63		<0.001
	+CPT1C	191.3 ± 9.47	67	0.4876	
GluA1(C811S)	–	146.5 ± 7.08	71		<0.001
	+CPT1C	198.2 ± 8.99	70	<0.001	
GluA1(C585,811S)	–	208.2 ± 11.55	35		< 0.001
	+CPT1C	208.1 ± 7.87	37	0.9014	

Transiently transfected tsA201 cells for different conditions were immunostained against surface and total GluA1 (see methods) and the ratio of GluA1 surface expression vs. GluA1 total expression was calculated and normalized to that of GluA1 control group. Three different immunofluorescence experiments were performed for each condition and the total number of analyzed cells is shown (n). Each p value is from a Mann–Whitney U-test comparing normalized fluorescence values of the distinct GluA1 receptors in the absence or presence of CPT1C ("vs. no CPT1C" column) or normalized fluorescence values of mutant versions of GluA1 against wild type GluA1 ("vs. GluA1" column).

Table 2 | Current density values for GluA1 mutants with or without CPT1C.

Receptor		Current density (pA/pF)	*n*	*p*-value (vs. no CPT1C)	*p*-value (vs. GluA1)
GluA1	–	85.86 ± 21.95	16		
	+CPT1C	155.5 ± 24.22	13	0.0334	
GluA1(C585S)	–	184.3 ± 19.19	13		0.0041
	+CPT1C	198.0 ± 41.25	11	0.7721	
GluA1(C811S)	–	97.32 ± 20.26	12		0.4166
	+CPT1C	213.0 ± 36.12	11	0.0210	
GluA1(C585,811S)	–	153.8 ± 14.58	10		0.0143
	+CPT1C	178.9 ± 34.89	10	0.8534	

Transiently transfected tsA201 cells for different conditions were whole-cell patch clamped and voltage ramps from −80 to +80 mV were applied (see methods for details). The maximum current at −80 mV was then normalized against the cell capacitance to obtain the current density values (-pA/pF). The total number of recorded cells is shown (n). Each p value is from a Mann–Whitney U-test comparing current density values for each GluA1 receptor in the absence or presence of CPT1C ("vs. no CPT1C" column) or current density values of mutant versions of GluA1 against wild type GluA1 ("vs. GluA1" column).

Specifically, CPT1C did not further increase the surface expression of GluA1(C585S) ($p = 0.4876$, **Figure 6D** and **Table 1**) or the current density of GluA1(C585S) ($p = 0.7721$, **Figure 5E** and **Table 2**). Likewise CPT1C did not vary the high surface expression or further enhance current density of the double mutant GluA1(C585,811S) ($p = 0.9014$ and $p = 0.8534$, **Tables 1**, **2**). This suggests that C585 might be crucial in the CPT1C effect on trafficking of GluA1.

In addition, GluA1(C811S) surface expression was also enhanced compared to GluA1 ($p < 0.0001$; **Figure 6D** and **Table 1**). Nevertheless, although significantly different from GluA1, GluA1(C811S) seems to be less efficiently expressed at the membrane surface compared the other GluA1 mutants ($p < 0.001$ for GluA1(C811S) vs. GluA1(C585S) or GluA1(C585,811S); Mann–Whitney *U*-test for both comparisons) and clearly GluA1(C811S) was not able to increase current density compared with GluA1 ($p = 0.4166$, **Figure 6E** and **Table 2**). However CPT1C co-expression does have an effect on both GluA1(C811S) surface expression (146.5 ± 7.08 % for GluA1(C811S) vs. 198.2 ± 8.99 % for GluA1(C811S)+CPT1C; $p < 0.001$; **Figure 6D** and **Table 1**) and current density (97.32 ± 20.26 pA/pF for GluA1(C811S) vs. 213.0 ± 36.12 pA/pF for GluA1(C811S)+CPT1C; $p = 0.0210$; **Figure 6E** and **Table 2**).

Finally, the effect of CPT1C on GluA1(C811S) is equivalent to the effect of the C585S mutation alone in terms of both, surface expression (**Figure 6D**; $p = 0.9623$) and current density (**Figure 6E**; $p = 0.6430$). These results suggest that the enhancing effect of CPT1C on surface expression and current density is mediated by a modification of cysteine 585 of GluA1 subunits.

PALMITOYLATION STATE OF GluA1 IS UNAFFECTED BY CPT1C OVEREXPRESSION

It has been previously demonstrated that palmitoylation of GluA1 at the C585 residue retains AMPARs in the Golgi Apparatus (Hayashi et al., 2005) implying that depalmitoylated GluA1 at C585 traffics more efficiently to the plasma membrane. Our data corroborate these findings since the number of receptors at the surface in the mutant C585S (where cysteine 585 cannot be palmitoylated) is increased. Interestingly, the surface expression of GluA1(C585S) is approximately the same as GluA1(C811S) expressed with CPT1C (where CPT1C effect can only be on the intact cysteine 585). This result seems to point to CPT1C being a potential depalmitoylating enzyme of GluA1.

To test this hypothesis we analyzed the palmitoylation level of GluA1 when expressed in the absence or presence of CPT1C. We performed the Acyl Biotin Exchange assay as described in Brigidi and Bamji (2013) (see methods). This assay allows the replacement of a pre-existing palmitate bound to cysteines of a given protein with a biotin group. The biotin is subsequently detected with streptavidin to give a read-out of palmitoylation levels. Therefore we transfected tsA201 cells with GluA1 alone or GluA1 plus CPT1C-GFP. Palmitoylation levels of GluA1 in the absence of CPT1C were equivalent to GluA1 palmitoylation levels in the presence of CPT1C (**Figure 7A**; upper panel, second and forth lane and **Figure 7B**). Immunoprecipitated GluA1 was quantified to normalize palmitoylation levels (**Figure 7A**; lower panel).

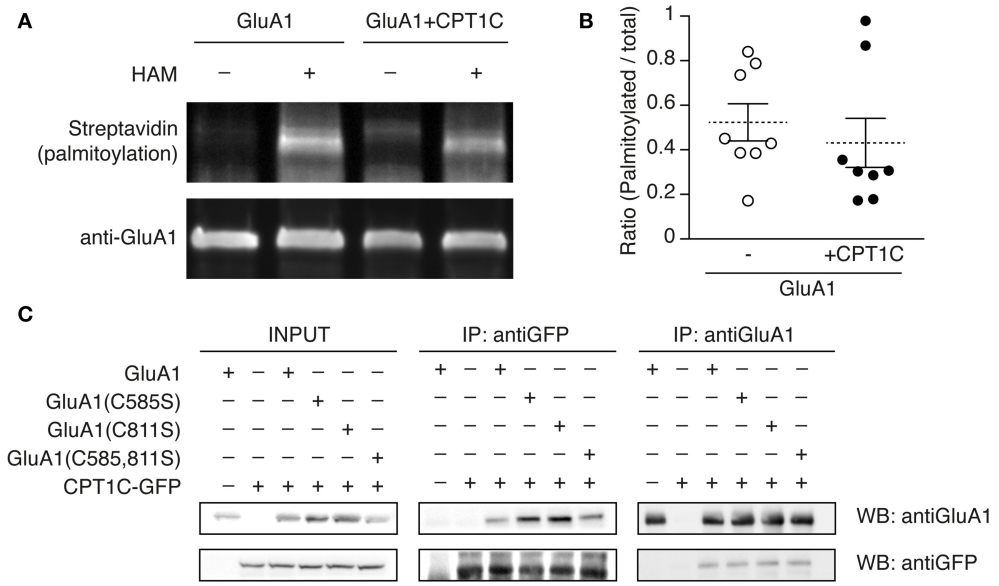

FIGURE 7 | GluA1 palmitoylation state is not altered by CPT1C and does not affect the interaction with GluA1. (A) Palmitoylation levels of GluA1 alone (GluA1) and together with CPT1C-GFP (GluA1+CPT1C), in transfected tsA201 cells, detected by means of Acyl-Biotin Exchange (ABE). The thiol-biotinylated immunoprecipitates of GluA1 following the ABE assay for both transfected conditions were subjected to SDS-PAGE. Palmitoylation of GluA1 can only be detected in plus-hydroxilamine (+HAM) samples. Minus -HAM samples control non-specific incorporation of biotin. GluA1 palmitoylation levels (top) were detected by Western blotting with streptavidin-HRP (palmitoylation). After stripping the membranes the total amount of immunoprecipitated GluA1 was detected by Western blotting with anti-GluA1-NT antibody (anti-GluA1, bottom). **(B)** Quantification of palmitoylation levels for GluA1 alone (open circles) or GluA1 plus CPT1C (filled circles) in tsA201 cells. Ratio of palmitoylated GluA1 to total GluA1 for each single experiment is shown together with mean (discontinuous horizontal lines) and SEM (continuous vertical lines) ($p > 0.05$; Mann–Whitney *U*-test; $n = 8$ for both). **(C)** Co-IP of the membranous fraction of tsA201 cells co-expressing GluA1 wild type or non-palmitoylable mutants—GluA1(C585S), GluA1(C811S), and GluA1(C585,811S)—together with CPT1C-GFP. The interaction between CPT1C and GluA1 is not dependent on palmitoylation of C585 or C811 residues. As negative controls GluA1 was co-expressed with an empty plasmid expressing GFP alone (first lanes of the boxes) and CPT1C-GFP was co-expressed with an empty pDsRed (second lanes from the boxes). Transfected cells were lysed and membranes were solubilized as described in **Figure 1** and methods. An input sample collected prior to immunoprecipitation of these extracts is shown as "INPUT." Inputs and immunoprecipitated samples were separated and Western Blotted as described in **Figure 1**. Immunoprecipitations were replicated three times.

Figure 7B display the single experiments ratio values of palmitoylated GluA1 vs. total GluA1 for both conditions where it can be observed there is no significant difference (0.523 ± 0.084 for GluA1 vs. 0.431 ± 0.11 for GluA1+CPT1C; $p = 0.3228$; $n = 8$ for both).

Thus, by using this methodology we could not confirm the role of CPT1C as a depalmitoylating enzyme.

Given that C585 seems critical to favor AMPARs trafficking but its palmitoylation state is not changed by CPT1C we wondered if the lack of palmitate group could perhaps interfere with the physical interaction between GluA1 and CPT1C. In order to check if CPT1C ability to interact with GluA1 was eliminated when residue 585 was non-palmitoylated, we did co-IP assays with the GluA1(C585S) and CPT1C. As shown in **Figure 7C**, GluA1 and CPT1C retained the ability to interact even when cysteines 585, 811 or both were mutated to non-palmitoylable forms. This result indicates that the binding of both proteins depends on other domains/residues and that palmitoylable C585 does not determine this interaction despite its importance in CPT1C effect on trafficking properties of AMPARs.

DISCUSSION

In this study we describe a novel function of CPT1C in regulating AMPAR surface expression in both heterologous cells and neurons. In tsA201 cells CPT1C increases whole-cell glutamate-evoked currents of homomeric GluA1 and heteromeric GluA1/GluA2 AMPARs. Moreover, CPT1C overexpression enhances the number of endogenous AMPARs trafficked to the dendritic surface in rat cortical neurons. This trafficking effect is specific to the brain isoform CPT1C since the canonical CPT1 isoform also expressed in neurons (CPT1A) is not able to increase GluA1 mediated currents. Additionally, CPT1C modulation seems to be subunit specific since GluA2 homomeric AMPARs are unaffected by CPT1C co-expression. Despite GluA1 and CPT1C coimmunoprecipitating, both proteins do not co-localize at the plasma membrane level and no further biophysical modulation of AMPARs by CPT1C exists. Finally, the palmitoylable cysteine 585 of the GluA1 subunit seems to be crucial for the CPT1C alteration of AMPARs trafficking properties although no changes in the palmitoylation state of the receptor seem to occur.

ROLE OF CPT1C IN AMPARs TRAFFICKING

In our experiments, both current density and cell surface expression are increased when CPT1C is co-expressed with GluA1. Despite this, GluA2 homomeric AMPARs seem not to be regulated by CPT1C, while GluA2-containing AMPARs, which are the most abundant form of AMPARs in neurons, are also sensitive to CPT1C. This points to a significant role of CPT1C in the delivery of GluA1 to the membrane in neurons. Indeed, the important role of CPT1C in synaptic transmission is evident since CPT1C knock-out mice have spatial learning problems, motor impairment and hypoactivity (Carrasco et al., 2012, 2013). Alterations in AMPAR-mediated signaling might underlie these phenotypes since in immature spines AMPAR content is low compared with mature synapses (Petralia et al., 1999). In fact, CPT1C KO animals show poor dendritic spine maturation in hippocampal neurons (Carrasco et al., 2012). In agreement with low AMPAR content at synapse level, recent unpublished electrophysiological data by our group has proved that synaptic transmission is altered in CPT1C KO animals (*submitted manuscript*). In these animals the lack of CPT1C translates to less efficient synaptic trafficking since AMPAR-mediated mEPSCs in pyramidal hippocampal neurons are diminished. All these findings reveal an important relationship between CPT1C and AMPARs. Our data and the functional evidence from the KO studies together with the ability of CPT1C to interact with AMPARs (**Figure 1** and Schwenk et al., 2012) makes CPT1C a suitable candidate to be a regulating partner of AMPARs and an important protein for the correct function of AMPARs.

SUBUNIT SPECIFICITY OF CPT1C MODULATION

Our coimmunoprecipitation experiments showed direct interaction of CPT1C with both GluA1 and GluA2 subunits. Nevertheless the observed effects of CPT1C on whole-cell currents and surface expression were specific for GluA1-containing AMPARs since the results were not replicated in cells expressing GluA2 homomeric AMPARs. GluA1 and GluA2 subunits have distinct features in their structure including the Q/R site and the intracellular C-terminal domain, which translate to important functional differences (Traynelis et al., 2010). Remarkably, the important C585 palmitoylable residue in GluA1 (C610 in GluA2) for CPT1C effect is located +3 aminoacids from the crucial Q/R site. Another significant difference is the short C-terminal domain of GluA2. In fact, variations in the C-tails and the Q/R site between both isoforms determine different trafficking properties of GluA1 and GluA2 (Greger et al., 2002; Henley and Wilkinson, 2013). Therefore, it could be possible that a differential modulation by CPT1C was dependent on the C-tail length, the specific C-terminal aminoacid composition or on Q/R site editing state of AMPARs. The majority of AMPARs in the CNS are heteromeric combinations (Lu et al., 2009; Traynelis et al., 2010) and trafficking properties are determined by the dominant effect of long forms of AMPARs (Henley and Wilkinson, 2013). Our results are in line with this dominant effect of long forms. However, it remains to be studied whether other features of GluAs account for the subunit selectivity. Future experiments with other AMPAR forms might unravel the subunit features accounting for the specific modulation of CPT1C. Moreover, it would be interesting to investigate whether CPT1C protein similarly modulates AMPARs together with auxiliary subunits.

It is noteworthy that the effect of CPT1C on GluA2-containing heteromeric AMPARs and native AMPARs—mostly heteromeric combinations containing GluA2—is less pronounced than the one observed for GluA1 homomeric AMPARs. Perhaps the 2-fold enhancement of GluA1 density at the cell membrane could be partially occluded in GluA2-containing receptors due to generally better trafficking properties of heteromeric combinations. Indeed, the current density values we obtained for heteromeric receptors were higher than for homomeric receptors (either GluA1 or GluA2). This reflects the fact that heteromeric combinations of AMPARs are favored at the expense of homomeric receptors when both subunits are present during the synthesis process at the ER (Cull-Candy et al., 2006). The enhanced trafficking of heteromeric combinations might translate into a less evident CPT1C influence on GluA2-containing receptors. Alternatively, stoichiometry might be an important determinant in CPT1C effect. This possibility could be studied in the future.

CPT1C IS NOT A GENUINE AUXILIARY SUBUNIT OF AMPARs

From our results, it looks like that this new AMPAR interactor has a putative role in the delivery of AMPAR subunits to the cell surface. It has been described that many other AMPAR interacting proteins control AMPAR trafficking (Palmer et al., 2005; Anggono and Huganir, 2012; Lu and Roche, 2012). This is the case for auxiliary AMPAR subunits such as the TARPs that affect the channel properties of AMPARs while also playing an important role in surface trafficking (Nicoll et al., 2006). CNIH proteins also increase AMPARs surface expression (Schwenk et al., 2009) and modify the behavior of AMPARs both in expression systems and in neurons (Kato et al., 2010b; Coombs et al., 2012). Conversely this is not the case for CPT1C, as we have shown that this protein does not alter GluA1 channel properties. This fact is supported by the confocal imaging experiments where we could not see any co-localization between CPT1C and surface GluA1. Consequently CPT1C cannot be considered a TARP-like "bona fide" auxiliary subunit and it seems that its role is restricted to controlling AMPARs trafficking.

CPT1C AND AMPARs INTERACT AT THE ER LEVEL

Even though CPT1C does not associate with AMPAR subunits at the plasma membrane level, it is clear that both proteins interact at some stage of the AMPAR synthesis pathway (Schwenk et al., 2012 and **Figures 1**, **4**). The fact that CPT1C shows a clear ER pattern (**Figures 4C–D**; Sierra et al., 2008; Carrasco et al., 2012) makes this organelle a meeting point for both proteins where CPT1C could posttranslationally modify AMPARs accounting for the increased traffic to plasma membrane. Further, our co-localization studies also demonstrate that CPT1C does not seem to interact with GluA1 outside of the ER at all, as CPT1C does not co-localize with the Golgi Apparatus marker GM-130. Thus, our results suggest that the effect of the interaction of CPT1C/GluA1 might take place exclusively at the ER level. They also point out that the complex CPT1C-AMPAR would dissociate at some stage

before AMPARs subunits move forward to the Golgi during their biosynthesis.

MECHANISMS UNDERLYING CPT1C MODULATION OF AMPARs: ROLE OF CYSTEINE 585 OF GluA1

AMPAR subunits are subject to several posttranslational modifications during biosynthesis that affect the trafficking of the receptors to the cell surface. This is the case for the reversible palmitoylation of AMPARs. All four AMPA receptor subunits are palmitoylated at two conserved sites, (C585 and C811 in GluA1) and palmitoylation/depalmitoylation of these two residues determine AMPARs trafficking properties (Hayashi et al., 2005; Lin et al., 2009; Yang et al., 2009). Given that CPT1s have palmitoylCoA as a substrate it seemed plausible to consider whether CPT1C was involved in a modification such as protein palmitoylation/depalmitoylation, thus potentially affecting AMPARs surface expression.

When studying the role of cysteine residues in CPT1C effect, we found that C585S mutation alone increased whole-cell currents and surface expression of GluA1 by 2-fold. These results are in accordance with previous ones demonstrating that depalmitoylation of AMPARs at C585 acts as a triggering signal for receptor forward trafficking (Hayashi et al., 2005). Interestingly, in the presence of CPT1C, the GluA1(C585S) no longer increased receptor trafficking. This points toward a crucial role of C585 residue for the CPT1C effect. This is confirmed by the fact that the GluA1(C811S) mutant is modulated by CPT1C to the same degree as GluA1 ruling out the involvement of C811 residue. Moreover the fact that CPT1C increases GluA1(C811S) trafficking to the same extent as GluA1(C585S) alone or with CPT1C suggests that the effect of CPT1C is dependent on the C585 residue.

Finally, our findings show that GluA1(C811S) increases surface expression when detected by immunofluorescence but current density is not increased in the same extent. This might be explained due to a different number of cells analyzed with each technique. Despite that discrepancy, the significant increment in both parameters when CPT1C is together with GluA1 (C811S) indicates that GluA1 C811 is not crucial for the CPT1C effect.

CPT1C DOES NOT ALTER GluA1 PALMITOYLATION STATE

Given that CPT1C produces an increase in GluA1 surface expression to the same extent as the non-palmitoylable form of GluA1(C585S), we hypothesized that the effect of CPT1C on GluA1 subunits could be via depalmitoylation of C585. However, the palmitoylation state of GluA1 seems to be unaffected by co-expression with CPT1C, at least when detecting palmitoylation levels with the ABE assay. A possible issue with this methodology might be that the ABE assay not only detects palmitoylation but also other S-acylation modifications of GluA1, which have not yet been described. Therefore other techniques might be necessary to detect the palmitoylation levels of GluA1 C585 unambiguously. Alternatively, a necessary depalmitoylation process performed in the ER by CPT1C could be counteracted by additional palmitoylation of the receptor at other cell locations, thus making it difficult to detect changes in the palmitoylation state. Therefore, this hypothesis should be closely examined with future specific refinements of palmitoylation assays.

HOW CAN CPT1C MODULATE AMPARs TRAFFICKING?

Though it appears that CPT1C does not depalmitoylate GluA1, the involvement of cysteine 585 is clear. It is possible therefore that the role of C585 is not related to the palmitoylation capacity of the amino acid. Perhaps the physical interaction of CPT1C with GluA1 masks the palmitate on this residue (or produces a conformational change in GluA1) facilitating its exit from the ER and the forward movement toward the Golgi. If that was the case, the masking would not be due to a direct interaction of CPT1C with the palmitate of C585 from GluA1, as both proteins still interact in the absence of this palmitate group. Otherwise, it may be possible that CPT1C acts as a chaperone during the synthesis of GluA1 and this chaperone effect could be related to the ER palmitoylation of C585. It has been described for other proteins (for instance the yeast polytopic membrane protein chitin synthase—Chs3) that the parallel action of palmitoyl acyl thioesterases and chaperones is necessary to achieve the correct folding and export from the ER (Lam et al., 2006) suggesting a relation between palmitoylation state and chaperone activity.

To gain insight into CPT1C modulation of AMPAR it would be important to elucidate the domains participating in the interaction. The topology of CPT1A shows that N- and C-terminal domains face the cytoplasm (Fraser et al., 1997). Presumably CPT1C displays the same topology thus restricting the interaction with AMPAR subunits to their C-terminal tail or transmembrane domains.

CPT1C AND DISEASE

Even though CPT1C expression is restricted to the brain in healthy individuals, it functions as a stress-responsive gene under a variety of conditions, as its mRNA is up-regulated in cell lines from several different tissues as well as in mice following exposure to any one of a number of p53-activating stresses (Reilly and Mak, 2012). Therefore, it is suggested that CPT1C promotes cancer cell survival and tumor growth and it has been proposed as a new therapeutic target in cancer treatment. It is noteworthy that recent studies report an aberrant expression of CPT1C in gliomas (Cirillo et al., 2014; Wakamiya et al., 2014). These findings highlight the importance of unraveling the molecular mechanisms of CPT1C, which could be of great interest across a range of fields, for example in the study of new anticancer therapies and new diagnostic or prognostic markers.

AUTHOR CONTRIBUTIONS

Esther Gratacòs-Batlle and David Soto designed the work; Esther Gratacòs-Batlle, Natalia Yefimenko, Helena Cascos-García and David Soto performed the research and analyzed the data; Esther Gratacòs-Batlle and David Soto interpreted the data and wrote the paper.

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REVIEW
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SYNGAP1: Mind the Gap

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A cardinal feature of early stages of human brain development centers on the sensory, cognitive, and emotional experiences that shape neuronal-circuit formation and refinement. Consequently, alterations in these processes account for many psychiatric and neurodevelopmental disorders. Neurodevelopment disorders affect 3–4% of the world population. The impact of these disorders presents a major challenge to clinicians, geneticists, and neuroscientists. Mutations that cause neurodevelopmental disorders are commonly found in genes encoding proteins that regulate synaptic function. Investigation of the underlying mechanisms using gain or loss of function approaches has revealed alterations in dendritic spine structure, function, and plasticity, consequently modulating the neuronal circuit formation and thereby raising the possibility of neurodevelopmental disorders resulting from synaptopathies. One such gene, *SYNGAP1* (Synaptic Ras-GTPase-activating protein) has been shown to cause Intellectual Disability (ID) with comorbid Autism Spectrum Disorder (ASD) and epilepsy in children. SYNGAP1 is a negative regulator of Ras, Rap and of AMPA receptor trafficking to the postsynaptic membrane, thereby regulating not only synaptic plasticity, but also neuronal homeostasis. Recent studies on the neurophysiology of SYNGAP1, using *Syngap1* mouse models, have provided deeper insights into how downstream signaling proteins and synaptic plasticity are regulated by SYNGAP1. This knowledge has led to a better understanding of the function of SYNGAP1 and suggests a potential target during critical period of development when the brain is more susceptible to therapeutic intervention.

Keywords: SYNGAP, synaptic plasticity, intellectual disability, autism spectrum disorders, learning and memory, neurodevelopmental disorders

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INTRODUCTION

The brain is the center of the nervous system and is the most complex organ in the body. All day-to-day activities including executive decisions, memories, emotions, and cognitive tasks are mediated by the cerebral activity. Apart from coordinating the ability to smell, touch, hear, taste, and see, the brain enables people to form words, perform mathematical calculations, communicate using different languages, grasp and appreciate music, make decisions, organize and plan everyday activities and above all, imagine. Therefore, normal development of brain is imperative for performing these and other essential functions. A cardinal feature of early stages of human brain development centers on the sensory, cognitive, and emotional experiences that shape neuronal-circuit formation and refinement. Consequently, alteration in any of these features accounts for many psychiatric and neurological disorders (Spooren et al., 2012; Kroon et al., 2013).

The human brain consists of 86 billion neurons and 85 billion non-neuronal cells (Azevedo et al., 2009), which play a vital role in information processing and transmission in the form of electrical signals through specialized junctions called synapses. Neuroscientists have made great progress in unraveling the cellular and molecular mechanisms of dendritic spine synapse formation and function, which is considered as one of the most remarkable developments in biology in the last three decades. A precise control of synaptic development and neuronal connectivity has been found to be necessary for normal brain development. Conversely, abnormal dendritic spine morphology and function can lead to disruption of neuronal circuits, and consequently can result in various psychiatric and neurodevelopmental disorders (NDDs; Melom and Littleton, 2011).

Altered dendritic spine function and neuronal circuit formation account for one of the major underlying mechanisms of Intellectual Disability (ID) and Autism Spectrum Disorder (ASD; Chechlacz and Gleeson, 2003; Kroon et al., 2013), which are often co-diagnosed in young children with NDDs and affect 1–3% of the general population. Due to high rates of co-morbidity of these NDDs, it has been broadly hypothesized that ID and ASD share common neurodevelopmental pathologies that lead to various behavioral and cognitive symptoms that define these disorders. The underlying cause of these NDDs are believed to be mutations in genes, parental drug use and aging process, viral infections and other environmental factors (van Spronsen and Hoogenraad, 2010).

Recent evidences from many animal models of ID and ASD suggest that mutations that cause NDDs occur in genes encoding the proteins that regulate synaptic function and/or structure (Boda et al., 2002; Bear et al., 2004; Ramocki and Zoghbi, 2008; Südhof, 2008; Gauthier et al., 2011; Penzes et al., 2011). Mutations in many of these single-genes are the major cause of syndromic and non-syndromic ID (NSID; Bhakar et al., 2012; Zoghbi and Bear, 2012). The most common single-gene mutations in ASD with ID are associated with Fragile X syndrome (FXS; *FMRI*), Tuberous Sclerosis (*TSC1*, *TSC2*), Angelman Syndrome (*UBE3A*), Rett Syndrome (*MECP2*), and Phlean-McDermid syndrome (*SHANK3*). Rare mutations in single-genes, such as those encoding for Neuroligin (*NLGN3, NLGN2*) and Neurexin (*NRXN1*), are also implicated in ID and ASD. These genes are just a few of many implicated in NDDs, suggesting that highly penetrant mutations of genes play an important role in regulating synaptic function. Heterozygous mutation in *SYNGAP1* cause ID and ASD, and whose product is now established as a major regulator of synaptic function.

Numerous studies have shown that a major share of dendritic spine synapses utilize the excitatory neurotransmitter, glutamate, to activate N-methyl D-aspartate receptors (NMDARs), which are associated with a vast array of transmembrane proteins, scaffolding proteins and many signaling proteins (Pèrez-Otaño and Ehlers, 2004; Lau and Zukin, 2007; Kerchner and Nicoll, 2008; Lai and Ip, 2013; Fan et al., 2014). SYNGAP1, is a downstream component of NMDAR-associated signaling complex that negatively regulates activation of small GTP-ase (Ras- and Rap-GAP) and of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking to excitatory postsynaptic membrane (Rumbaugh et al., 2006; Huang, 2009; Walkup et al., 2015). *SYNGAP1* is a ∼140 kDa protein located on Chromosome 6p21.3[1] (**Figure 1**; Husi et al., 2000). Phosphorylation of SYNGAP1 is regulated by CaMKII, which reduces SYNGAP1's control of Ras-GTPase, leading to Ras activation by increasing the GTP-bound form of Ras. It is now established as a major signaling protein that plays a pivotal role in regulating fundamental molecular changes in dendritic spine synaptic morphological and functional modifications. Moreover, mutations in SYNGAP1 are established as relevant for human pathology, because they have been associated with ID comorbid with ASD in children (Hamdan et al., 2009, 2011a,b, 2014; Gauthier et al., 2011; Berryer et al., 2013).

Until a few years ago, the neurophysiological mechanism that causes ID in patients with *SYNGAP1* mutation was not clear. Using mouse models of *Syngap1* heterozygous mutations (*Syngap1*$^{-/+}$), several labs have shown that 50% reduction of the level of SYNGAP1 is sufficient to cause significant increases in the presence of mushroom-shaped dendritic spines during early stages of development resulting from a lack of inhibition of Ras-GTPase, which in turn allows more AMPARs to be transported to the postsynaptic membrane. Moreover, it has been shown that in *Syngap1*$^{-/+}$ models certain critical periods of neuronal growth and maturation are disrupted, leading to developmental brain disorders, that in turn cause cognitive and social dysfunctions (Guo et al., 2009).

Syngap1 EXPRESSION, FUNCTIONAL DOMAINS, AND ISOFORMS

SYNGAP1 is a ∼140 kDa protein, first characterized by Chen et al. (1998) using a mouse model of *Syngap1*$^{-/+}$ mutation and followed by Kim et al. (1998) who developed a truncated form of SYNGAP1 using yeast two-hybrid system (See **Table 1** for Historical perspective). Based on these studies, it can be understood that the N-terminal half of SYNGAP1 has a Ras-GAP domain, along with the region that is loosely homologous to Pleckstrin homology and a C2 domain which is potentially involved in binding of $Ca^{2+}$and phospholipids. Interestingly, the alignment of GAP domain of SYNGAP1 with other Ras-GAPs suggests that the amino acids in GAP domain are vital for interaction with Ras and for the stimulation of Ras-GTPase activity (Chen et al., 1998; Kim et al., 1998). Given the Ras-GAP-interacting domain of a newly discovered protein and its presence in excitatory synapses (Chen et al., 1998; Kim et al., 1998), this protein was termed SYNGAP1 (Synaptic Ras-GAP activator protein). Moreover, studies have shown that SYNGAP1 is expressed only in brain tissue and not in other tissues (Chen et al., 1998; Kim et al., 1998). In the brain, it is primarily expressed in the excitatory neurons, where it is localized to synapses. On the contrary, SYNGAP1 was absent

[1]http://www.ncbi.nlm.nih.gov/gene/8831

SYGP1_HUMAN	MSRSRASIHRGSIPAMSYAPFRDVRGPSMHRTQYVHSPYDRPGWNPRFCIISGNQLLMLD	60
SYGP1_RAT	MSRSRASIHRGSIPAMSYAPFRDVRGPPMHRTQYVHSPYDRPGWNPRFCIISGNQLLMLD	
SYGP1_MOUSE	MSRSRASIHRGSIPAMSYAPFRDVRGPPMHRTQYVHSPYDRPGWNPRFCIISGNQLLMLD	
SYGP1_HUMAN	EDEIHPLLIRDRRSESSRNKLLRRTVSVPVEGRPHGEHEYHLGRSRRKSVPGGKQYSMEG	120
SYGP1_RAT	EDEIHPLLIRDRRSESSRNKLLRRTVSVPVEGRPHGEHEYHLGRSRRKSVPGGKQYSMEA	
SYGP1_MOUSE	EDEIHPLLIRDRRSESSRNKLLRRTVSVPVEGRPHGEHEYHLGRSRRKSVPGGKQYSMEA	
SYGP1_HUMAN	ISTALRNPNIQRQPSRQSERPRPQPVVLRGPSAEMQGYMMRDLNSSIDLQSFMARGLNSS	780
SYGP1_RAT	ISTALRNPNIQRQPSRQSERARSQPMVLRGPSAEMQGYMMRDLNSSIDLQSFMARGLNSS	
SYGP1_MOUSE	ISTALRNPNIQRQPSRQSERTRSQPMVLRGPSAEMQGYMMRDLNSSIDLQSFMARGLNSS	
SYGP1_HUMAN	AAGMRLSQMGVTTDGVPAQQLRIPLSFQNPLFHMAADGPGPPGGHGGGGGHGPPSSHHHH	960
SYGP1_RAT	AAGMRLSQMGVTTDGVPAQQLRIPLSFQNPLFHMAADGPGPPAGHGGSSGHGPPSSHHHH	
SYGP1_MOUSE	AAGMRLSQMGVTTDGVPAQQLRIPLSFQNPLFHMAADGPGPPAGHGGSSGHGPPSSHHHH	
SYGP1_HUMAN	HHHHHHRGGEPPGDTFAPFHGYSKSEDLSSGVPKPPAASILHSHSYSDEFGPSGTDFTRR	1020
SYGP1_RAT	HHHHHHRGGEPPGDTFAPFHGYSKSEDLSTGVPKPPAASILHSHSYSDEFGPSGTDFTRR	
SYGP1_MOUSE	HHHHHHRGGEPPGDTFAPFHGYSKSEDLSSGVPKPPAASILHSHSYSDEFGPSGTDFTRR	
SYGP1_HUMAN	QLSLQDNLQHMLSPPQITIGPQRPAPSGPGGGSGGGSGGGGGGQPPPLQRGKSQQLTVSA	1080
SYGP1_RAT	QLSLQDNLQHMLSPPQITIGPQRPAPSGPGGGSGGGSGGGGGGQPPPLQRGKSQQLTVSA	
SYGP1_MOUSE	QLSLQDSLQHMLSPPQITIGPQRPAPSGPGGGSGGGS---GGGQPPPLQRGKSQQLTVSA	1077
SYGP1_HUMAN	EIHSLKERLHMSNRKLEEYERRLLSQEEQTSKILMQYQARLEQSEKRLRQQQAEKDSQIK	1260
SYGP1_RAT	EIHSLKERLHMSNRKLEEYERRLLSQEEQTSKILMQYQARLEQSEKRLRQQQVEKDSQIK	
SYGP1_MOUSE	EIHSLKERLHMSNRKLEEYERRLLSQEEQTSKILMQYQARLEQSEKRLRQQQVEKDSQIK	1257
SYGP1_HUMAN	SIIGRLMLVEEELRRDHPAMAEPLPEPKKRLLDAQERQLPPLGPTNPRVTLAPPWNGLAP	1320
SYGP1_RAT	SIIGRLMLVEEELRRDHPAMAEPLPEPKKRLLDAQRGSFPPWVQQTRV----------	1308
SYGP1_MOUSE	SIIGRLMLVEEELRRDHPAMAEPLPEPKKRLLDAQERQLPPLGPTNPRVTLAPPWNGLAP	1317

FIGURE 1 | Amino acid sequence of human SYNGAP1 and its difference with mouse SYNGAP1. Amino acid sequence differences of SYNGAP1 between *Homo* sapiens (SYGP1_Human), *Rattus rattus* (SYGP1_RAT) and *Mus musculus* (SYGP1_MOUSE). Variations in the sequences were indicated in red colored fonts. Dashed line indicates empty sequences.

in inhibitory neurons. Chen et al. (1998) have shown that the carboxyl terminal tail of SYNGAP1 interacts with postsynaptic density protein (PSD-95), as confirmed by their coprecipitation (Kim et al., 1998). In addition, Kim et al. (1998) have shown that the C-terminal half consists of a repeat of 10 histidines that may be involved in metal chelation, several potential serine and tyrosine phosphorylation sites and a T/SXV motif necessary for interaction with SAP102 and PSD-95. This study further suggested that SYNGAP1 is a cytosolic protein without a signal peptide or any transmembrane domain (Kim et al., 1998).

Studies have shown that functionally distinct proteins may be produced via regulated alternate splicing of mRNA (Lipscombe, 2005; Li et al., 2007; Grabowski, 2011; Raj and Blencowe, 2015). It is evident that *Syngap1* is a complex gene that gives rise to multiple protein domains. This further implies that *Syngap1* may be spliced differentially, which can lead to different isoforms. Indeed, Chen et al. (1998) showed that two splice variants were observed, one at the amino terminus and one at carboxyl terminus, which further encode four variants with molecular weights of 134, 137, 140 and 143 KDa. The amino acid terminal contains a putative Pleckstrin homology (PH) domain (Chen et al., 1998), which may attach the protein to the membrane (Lemmon and Ferguson, 2000). In support of this finding, a recent study has identified distinct isoforms of SYNGAP1 (**Figure 2**), differing in their N- and C-terminals (McMahon et al., 2012). The existence of different isoforms was further confirmed when anti-SYNGAP1 antibody recognized doublet or triplet of proteins at 130 kDa only in the brain, with no detection of SYNGAP1 and its isoforms in any other parts of the body such as kidney, heart or lung (Chen et al., 1998; Kim et al., 2003). Each isoform contains a central GAP domain to regulate the activity of GTPase in small GTPases such as Ras and Rap. Three distinct *Syngap1* isoforms, SYNGAP A, B and C, differing in their N-termini arising from different promoter regions have been identified (McMahon et al., 2012). A and B isoforms contain unique peptide sequence and a complete PH domain, whereas isoform C is a shorter, truncated protein with no unique peptide sequence and no PH domain. Furthermore, SYNGAP A, B, and C isoforms

TABLE 1 | Historical perspective of major findings of *SYNGAP1*.

Observation/findings of SYNGAP1	Referrences	Model/samples
First *Syngap1* Het mouse model	Chen et al. (1998)	Mouse
Binds to CaMKII /PSD95	Chen et al. (1998)	Mouse
	Kim et al. (1998)	Yeast two-hybrid hippocampal cDNA library
Amino acid sequences and molecular weight	Chen et al. (1998)	Mouse
	Kim et al. (1998)	Yeast two-hybrid hippocampal cDNA library
	Husi et al. (2000)	Mouse
	McMahon et al. (2012)	Mouse
Domain structure of SYNGAP1 and Isoforms	Chen et al. (1998)	Mouse
	McMahon et al. (2012)	Mouse
Localized in excitatory neurons	Chen et al. (1998)	Mouse
	Kim et al. (1998)	
	Kim et al. (1998)	Yeast two-hybrid hippocampal cDNA library
Syngap1 Homozygous mice die within a week	Komiyama et al. (2002)	Mouse/Primary neuronal culture
	Kim et al. (2003)	Mouse/cortical culture
Synaptic transmission and LTP	Komiyama et al. (2002)	Mouse/Primary neuronal culture
	Kim et al. (2003)	Mouse/cortical culture
Learning and Memory deficits	Komiyama et al. (2002)	Mouse/Primary neuronal culture
Altered ERK, Ras, Rac p-Cofilin	Komiyama et al. (2002)	Mouse/Primary neuronal culture (ERK)
	Carlisle et al. (2008)	Mouse/Hippocampi neuronal culture
Dendritic spine structure	Vazquez et al. (2004)	Mouse/primary neuronal culture
	Carlisle et al. (2008)	Mouse/Hippocampi neuronal culture
Cognitive and social dysfunction	Guo et al. (2009)	Mouse
	Muhia et al. (2010)	Mouse
Intellectual disability in children	Hamdan et al. (2009)	Human
Prematuration of dendritic spines	Clement et al. (2012)	Mouse
	Aceti et al. (2015)	Mouse (*in vivo*)
Altered critical period of plasticity	Clement et al. (2013)	Mouse

This table summarizes the major findings/observations of function of SYNGAP1 by various groups.

can be subdivided based on transcription start sites (A1–A11; B1; C1–C8). To determine whether the multiple promoters were also present in humans, a sequence comparison with mouse and rat revealed a highly conserved regions with no predicted functional moieties (McMahon et al., 2012). Finally, alternate splicing of *Syngap1* mRNA leads to multiple isoforms of C-termini, designated as α, β and γ. Of these, the most studied C-terminus isoform is SYGNAP1 α1, which contains the PDZ-binding domain and mediates binding to scaffolding proteins of PSD.

The expression of various genes that encode proteins regulating synaptic formation and function are shown to reach their peak level of expression during early stages of development. Moreover, several of these proteins have recently been implicated in ID (State and Levitt, 2011; State and Sestan, 2012). Indeed, expression of SYNGAP1 in neurons reaches its peak 14 days after birth, i.e., at Postnatal day 14 (PND 14 days) in rodents (Kim et al., 1998; Clement et al., 2012), but steadily decreases to adult level by 2 months of age (Porter et al., 2005). Further, SYNGAP1 is highly expressed in hippocampus and cortex, and less in striatum (Komiyama et al., 2002). The expression of SYNGAP1 was reduced by 50% in *Syngap1*$^{-/+}$ mice (Komiyama et al., 2002; Kim et al., 2003; Clement et al., 2012). However, no abnormal gross development of brain was observed in *Syngap1*$^{-/+}$ mice (Kim et al., 2003).

It is well known that development and synaptic activity plays a major role in differential splicing of genes involved in synaptic function. The N-termini SYNGAP1 isoforms, A and B had a pattern of regulation during development (gradual increase in expression till PND14), while isoform C was expressed at very low level till PND14 (McMahon et al., 2012). However, all three isoforms, A, B, and C reached their peak expression at PND14. Further, it is shown that Syngap B and C were up-regulated two-fold after 4 h of bicuculline treatment, whereas SYNGAP A was down-regulated (McMahon et al., 2012). These changes were inhibited in the presence of tetrodotoxin. This confirms that differential splicing of *Syngap1* occurs based on neuronal activity, which leads to opposing functional effects of *Syngap1* isoforms; SYNGAP A had silencing effect, while SYNGAP B and C had positive effect on synaptic transmission and strength. Unlike N-termini isoforms, the protein levels of C-termini isoforms did not change when stimulated with bicuculline. Importantly, it is not clear from previous studies which combination of N- and C- termini isoforms exists in neurons. Using isoform-specific antibodies, it is clear that both C-terminal isoforms exist in hippocampus and cortex (McMahon et al., 2012; Yang et al., 2013) and that α2-containing isoforms localize to PSDs, although they do not contain a QTRV region to bind to PDZ domain. This could be because, when non-phosphorylated CaMKII is inactive at rest, both isoforms are localized within PSD core and α1 binding to PSD-95 serves a distinct function, that of blocking the other portions from binding to PSD-95. Furthermore, upon activation of NMDARs, both α1 and α2 isoforms move out of the PSD core, but this change was reversed within 30–45 min following the NMDAR activation.

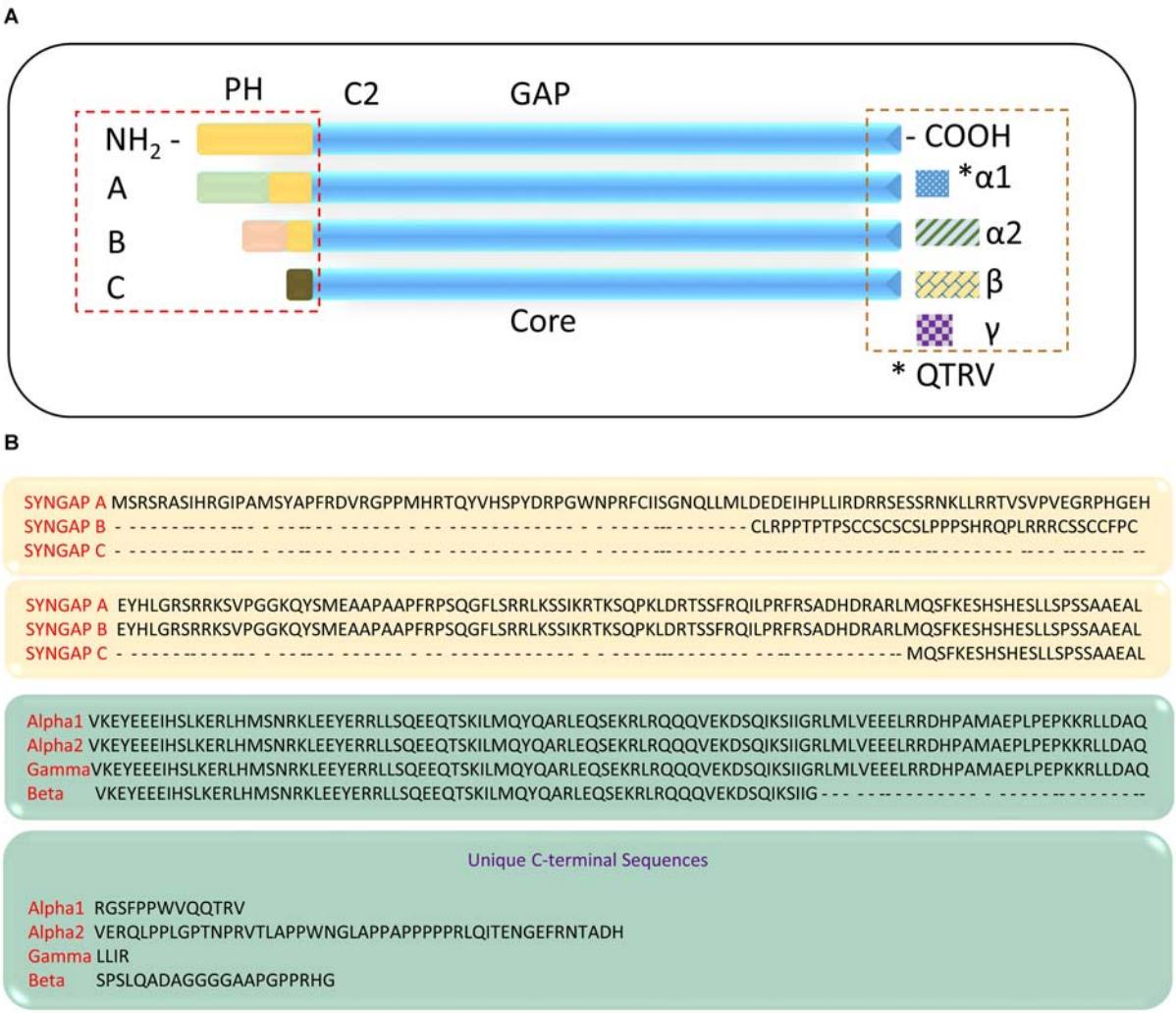

FIGURE 2 | SYNGAP1 isoforms arising from alternate splicing. (A) A schematic illustration of potential SYNGAP1 isoforms, which vary in both N- and C-termini. **(B)** Amino acid sequences that are unique to different N- and C-termini isoforms identified by mass spectrometry (McMahon et al., 2012).

This movement of SYNGAP1 α1 and α2 out of PSD core could have two significant effects. The first major effect could be a displacement of GAP activity and thus induces activity-dependent synaptic modification (Yang et al., 2013; Araki et al., 2015). The second major effect of SYNGAP1 α1 and α2 isoforms moving out of the PSD core is to create an empty slot for the association of an AMPAR (Yang et al., 2013), thereby regulating synaptic strength. In fact, overexpression of SYNGAP1 α1 isoform reduces AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), whereas overexpression of SYNGAP1 α2 isoform enhances mEPSCs. Nevertheless, these studies illustrate the fact that different isoforms exert opposing effects on synaptic strength and that both full peptide sequences, N- and C-terminal, and their isoforms must be considered when examining the functional properties of the protein.

SYNGAP1 MUTATIONS IN INTELLECTUAL DISABILITY

ID, formerly characterized as Mental Retardation, is defined by three criteria: (a) an intelligent quotation of less than 70; (b) limitations in two or more adaptive behaviors such as communication, self-care and social skills, community use, self-direction, health, and safety; and (c) evidence that the mental manifestations began before the age of 18 (van Bokhoven, 2011). ID comprises a diverse collection of syndromic and non-syndromic disorders. Unlike NSID, which is characterized by intellectual deficits as the only clinical feature, syndromic ID patients typically exhibit other abnormal clinical features, such as cranial, facial, and skeletal dysmorphisms. Major causes of ID and ASD stem from mutations of genes encoding for proteins that are critical regulators of synaptic function. *De novo* mutations in individual genes explain an important aspect of

NSID characterized by the absence of associated morphologic, radiologic and metabolic features, as opposed to ID associated with more complex chromosomal aberrations (Ropers and Hamel, 2005). The genetic factors involved in NSID are not clearly known. So far, 29 X-linked and five autosomal recessive genes have been identified by linkage and cytogenetic analysis (Bienvenu and Chelly, 2006; Chelly et al., 2006; Gécz et al., 2009). In addition, mutations in autosomal dominant genes are still to be identified in NDDs, as ID and ASD result in lower reproductive probability, which further reduces the chances of identifying families that are open to linkage analysis. Yet, *de novo* mutations are the most commonly recognized cause of ID, which suggests that monoallelic lesions are sufficient to cause this disorder. In fact, studies have shown that one to three *de novo* mutations per zygote affect amino acid sequences (Crow, 2000a,b, 2006).

In the past decade, novel autosomal *de novo* mutations were identified in genes encoding for proteins involved in synaptic plasticity. In relation to that, recent studies from children have shown that *de novo* truncating mutations in *SYNGAP1* cause NSID (Hamdan et al., 2009, 2011a,b, 2014; Berryer et al., 2013). Hamdan et al. (2009) identified protein-truncating *de novo* mutations in *SYNGAP1* in 3 of 94 patients with NSID. These patients ranged from 4–11 years and had similar clinical features as described in the Mullen Scale of Early Learning and the Vineland Adaptive Behavior Scales (Hamdan et al., 2009). Of those three patients, two patients were heterozygous for nonsense mutations, while the third patient was heterozygous for a frame-shift mutation starting at codon 813, producing a premature stop codon at 835. All these children were born to non-consanguineous parents. During early stages of development, these children presented with hypotonia and global delay of development with the onset of walking at 2-years. Apart from these defects, two of these children had presented with tonic-clonic and myoclonic seizures.

In order to further explore the association of *SYNGAP1* to ID, particularly to understand whether patients with epilepsy and ASD had *SYNGAP*1 *de novo* mutations, Hamdan et al. (2011a) sequenced exons of *SYNGAP1* from additional cohorts of patients. *De novo* out-of-frame deletions were identified in two patients with NSID presented with microcephaly and generalized epilepsy. The authors also described a *de novo* splicing mutation in a patient with autism that had not acquired microcephaly or epilepsy (Refer to **Table 2**). Moreover, missense mutations in *SYNGAP1* were detected in three patients. Surprisingly, all these patients were born to non-consanguineous parents. Furthermore, other studies (Krepischi et al., 2010; Zollino et al., 2011; Writzl and Knegt, 2013) have identified *de novo* microdeletions in chromosome region 6p21.3 in patients with ID, epilepsy and severe language impairment. Therefore, it is evident from the studies that heterozygous mutations in *SYNGAP1* are the major cause of NSID.

SYNGAP1 IN SCHIZOPHRENIA

Although several studies have shown the role of *Syngap1* mutations in neurodevelopmental disorders, a little is known about its relevance to schizophrenia. Converging evidence suggest that dysfunction of NMDARs and the signaling complex associated with them is now considered to be one of the major causes of schizophrenia (Belsham, 2001). Hypofunction of NMDARs was first implicated when reduced concentration of glutamate were found in the cerebrospinal fluid of patients with schizophrenia (Kim et al., 1980). The alteration in the function of activation-ready NMDAR complexes localized in the PSD can lead to a defect in downstream signaling pathways. It has been shown that a major function of SYNGAP1 is to transduce the activation of synaptic NMDA receptors to a biochemical signal that is necessary for proper neuronal function. Therefore, SYNGAP1 and its interacting proteins may be abnormal in patients with schizophrenia.

A study by Funk et al. (2009) has shown that SYNGAP1 and its interacting proteins, such as PSD95, were reduced in patients with schizophrenia. Interestingly, patients who were non-medicated for 6 weeks prior to the time of their death showed decreased levels of SYNGAP1 compared to medicated patients. A similar observation was made for SYNGAP1-interacting proteins such as PSD-95. This study hypothesized that *SYNGAP1*$^{-/+}$ are associated with schizophrenia-like behavioral phenotypes. Indeed, reduced expression of *SYNGAP1* results in abnormal behaviors that are strikingly similar to that reported in mice with reduced NMDAR function (Guo et al., 2009). This suggests that proteins downstream of NMDAR, including SYNGAP1, participate in a common pathway that may be dysfunctional in people with schizophrenia. However, other studies (Hamdan et al., 2009) did not find any *de novo* mutations, splicing or truncating, in their patients with schizophrenia. As the sample number of patients with schizophrenia studied in their work is low, more samples are needed to confirm the role of SYNGAP1 Het in schizophrenia.

MOUSE MODELS OF *Syngap1*

The recent advances in genomic science and the development of transgenic technology in mice have advanced research into the effect of monoallelic mutations in genes that are associated with synaptic transmission and neuronal circuit formation. *SYNGAP1* is highly conserved across species (McMahon et al., 2012), which has allowed for the development of different animal models of *Syngap1*$^{-/+}$ mice (Komiyama et al., 2002; Kim et al., 2003; Vazquez et al., 2004; Muhia et al., 2010). The *SYNGAP1* mouse ortholog, *Syngap1*, is located on chromosome 17[2]. Interestingly, *Syngap1*$^{-/-}$ mutant mice do not survive for more than a week (Kim et al., 2003). This is due to increased levels of caspase-3 activation in *Syngap1*$^{-/-}$, which suggests that apoptosis is enhanced by reduction of SYGNAP1 (Knuesel et al., 2005). The different animal models of *Syngap1*$^{-/+}$ are extensively discussed in a recent review (Ogden et al., 2015).

[2]http://www.ncbi.nlm.nih.gov/gene/240057

TABLE 2 | Clinical features observed in patients with *SYNGAP1* heterozygous mutation.

Patient no	Gene	DNM	Age	Sex	ID	Epilepsy	Cranial MRI/CT
1	SYNGAP1	p.Lys138X	4 yrs. 5 months	F	++/+++	GN	Normal/ND
2	SYNGAP1	p.Arg579X	5 yrs. 10 months	F	++/+++	GN	Normal/ND
3	SYNGAP1	p.Leu813ArgfsX23	12 yrs. 10 months	F	++/+++	–	ND/Normal
4	STXBP1	p.Arg388X	15 yrs.	F	+++	PC	Normal/Normal
5	STXBP1	c.169–1G>A	27 yrs.	F	+++	PC	ND/Normal
6	SHANK3	c.601–1G>A	15 yrs.	M	+	–	ND/Normal
7	KIF1A	p.Thr99Met	3 yrs. 5 months	F	+++	–	Atrophy/ND
8	GRIN1	p.Glu662Lys	10 yrs.	F	++	–	ND/Normal
9	GRIN1	p.Ser560dup	7 yrs. 6 months	M	+++	PC	Normal/ND
10	EPB41L1	p.Pro854Ser	6 yrs.	M	+++	–	Normal/ND
11	CACNG2	p.Val143Leu	8 yrs.	M	++	–	Normal/ND
12	KIFC1, PHF1, CUTA, SYNGAP1	–	6 yrs.	M	++	–	Normal/ND
13	SYNGAP1, CUTA, PHF1	–	5 yrs.	F	+++	GN	Normal/ND
14	SYNGAP1, CUTA, PHF1	–	9 yrs.	M	++	PC	Normal/ND

Summary of clinical features observed in patients with SYNGAP1 heterozygous mutation. ID Scale: + denotes mild, ++ moderate, +++ severe. Abbreviations used ND, not determined; PC, Partial complex epilepsy; GN, Generalized epilepsy. These features are based on different sources (Hamdan et al., 2009, 2011a,b, 2014; Krepischi et al., 2010; Zollino et al., 2011; Berryer et al., 2013; Writzl and Knegt, 2013).

Due to a rapid increase in the availability of the types of genetically modified mice (Branchi et al., 2003), it is critical to meticulously characterize their biochemical, pathological and behavioral features and compare them with human phenotypes (Bailey et al., 2006; Crawley, 2008). Generally, laboratories involved in testing the phenotypes of genetically modified mice subject them to a battery of behavioral features to assess cognitive, motor, and sensory functions. In addition, to consider a genetically modified mouse as a disease model, the transgenic animal must fulfil at least two levels of validity to judge its psychopharmacology (van der Staay et al., 2009). An animal model should score high on the following validities: *face validity*, i.e., resemblances of behavioral phenotypes of mouse model to that of human disorder; *construct validity*, i.e., closely reconstructs and mimics the underlying cause of the disease or disorder; and *predictive validity*, i.e., drug treatments alleviate symptoms in mouse and human. A mouse model should fulfil at least face and construct validity. Indeed, various mouse models of *Syngap1*$^{-/+}$ mice satisfied face validity (Komiyama et al., 2002; Kim et al., 2003; Guo et al., 2009; Muhia et al., 2010). These various *Syngap1*$^{-/+}$ mouse models recapitulated many of the phenotypes observed in humans. For example, Komiyama et al. (2002) were the first to observe learning and memory deficits in Syngap1$^{-/+}$ mice. Using a different model described by Kim et al. (2003), *Syngap1*$^{-/+}$ mice displayed altered social/conspecific interaction, abnormal spatial working memory, decreased anxiety-related response, hyperactivity, impaired cued conditioning behavior, increased startle reflex, increased horizontal stereotypic behavior and reduced prepulse inhibition, as well as learning deficits (Guo et al., 2009). Later, using another genetic model of *Syngap1*$^{-/+}$ mice (Muhia et al., 2010), Muhia et al. observed cognitive dysfunctions similar to the Guo et al. (2009) study. As mentioned previously, epilepsy is a prominent clinical feature observed in *SYNGAP1* patients. Accordingly, *Syngap1*$^{-/+}$ mice are prone to audiogenic seizures and have reduced seizure threshold and altered electroencephalogram (EEG; Clement et al., 2012; Ozkan et al., 2014). Therefore, based on these studies, it is clear that mouse models of *Syngap1*$^{-/+}$ (Ogden et al., 2015) mutation phenocopy the deficits observed in *SYNGAP1* patients, thereby allowing a better understanding of *SYNGAP1*$^{-/+}$ mutation in neuronal function and its consequence in ID.

ROLE OF *Syngap1* IN NEUROLOGICAL PATHWAY

For nearly two decades, neuroscientists have studied SYNGAP1-related signaling pathways. Synapses are extremely ordered structures that facilitate the transmission of information from presynaptic terminal to the postsynaptic membrane and, subsequently, activate signal transduction cascades that lead to suitable cellular events. In the postsynaptic membrane, two major ionotropic glutamate receptor subtypes are present—NMDARs and AMPARs. NMDARs are glutamate-sensitive ion channels that open up when glutamate and its co-agonist are bound to them. However, the actual permeation of ions through NMDAR channels occurs after the removal of Mg^{2+} block achieved by depolarization of the postsynaptic membrane. This depolarization is induced by glutamate binding to AMPARs. Subsequently to activation of NMDARs in the postsynaptic membrane, Ca^{2+} enters the dendritic spine, triggering activation of kinase cascades and thereby mediating various synaptic functions (Fan et al., 2014). NMDARs are an integral component of the PSD and bind to several PSD-enriched scaffold and signaling molecules, resulting in creation of a vast protein complex (Niethammer et al., 1996; Kennedy, 1997; Xu, 2011). This NMDAR-PSD protein complex is believed to play a vital role in the precise tuning of synapses in response to changing input stimuli pattern (Grant and O'dell, 2001; Yashiro and Philpot, 2008). SYNGAP1, one of the most abundant proteins in the PSD, is associated with NMDAR protein complex (**Figure 3**), which was first shown by Chen et al. (1998) and followed by Kim et al. (1998). Establishing SYNGAP1's role in the

NMDAR-mediated protein complex and signaling cascade is important to further our understanding of the etiology of SYNGAP1-mediated ID and ASD (**Figure 3**). SYNGAP1 has been shown to co-immunoprecipitate with PSD-95 protein complex from deoxycholate-solubilized mouse brain membrane preparations (Chen et al., 1998; Kim et al., 1998).

The GAP domain of SYNGAP1 is homologous to that of p120GAP and neurofibromin, two canonical Ras-GAPs that do not regulate Rap (Chen et al., 1998; Kim et al., 1998). However, SYNGAP1 has been shown to regulate Rap-GTPase more potently than Ras-GTPase (Pena et al., 2008). A recent study by Walkup et al. (2015) has shown that recombinant SYNGAP1 lacking 102 residues at the N-terminus is phosphorylated by cyclin-dependent kinase 5 (CDK5), as well as by CaMKII. Interestingly, phosphorylation of SYNGAP1 by CDK5 and CaMKII increases overall SYNGAP1 activity, but also alters the ratio of its GAP activity towards Ras- and Rap-GTPases. Phosphorylation of SYNGAP1 by CaMKII increases its Ras-GAP activity by 25% and its Rap-GAP activity by 76%. CDK5 increases recombinant SYNGAP1 activity on Ras-GAP by 98% and its Rap-GAP activity by 20%. Furthermore, upon NMDAR stimulation, Ca^{2+} entering the synapse dissociates CaMKII from SYNGAP1 and phosphorylates SYNGAP1 (pSYNGAP1). This leads to activation of Ras that activates proteins downstream, consequently to AMPAR insertion in the postsynaptic membrane. Therefore, the phosphorylation of SYNGAP1 is believed to be important in regulating transient changes in the number of surface AMPA receptors or gradually adjusting their steady-state level.

By biochemical analysis of proteins containing the GAP, Krapivinsky et al. (2004) have identified the C2 domain as essential for the Rap-GAP activity of SYNGAP1, which is in line with the recent observation (Walkup et al., 2015). The homology of SYNGAP1 with other Ras-GAP domains across species and *in vivo* associations of SYNGAP1 with the NMDA receptor complex indicate that SYNGAP1 plays a role in Ras-mediated signaling in excitatory synapses, particularly in response to Ca^{2+} (Kim et al., 1998; Carlisle et al., 2008). Apart from regulating Ras-mediated signaling, SYNGAP1 has been shown to mediate the activity of other major signaling proteins, such as Rac, p-Cofilin, p21-activated kinase (PAK) and LIMK (Carlisle et al., 2008) and these proteins were shown to be elevated at basal conditions in *Syngap1*$^{-/+}$ mice.

ROLE OF *SYNGAP1* IN REGULATING DENDRITIC SPINE MORPHOLOGY AND PLASTICITY

The neuronal signaling cascades underlying synaptic plasticity and dendritic spine structure have been intensely studied and a multitude of signaling molecules have been identified (Kennedy et al., 2005; Patterson and Yasuda, 2011). Initial stages of dendritic spine formation and neuronal connections depend on cytoskeleton protein, F-actin, which is regulated by Ras- and Rac-GTPases. Therefore, it is possible that the dendritic spine structure and function would be altered in *Syngap1*$^{-/+}$ mutations, which may explain the behavioral deficits observed in *SYNGAP1*$^{-/+}$ patients.

Ras- and Rac-mediated signaling cascade, including ERK and MAPK, has also been shown to play a major role in normal synaptic transmission and in long-term potentiation (LTP; Carlisle and Kennedy, 2005; Kennedy et al., 2005) by modulating insertion of AMPARs into the postsynaptic membrane (**Figure 3**). On the contrary, opposite effects were observed in *Syngap1* knockout in neuronal cultures using small interfering RNA. Studies from neuronal culture have demonstrated that overexpression of *Syngap1* resulted in a remarkable down regulation of AMPAR-mediated currents (Rumbaugh et al., 2006). In contrast, AMPAR-mediated currents were increased in *Syngap1*$^{-/+}$ in neuronal cultures treated with small interfering RNA.

Under basal conditions, when fEPSPs, which are predominantly mediated by AMPARs, were measured from adult mouse CA1 hippocampal pyramidal region, *Syngap1*$^{-/+}$ mice did not show any abnormal excitatory synaptic responses (Komiyama et al., 2002). Furthermore, the presynaptic fibers were required to evoke an equivalent postsynaptic response in slices from wild type (WT), and *Syngap1*$^{-/+}$ mouse responses were not altered suggesting that the activity of postsynaptic AMPARs were unchanged. However, these experiments were performed in adults and the genes encoding the proteins that regulate synaptic function reach their peak level of expression during early stages of development (State and Levitt, 2011; State and Sestan, 2012). Indeed, *Syngap1* Het mutations can affect synaptic transmission during early developmental period. Thus, Vazquez et al. (2004) have reported that the *Syngap1*$^{-/+}$ mice form dendritic spines and synapses prematurely, and that spines ultimately become larger in *Syngap1*$^{-/+}$. In addition, Clement et al. (2012, 2013) have shown elevated input-output relationship in extracellular field recording and AMPAR-mediated mEPSCs, which reached WT adult level 2 weeks after birth (PND14), confirming the earlier findings (Vazquez et al., 2004). These studies suggest that SYNGAP1 controls the trajectory of synapse maturation during a particular period of development by controlling protein synthesis and homeostatic synaptic plasticity during development (Wang et al., 2013).

In hippocampal pyramidal neurons, spine structure is tightly correlated with synaptic function (Noguchi et al., 2011). *Syngap1*$^{-/+}$ mutation disrupts proper development of dendritic spine structures. *Syngap1*$^{-/+}$ mice have more mature, mushroom-shaped spines during early stages of development (PND14) suggesting precarious prematuration of dendritic excitatory spine structures (Clement et al., 2012, 2013). Further, accelerated spine formation and premature spine pruning have been observed in developing neocortical tissue of *Syngap1*$^{-/+}$ mice (Aceti et al., 2015). These abnormalities observed during development in *Syngap1*$^{-/+}$ mutation persisted into adulthood (Vazquez et al., 2004; Carlisle et al., 2008; Clement et al., 2012), consistent with the spine dysfunction theory of cognitive disorders.

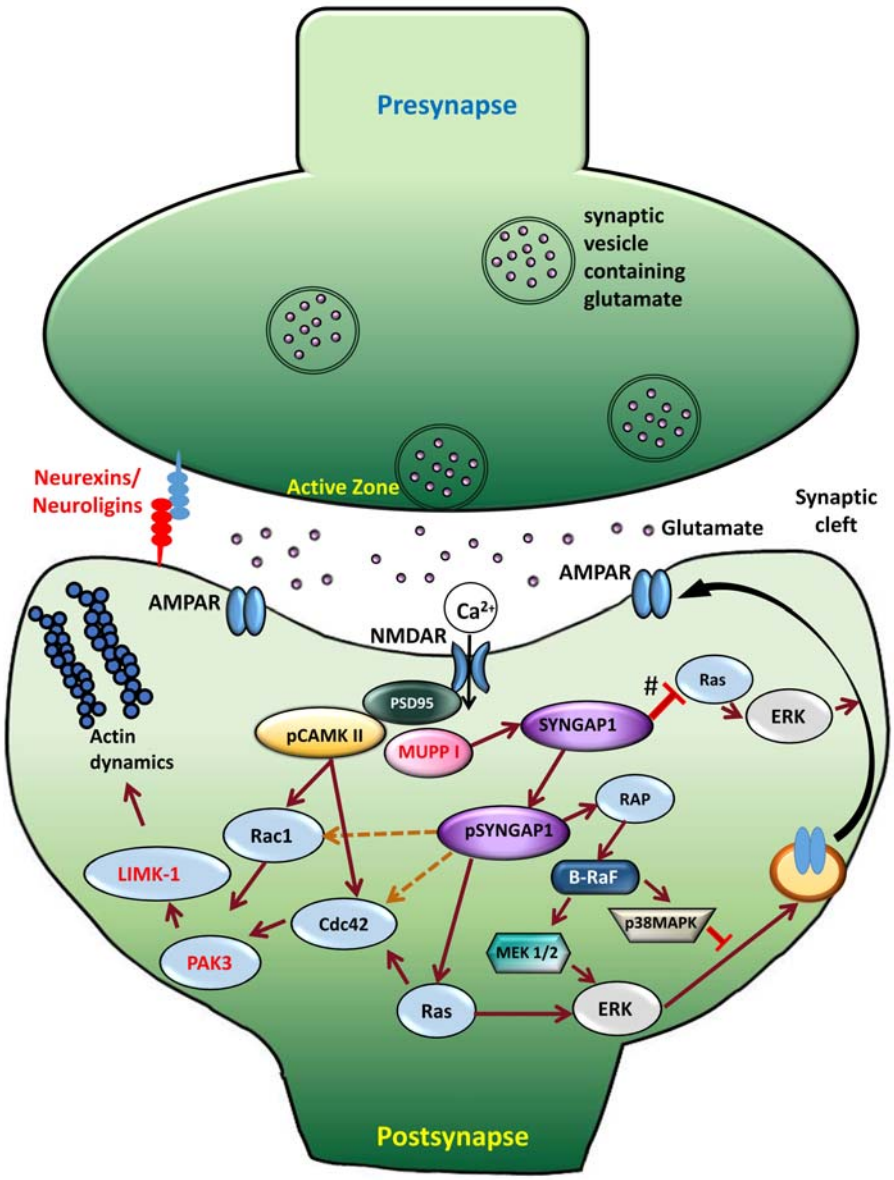

FIGURE 3 | Signaling mechanism upon phosphorylation of SYNGAP1. Schematic model of the cellular events that link CaMKII activity to phosphorylation of SYNGAP1 and its regulation of downstream molecules. Glutamate receptors, such as NMDAR and AMPAR, are clustered at the postsynaptic active zone with a dense matrix called PSD. Upon NMDAR activation, Ca^{2+} enters the postsynaptic cytosol, triggering phosphorylation of CaMKII, which in turn phosphorylates SYNGAP1 (pSYNGAP1). pSYNGAP1 regulates Ras-GTPases controlling actin dynamics and AMPARs insertion into the postsynaptic membrane. In *Syngap1* Heterozygous mutation, the inhibition of Ras activation by SYNGAP1 (shown as #) is lost, which increases Ras activity, thereby increasing AMPAR exocytosis to the postsynaptic membrane. Phosphorylation of SYNGAP1 by cyclin-dependent kinase 5 (CDK5) activates Rap1 that increases endocytosis of AMPAR. It is not clear how pSYNGAP1 regulates other SYNGAP1-associated proteins such as Cdc42, Rac1 (dotted orange lines), which are yet to be studied.

Altered maturation of dendritic spine morphology and function can lead to various learning and memory deficits (Peca et al., 2011; Goncalves et al., 2013). Indeed, patients with *SYNGAP1* Het mutations were observed to have learning and memory deficits (Hamdan et al., 2009, 2011a). To understand the impact of early maturation of dendritic spine morphology and function on learning and memory in *Syngap1*$^{-/+}$ mice, synaptic plasticity studies were carried out by various labs. There are two major forms of synaptic plasticity, LTP and LTD (long term depression), which have been considered to represent the cellular correlates of learning and memory and are both dependent on local protein synthesis (Volianskis et al., 2015). Deficits in LTP have been observed in many animal models of ID and ASD (Volk et al., 2007, 2015; Pavlowsky et al., 2012). Alterations in signaling proteins function can lead to anomalous synaptic plasticity and dendritic spine structure and can correlate with

cognitive impairments in patients with ID and ASD (McKinney, 2005; Penzes et al., 2011; van Bokhoven, 2011; Kroon et al., 2013). Interestingly, adult *Syngap1*$^{-/+}$ mice do show reduced LTP in the CA1 hippocampal region, which is likely due to reduced activation of Ras and ERK during LTP (Komiyama et al., 2002; Kim et al., 2003; Ozkan et al., 2014), suggesting that reduced level of SYNGAP1 derepresses the resting levels of activated Ras and ERK. Additionally, SYNGAP1 has been shown to rapidly disperse from spines during and after LTP because of the phosphorylation of SYNGAP at Ser1108/1138 by CaMKII. Subsequently, SYNGAP1 activates Ras, which triggers long-term changes in spine size, suggesting the inhibition of stable LTP by SYNGAP1 (Araki et al., 2015).

CRITICAL PERIOD OF PLASTICITY

Steady increase of synaptic AMPARs and subsequent functional unsilencing of glutamatergic inputs are characteristics of early postnatal development (Kerchner and Nicoll, 2008). Premature acquisition of functional AMPARs during development is suggestive of an acceleration of neurodevelopmental pattern during a critical period of development. A critical period is a regulated time window during which the sensory experience and intrinsic neuronal activity provide information that are essential for normal development and refinement of neuronal circuits (Meredith et al., 2012). Any alteration to dendritic spine structure and function during this critical period can have a lasting effect on cognitive functions, the development of which requires the formation and refinement of synaptic networks of neurons in the brain. Precariously high AMPAR/NMDAR ratios observed in *Syngap1*$^{-/+}$mice could lead to altered duration of plasticity-related critical periods. In the thalamocortical pathway, generation of LTP becomes difficult towards the end of the first postnatal week (Crair and Malenka, 1995). However, high frequency stimulation failed to elicit LTP in PND4 and PND7 in *Syngap1*$^{-/+}$ mice, while LTP was generated in PND4 WT animals. Given that SYNGAP1 has been shown to suppress AMPAR insertion in the postsynaptic membrane, the main explanation for LTP failure at synapses would be a precocious unsilencing of the developing thalamocortical pathway. Indeed, *Syngap1*$^{-/+}$ mice have altered unsilencing of post-synapses during early stages of development in the thalamocortical pathway (Clement et al., 2013) and altered formation and elimination of dendritic spines (Aceti et al., 2015). These studies further confirm the hypothesis that prematuration of dendritic spine structure due to accumulation of AMPAR at synapses shortens the duration of the critical window of plasticity leading to altered behavioral function (**Figure 4**).

In addition to LTP, another form of synaptic plasticity, LTD is important for proper formation and refinement of neuronal connections (Feldman and Knudsen, 1998; Hensch, 2005). Thus, it would be predicted that thalamocortical synapses exhibits LTP as well as LTD. Similar to LTP, the amount of LTD induced in thalamocortical connections exhibited a developmental reduction with little or no depression remaining by P10–12 (Feldman et al., 1998). This signals the end of critical period of plasticity in thalamocortical synapses. In addition, this study suggests that NMDAR dependent LTD modulates the efficacy of synapses previously unsilenced by LTP (Daw et al., 2007), thereby, allowing the synapses to modulate the connections based on the input specific activity. However, it is not clear LTD can induce resilencing of functional synapses during critical period of development. Given the importance of LTD in critical period of plasticity, it is not clear whether LTD is altered during critical period of development in *Syngap1*$^{-/+}$mice.

PROPOSED MODEL OF *Syngap1*$^{-/+}$ MUTATION IN NEURONAL DEVELOPMENT AND MATURATION

One of the common features observed in most forms of ID is the inability to develop and maintain normal dendritic spine architecture and proper function at synapses, which lead to abnormal neuronal connections. Based on the above discussion, it is evident that *Syngap1*$^{-/+}$ mutations lead to abnormal dendritic spine maturation during development. All mutations in *Syngap1* are predicted to truncate the protein, thus decreasing the ability of SYNGAP1 to bind to the molecules downstream in the signaling pathway. Phosphorylation of SYNGAP1 is regulated by CaMKII, which reduces SYNGAP1's control of Ras-GTPase, leading to Ras activation by increasing the GTP-bound form of Ras. Further, phosphorylation of SYNGAP1 by CaMKII increases the ratio of Rap1-GAP to Ras-GAP (Walkup et al., 2015). This would shift the steady-state balance of AMPAR trafficking at the synapse towards exocytosis by decreasing the level of active Rap1 compared to active Ras, which would result in an increased surface AMPAR. In contrast, phosphorylation of SYNGAP1 by CDK5 decreases its ratio of Rap1-GAP to Ras-GAP activity, which would allow more AMPAR to be endocytosed from the postsynaptic surface. Therefore, SYNGAP1 negatively regulates Ras activation and insertion of AMPA receptors in the postsynaptic membrane. Phosphorylation of SYNGAP1 creates transient changes in the number of AMPARs and gradually adjusts the steady-state level of AMPARs in the postsynaptic membrane (**Figure 3**). However, due to *Syngap1*$^{-/+}$ mutation, truncated SYGNAP1 fails to inhibit Ras activity, thereby facilitating conversion of inactive, GDP-bound Ras to an active, GTP-bound form and increasing the level of Ras activation. Ras is one of the important components of the signaling pathway underlying NMDA receptor mediated activation of ERK (Iida et al., 2001). Thus, increase in Ras activity elevates the level of phosphorylated ERK, which further facilitates the insertion of AMPARs to the postsynaptic membrane (Derkach et al., 2007). In this aspect, SYNGAP1 is a key molecule that facilitates a cross talk between CaMKII and Ras/MAPK signaling pathways that leads to AMPAR trafficking, thereby controlling the excitatory synaptic strength, particularly in developing neurons. However, SYNGAP1's control of excitatory synaptic strength during development is lacking in ID patients or in the *Syngap1*$^{-/+}$ mice model.

Furthermore, increased level of Ras activation leads to activation of LIMKII, CDC42 and p-cofilin, which regulate actin

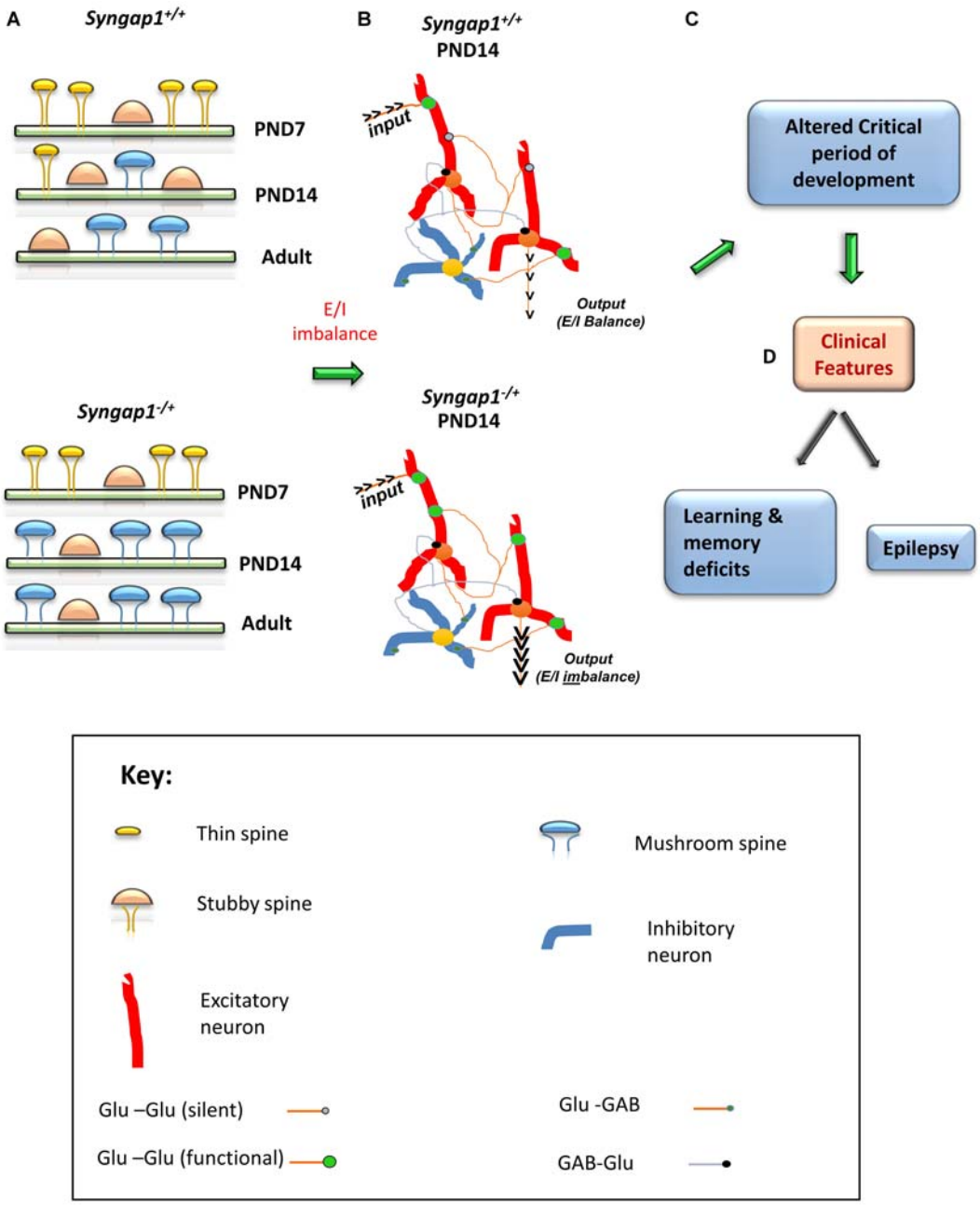

FIGURE 4 | Schematic model of the impact of Syngap1 haploinsufficiency on neuronal circuit function. This figure illustrates the impact of *Syngap1* heterozygous mutation on dendritic spine morphology, neuronal connection organization, and behavioral phenotypes. Heterozygous mutations in *Syngap1* lead to prematuration of dendritic spine morphology during early stages of development **(A)**. This causes abnormal formation and elimination of spines that leads to altered spine density and excitatory neuronal connections during development in the cortex (Aceti et al., 2015). Further, the abnormal cortical excitatory neuronal connections lead to E/I imbalance during early stages of development, which persists, into adult stages in *Syngap1* Hets **(B)**. Consequently, these abnormalities bring about altered duration of critical period of development **(C)**, which leads to cognitive and social dysfunction **(D)**. PND, Post-natal Day. The gene products implicated in intellectual disability (ID) and/or autism spectrum disorder (ASD) are marked in Red color text. Some features are modified with permission based on Clement et al. (2012).

cytoskeleton (**Figure 3**). Actin is the major cytoskeletal element in dendritic spines, where it serves both as framework for the spine structure and as a scaffold for postsynaptic proteins (Dillon and Goda, 2005). Cofilin is best known as a regulator of actin whose assembly and disassembly depends upon the concentration of cofilin. In *Syngap1*$^{-/+}$ mice, increased levels of p-Cofilin shift the equilibrium towards the more stable actin form, F-actin. This makes the dendritic spine more stable at

an early stage of development. Thus, more stable form of actin combined with an increase in insertion of AMPAR into the postsynaptic membrane during development (PND14–16 in hippocampus in Hets) causes the dendritic spines to mature into mushroom shaped spines, which occurs earlier in *Syngap1*$^{-/+}$ than in WT animals (**Figure 4**).

This leads to elevated excitatory synaptic transmission causing Excitatory/Inhibitory (E/I) imbalance, particularly during the critical period of development. Due to the altered E/I balance, humans, as well as *Syngap1*$^{-/+}$ mice, are prone to epileptic seizures. The altered E/I balance observed in *Syngap1*$^{-/+}$ mice is representative of an altered form of synaptic homeostasis that degrades the ability of mature neurons to optimally balance excitation relative to inhibition. Indeed, truncation of SYNGAP1 occludes the ability of neurons to scale up synaptic strength in response to activity, suggesting that SYNGAP1 associated signaling is necessary for maintaining homeostatic synaptic plasticity (Wang et al., 2013). In fact, altered dendritic spine morphology and function during the critical period of development causes a coordinated acceleration of dendritic elongation, spine formation, and elimination (pruning) in cortical neurons, which may result in altered neuronal connectivity and abnormal closure of critical period plasticity (**Figure 4**). Interestingly, E/I imbalance in *Syngap1*$^{-/+}$ mutation leads to altered pruning of spines, which in turn causes abnormal connections (non-target) in neurons and negatively affects the organization of neuronal circuits (Aceti et al., 2015). The abnormal pruning of spines and connections between neurons could be a consequence of an altered duration of critical period observed in *Syngap1*$^{-/+}$ mice. This would prevent the neuronal connections to be actively refined by the surrounding environment in which the individual exists.

There appear to be independent critical periods of development for different modalities, ranging from basic visual processing to language and social skills, which are observed to be affected in patients with *SYNGAP1* heterozygous mutation. *Syngap1*$^{-/+}$ mice displayed early closure of critical period of plasticity during development (Clement et al., 2013). The precise development of the timing of critical periods during cortical development is essential for the proper organization of synaptic connections and neuronal circuit formation. Thus, premature closure of plasticity window during development could contribute to altered refinement of cortical circuits that persist throughout the life of an animal and thus contributing to cognitive deficits in *Syngap1*$^{-/+}$ mice. Thus, transient neurodevelopmental events induced by *Syngap1* mutations could cause life-long disruptions to cognition and behavior that are difficult to treat in adulthood.

ROLE OF ASTROCYTES IN ID AND ASD

While neurons are considered as major players in brain function such as perception, social behavior and memory, astrocytes have been relegated to a far lesser supporting role. However, in recent years, emerging evidences suggest that signaling between astrocytes and neurons at the tripartite synapse plays an important role during the critical period of development (Stevens, 2008; Clarke and Barres, 2013). Although astrocytes were considered to play a passive onlooker in the synapse, but studies show that, they are necessary for neuronal maturation, function, and development of neurons. During early stages of development, astrocytes and neurons are formed from neuronal precursor cells (Freeman, 2010). Three-dimensonal reconstructions of dye-filled astrocytes reveal that astrocytes extend thousands of intricate processes that are organized into large, non-overlapping anatomical domains. It has been estimated that a single astrocyte can associate with multiple neurons and over 100,000 synapses (Bushong et al., 2002; Halassa et al., 2007a,b). While astrocytes are incapable of generating action potentials, they do secrete a wide array of gliotransmitters and express many of the same channels, receptors and cell surface molecules similar to neurons (Haydon, 2000; Fields and Stevens-Graham, 2002; Fiacco and Mccarthy, 2006).

Neurons rely on astrocytes to instruct the formation and elimination of their synapses lead to the possibility that astrocytes work in parallel with and interacts with, the neuronal processes that control circuit formation. One of the first evidences that astrocytes contribute majorly in critical period of development came from a study by Muller and Best (1989) that injection of immature astrocytes into the adult visual cortex reopened the window of ocular dominance plasticity. Further, a study from purified rodent ganglion cells (RGCs) suggested that RGCs formed very few syanpses in the absesnce of astrocytes. However, when cultured in the presence of astrocytes, or in a medium that had been conditioned with any other soluble signals released by astrocytes, RGCs can form ten-fold more excitatory synapses and synaptic functionality was increased (Pfrieger and Barres, 1997; Ullian et al., 2001). Not only astrocytes regulate the development, maturation, and function of excitatory neurons, they are a requisite for the development of inhibitory synapses. Liu et al. (1996) showed that local contact between neurons and astrocytes significantly increased the amplitude and density of $GABA_A$ receptors in developing hippocampal neurons. In addition, astrocytes were shown to regulate chloride ion gradient in cultured spinal cord neurons and convert GABAergic neurons from excitatory to inhibitory (Li et al., 1998). These studies suggest that immature astrocytes are necessary for critical period of development and it is linked to maturation of astrocytes.

It is evident from the above mentioned studies that astrocytes play a major role in normal neuronal development and function, it would not be surprising that astrocytes contribute in some capacity to almost all pathological conditions of the nervous system (Lin and Koleske, 2010; Parpura et al., 2012). Consequently, astrocyte-dysregulated function has been linked with the progressive pathology of ischemic stroke, epilepsy and to a number of neurodegenerative disorders including amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, Rett syndrome, FXS, and autism (Yamamuro et al., 2015). FXS is one of the most common form of ID and affects 1 in 4000 males and 1 in 6000 females. Fragile X mental retardation protein (FMRP) is reported to be expressed in Oligodendritic precursor cells but not mature oligodendrocytes (Wang et al., 2004). However, a study by Pacey and Doering (2007) reported expression of FMRP in astrocytes. Further, they showed that WT neurons grown

on *Fmr1* KO astrocytes exhibited significantly altered dendritic arbor morphologies, whereas *Fmr1* KO neurons cultured with WT astrocytes, the alterations in dendritic morphologies and synaptic protein expression were prevented (Jacobs and Doering, 2010; Jacobs et al., 2010). These experiments were the first to suggest that astrocytes contribute to the normal development of dendritic spine morphology and function. Therefore, it is important to study the role of astrocytes in *Syngap1*$^{-/+}$ mutations. However, there are no studies to date to suggest expression of *Syngap1* in astrocytes or its role in ID due to *Syngap1*$^{-/+}$.

CONCLUSION

Basic research in ID and ASD using model organisms has been critical in advancing our understanding of many NDDs. Important insights into the neurophysiology of *Syngap1*$^{-/+}$ mutations, especially the regulation of dendritic spine formation and function, has been gained from the study of *Syngap1*$^{-/+}$ mouse models. Although it is clear from these studies that SYNGAP1 is a negative regulator of AMPAR insertion in the postsynaptic membrane that regulates dendritic spine structure and function, certain questions still remain unanswered, such as which downstream proteins are regulated by affected by *Syngap1*$^{-/+}$ mutations. The other major question is to find the precise window during development to address the symptoms observed in ID. In fact, repairing pathogenic *Syngap1*$^{-/+}$ mutation after the end of critical period of development failed to rescue neurophysiological and cognitive functions. Therefore, it is important to find the right period of development in order to rescue the cognitive deficits observed in *Syngap1*$^{-/+}$ mutation. One of the means of finding therapeutic targets is to find a protein which has been implicated in another ID and ASD that produces similar or opposite cellular and behavioral phenotypes as that of *Syngap1*$^{-/+}$ mutants. The opposing effects of these mutations may balance one another at synaptic and behavioral function (Auerbach et al., 2011). Understanding the effect of complementary pathways to rescue a gene of interest, for example *Syngap1*$^{-/+}$ mutation, would allow better therapeutic designs to alleviate ID symptoms (earlier the better). It is important to understand where an ID and ASD patient lies on the spectrum of synaptic and behavioral dysfunction to choose an appropriate therapy. Thus, continued study of various disorders that exhibit ID and ASD phenotypes may lead to better therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Cross-talk and regulation between glutamate and $GABA_B$ receptors

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Brain function depends on co-ordinated transmission of signals from both excitatory and inhibitory neurotransmitters acting upon target neurons. NMDA, AMPA and mGluR receptors are the major subclasses of glutamate receptors that are involved in excitatory transmission at synapses, mechanisms of activity dependent synaptic plasticity, brain development and many neurological diseases. In addition to canonical role of regulating presynaptic release and activating postsynaptic potassium channels, $GABA_B$ receptors also regulate glutamate receptors. There is increasing evidence that metabotropic $GABA_B$ receptors are now known to play an important role in modulating the excitability of circuits throughout the brain by directly influencing different types of postsynaptic glutamate receptors. Specifically, $GABA_B$ receptors affect the expression, activity and signaling of glutamate receptors under physiological and pathological conditions. Conversely, NMDA receptor activity differentially regulates $GABA_B$ receptor subunit expression, signaling and function. In this review I will describe how $GABA_B$ receptor activity influence glutamate receptor function and *vice versa*. Such a modulation has widespread implications for the control of neurotransmission, calcium-dependent neuronal function, pain pathways and in various psychiatric and neurodegenerative diseases.

Keywords: glutamate receptor, NMDAR, $GABA_BR$, AMPAR, AKAP, receptor regulation, receptor trafficking and mGluR

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Introduction

Most excitatory signals that a neuron receives are mediated via glutamate receptors whereas most inhibitory signals are mediated via γ-aminobutyric acid (GABA) receptors (Cherubini et al., 1991; Hollmann and Heinemann, 1994). Many factors influence the regulation of excitatory and inhibitory synaptic inputs on a given neuron. One important factor is the subtype of neurotransmitter receptors present at not only the correct location to receive the appropriate signals but also their abundance at synapses (Dingledine et al., 1999; Sheng and Kim, 2011). Thus the molecular mechanisms that regulate receptor expression and localization at specific sites are of considerable importance. This review will describe the recent advances in our understanding of the molecular mechanisms underlying glutamate and $GABA_B$ receptors cross-talk and discuss the roles of specific proteins that might control these processes.

Glutamate receptors are the major excitatory neurotransmitter receptors in the brain and play an important role in neural plasticity and development. Improper function of glutamate receptors is involved in various psychiatric and neurodegenerative diseases (Mattson, 2008; Musazzi et al., 2013). N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) and kainate receptors are

glutamate-gated ion channels, whereas metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors (GPCRs) that signal downstream via interaction with heterotrimeric G-proteins. Pharmacological and molecular biological studies have revealed that glutamate receptors exist as different subclasses, where receptor subtypes comprise multiple subunits such as NMDA receptors (GluN1 to GluN3), AMPA receptors (GluA1 to GluA4), kainate receptors (GluK1 to GluK5) and mGlu receptors (mGluR1 to mGluR8) (for reviews, see Nakanishi et al., 1998; Lodge, 2009; Nicoletti et al., 2011).

Conversely, GABA receptors are the primary proteins responsible for inhibitory responses in the brain. Metabotropic GABA receptors ($GABA_B$Rs) are GPCRs that can mediate slow inhibitory neurotransmission in the CNS. $GABA_B$Rs are located at both presynaptic and postsynaptic compartments and changes in their number, localization and activity affect the level of synaptic inhibition. Presynaptic $GABA_B$Rs inhibit release of neurotransmitter by inhibiting Ca^{2+} channels (Wu and Saggau, 1995; Takahashi et al., 1998). Activation of postsynaptic $GABA_B$Rs activates inwardly rectifying K^+ channels (GIRK) to generate slow inhibitory postsynaptic potentials (reviewed in Marshall et al., 1999; Bowery et al., 2002; Gainetdinov et al., 2004). The $GABA_B$R is a heteromeric GPCR consisting of $GABA_{B1}$ and $GABA_{B2}$ subunits that exert much longer lasting synaptic inhibition compared to $GABA_A$ ion channels (Marshall et al., 1999; Watanabe et al., 2002). The ligand-binding domain (Malitschek et al., 1999) is present in $GABA_{B1}$ subunit and G-proteins interact with $GABA_{B2}$ to regulate adenylate cyclase, GIRK channels and Ca^{2+} channels (Robbins et al., 2001). A large body of work over the last 20 years has demonstrated that $GABA_B$ receptors are regulated via mechanisms distinct from those utilized by many classical GPCRs such as the β_2-adrenergic receptor (Bettler and Tiao, 2006). For example, following agonist exposure most GPCRs are phosphorylated and endocytosed from the cell surface into intracellular compartments and then either down-regulated via lysosomal or proteasomal degradation or recycled back to the cell surface following agonist removal. In contrast, cell surface $GABA_B$ receptor levels are not significantly altered upon receptor stimulation in cultured cortical and hippocampal neurons (Fairfax et al., 2004; Bettler and Tiao, 2006). $GABA_B$ receptors are very stable at the plasma membrane even after agonist exposure with little internalization in cultured neurons. The absence of receptor endocytosis correlates with lack of arrestin recruitment and agonist-induced phosphorylation (Fairfax et al., 2004). Surprisingly, increased phosphorylation at serine 892 in $GABA_{B2}$ subunit decreased degradation rates and stabilizes surface $GABA_B$Rs in neurons (Couve et al., 2004; Fairfax et al., 2004).

The main regulatory sites on both glutamate receptors and $GABA_B$Rs are their intracellular C-terminal tails. Depending on the activity or stimulation received by the receptors, the C-terminal domains bind to various proteins including enzymes, scaffolds, and trafficking and signaling proteins (De La Rue and Henley, 2002). These sites sometimes also mediate complex formation during a cross-talk between the receptors. Many immunocytochemical and electron microscopy studies have demonstrated that glutamatergic synapses are enriched with $GABA_B$Rs (Fritschy et al., 1999; Luján and Shigemoto, 2006). There is also increasing evidence that NMDARs, AMPARs and mGluRs are modulated directly and sometimes indirectly by $GABA_B$Rs (Morrisett et al., 1991; Hirono et al., 2001; Otmakhova and Lisman, 2004; Tabata et al., 2004; Sun et al., 2006; Chalifoux and Carter, 2010; Gandal et al., 2012; Terunuma et al., 2014). Conversely, $GABA_B$R subunits are differentially regulated by glutamate receptor subtypes under various stimulation protocols (Vargas et al., 2008; Cimarosti et al., 2009; Guetg et al., 2010; Maier et al., 2010; Terunuma et al., 2010; Kantamneni et al., 2014). The sections below in this review will follow this theme of regulation or modulation between $GABA_B$ and glutamate receptors. This cross-talk provides important regulatory mechanisms, for example, in altering presynaptic release or changes to membrane potential, but also alters the function of glutamate receptors, which may prove useful in a therapeutic context.

$GABA_B$R-Mediated Regulation of Glutamate Receptor Function

$GABA_B$R Regulation of NMDAR-Dependent Post-Synaptic Calcium Signals

The major synaptic Ca^{2+} signals in the brain are mediated via NMDARs, which are crucial for activity-dependent changes in synaptic plasticity (Bliss and Collingridge, 1993; Mainen et al., 1999; Malenka and Bear, 2004). These Ca^{2+} signals are thought to be inhibited by $GABA_B$ receptors via modulation of K^+ channels, resulting in a hyperpolarization that decreases the Ca^{2+} influx and overall current by enhancing Mg^{2+} blockade of NMDARs. (Morrisett et al., 1991; Otmakhova and Lisman, 2004; Deng et al., 2009). Interestingly, it has also been demonstrated recently that Ca^{2+} influx via NMDARs is inhibited by $GABA_B$ receptor activation (Chalifoux and Carter, 2010). This effect on NMDARs is independent of K^+ channel and voltage sensitive Ca^{2+} channel activation, $G\beta\gamma$ subunits and internal Ca^{2+} stores. Via coupling to $G\alpha_i/G\alpha_o$ G proteins, $GABA_B$Rs inhibit adenylate cyclase to reduce PKA activity by decreasing cAMP levels. The Ca^{2+} influx via NMDA receptors is normally increased by PKA activity and reduction of PKA activity by $GABA_B$Rs inhibits Ca^{2+} signals (Chalifoux and Carter, 2010). $GABA_B$R-mediated postsynaptic modulation through the PKA pathway does not affect synaptic currents mediated by NMDA or AMPA receptors (Chalifoux and Carter, 2010). As outlined below, protein kinases such as PKA and phosphatases such as PP1/2 and calcineurin (CaN) are regulated via AKAPs (A Kinase Anchoring Proteins) and mediate signaling where they act as scaffold molecules (see below for further insights).

NMDAR and $GABA_B$R Cross-Talk in Disease

Recently it has been demonstrated that, there is clear interplay between $GABA_B$ and NMDA receptors not only in physiological functions but also in pathological situations. Altered NMDAR activity is observed in models of pain and neuropsychiatric disorders, but an interesting phenomenon is that these phenotypes can be rescued with $GABA_B$R ligands. For example,

in diabetic neuropathy, NMDAR expression is increased in spinal cord dorsal horn, while $GABA_B$ receptors are down regulated at protein level (Wang et al., 2011). Using streptozotocin (STZ)-induced diabetic neuropathy rat models (STZ), it has been found that intrathecal injection of the $GABA_BR$ agonist baclofen significantly increased paw withdrawal threshold. This effect was blocked with pre-treatment of CGP55845, a $GABA_BR$—selective antagonist (Bai et al., 2014). In STZ rats, changes in expression were observed in both cyclic AMP response element-binding protein (CREB) and GluN2B, which were significantly increased at the protein (CREB and GluN2B) and mRNA level (GluN2B) in spinal cord. The higher expression levels of both GluN2B and phosphorylated CREB proteins were significantly reduced by administration of baclofen (Liu et al., 2014). Importantly, baclofen-induced reduction of GluN2B and CREB expression was abolished when CGP55845 was pre-administered, suggesting that $GABA_BR$ activation in the spinal cord dorsal horn can normalize NMDAR expression levels in diabetic neuropathic pain (Wang et al., 2011; Bai et al., 2014; Liu et al., 2014).

In contrast, reduced NMDA receptor functionality has been observed in neuropsychiatric disorders like intellectual disability, autism and schizophrenia (Gonda, 2012). For example, a mouse model expressing a reduced amount of GluN1 subunit ($NR1^{neo-/-}$ mice) was characterized to mimic schizophrenic-like behavior (Mohn et al., 1999). These mice have increased power in the gamma (30–80 Hz) EEG range during rest, but show a reduced auditory-stimulus evoked gamma power (reduced gamma signal-to-noise), causing changes in excitatory/inhibitory balance, and express treatment resistant symptoms of autism and schizophrenia (Gandal et al., 2012). Treating $NR1^{neo-/-}$ mice with baclofen restored excitatory/inhibitory balance, neural synchrony and also improved social function and spatial memory deficits (Gandal et al., 2012). To summarize, diseases characterized by NMDA receptor dysfunction, have the additional possibility of using $GABA_B$ receptors as an appropriate target for therapy that could possibly pave the way to restore abnormalities in many other neurological diseases.

$GABA_BR$ Cross-Talk with AMPARs

Surface expression of AMPA receptors was increased in a knock-in mouse model in which wild-type $GABA_{B2}R$ was replaced with a S783A-mutated version which cannot be phosphorylated (Terunuma et al., 2014). The S783 on $GABA_{B2}$ subunit is phosphorylated by AMP-dependent protein kinase (AMPK), which in-turn enhances receptor coupling to GIRKs (Kuramoto et al., 2007). Activating NMDARs transiently results in increased phosphorylation whereas prolonged activation results in dephosphorylation of $GABA_BRs$ by protein phosphatase 2A (PP2A). $GABA_BRs$ stability at cell surface is due to high constitutive phosphorylation of $GABA_{B2}R$ and dephosphorylation of this subunit selectively targets the receptors for lysosomal degradation (Fairfax et al., 2004; Terunuma et al., 2010). The expression of $GABA_BR$ was increased with the mutation due to reduced degradation, leading to decreased level of Arc/Arg3.1 protein necessary for memory consolidation. This, in turn, increased the number of excitatory synapses, PSD95 protein expression and cell surface AMPA receptors. This cross-talk demonstrates a crucial role for $GABA_BRs$ in regulating excitatory synaptic transmission and neuronal architecture (Terunuma et al., 2014).

$GABA_BR$ Cross-Talk with mGluRs

Long-term depression (LTD) at cerebellar parallel fiber Purkinje cell synapses is a form of synaptic plasticity critical for cerebellar motor learning and requires the activation of the metabotropic glutamate receptor mGluR1 (Ichise et al., 2000; Ito, 2001). $GABA_BRs$ are concentrated at cerebellar parallel fiber Purkinje cell synapses and have many functions that are both dependent and independent of GABA. $GABA_BRs$ and mGluR1 are highly co-expressed in cerebellar Purkinje cells, and display very similar subcellular localizations throughout development (Ige et al., 2000; Luján and Shigemoto, 2006; Rives et al., 2009). Electrophysiological studies have shown that at Purkinje cell synapses, $GABA_BR$ activation inhibits neurotransmitter release by inhibiting calcium channels as well as affecting release processes (Dittman and Regehr, 1996, 1997; Vigot and Batini, 1997). Extracellular Ca^{2+} interacts with $GABA_BR$ in cerebellar Purkinje cells, leading to an increase in the glutamate sensitivity of mGluR1. This sensitization of mGluR1 to glutamate is specifically mediated by $GABA_BRs$ as it is absent in cells from $GABA_{B1}{}^{-/-}$ animals. It has also been shown that both GPCRs form a complex in cerebellum and that extracellular Ca^{2+}-mediated crosstalk is not mediated via $G_{i/o}$ proteins (Tabata et al., 2004). Activity-dependent $GABA_BR$ inhibition by selective antagonists reduces the magnitude of LTD at parallel fiber Purkinje cell synapses (Kamikubo et al., 2007; Rives et al., 2009). In summary $GABA_BRs$ not only mediate classical synaptic GABAergic neurotransmission but also regulate mGluR signaling and cerebellar synaptic plasticity.

NMDAR-Mediated Regulation of $GABA_BR$ Function

$GABA_BRs$ are very stable at cell surface in terms of agonist stimulation and the number of cell surface $GABA_BRs$ is primarily controlled by glutamate and not GABA in central neurons (Fairfax et al., 2004; Vargas et al., 2008). Sustained application of glutamate leads to $GABA_BR$ endocytosis, trafficking to lysosomes and subsequent degradation, resulting in a decrease in receptor expression at the cell membrane (Vargas et al., 2008; Maier et al., 2010). Further dissection of the effect of glutamate indicated that activation of AMPA and NMDA receptors is required for the down-regulation of $GABA_BRs$ and that this effect is enhanced by activation of the group I mGluRs (mGlu1 and mGlu5) (Maier et al., 2010). Activation of NMDARs alone leads to down-regulation and degradation of $GABA_{B1}$ and $GABA_{B2}$ subunits, thereby reducing cell surface expression (Guetg et al., 2010; Terunuma et al., 2010; Kantamneni et al., 2014). Mechanistically, NMDAR activation triggers $GABA_{B1}$ subunit phosphorylation on Ser867 by CaMKII, causing a CaMKII-dependent down regulation (Guetg et al., 2010). In both hippocampal and cortical cultured neurons NMDAR activation also alters the

phosphorylation state of $GABA_{B2}$ subunit on Ser783, resulting in endocytosis and lysosomal degradation of the receptor complex (Terunuma et al., 2010). The $GABA_{B2}$ subunit is also rapidly phosphorylated by AMPK upon NMDAR activation. Prolonged NMDAR activation subsequently results in $GABA_{B2}$ subunit dephosphorylation by PP2A, which decreases the number of cell surface receptors (Terunuma et al., 2010).

Recently it has been shown that selective activation of synaptic NMDARs using chemically induced LTP (long-term potentiation) protocol (chem-LTP) leads to an increase in surface $GABA_B$ receptors (Kantamneni et al., 2014). In the chem-LTP protocol, glycine (along with strychnine and bicuculline—to block glycine and $GABA_A$ receptors, respectively) was used to specifically activate synaptic NMDARs, leading to significant increase in surface expression of AMPARs (Lu et al., 2001; Park et al., 2004). Prolonged activation of extrasynaptic NMDARs promotes cell death, whereas activation of synaptic NMDARs mediates synaptic plasticity and is thought to be involved in neuroprotection via modulation of nuclear Ca^{2+} signaling (Hardingham and Bading, 2010). Using the chem-LTP method, both $GABA_{B1}$ and $GABA_{B2}$ receptor subunit expression on the cell surface were increased in cultured rat hippocampal neurons due to enhanced receptor recycling from intracellular pools (Kantamneni et al., 2014).

$GABA_BR$ subunits are differentially regulated under oxygen/glucose deprivation (OGD) conditions, which stimulates release of excess glutamate resulting in excitotoxic activation of NMDARs (Papadia and Hardingham, 2007; Cimarosti et al., 2009; Kantamneni et al., 2014). After OGD, expression of $GABA_{B1}$ subunits at the cell surface is increased via enhanced recycling, while total cellular and cell surface expression levels of $GABA_{B2}$ subunits are decreased due to reduced recycling (Cimarosti et al., 2009; Kantamneni et al., 2014; Maier et al., 2014). Removing $GABA_{B2}$ subunit will decrease the number of functional $GABA_BRs$, as both subunits are required for normal signaling. In conclusion, the above findings demonstrate that the expression and regulation of $GABA_BR$ subunits are dynamically regulated in response to synaptic and prolonged/global stimulation of NMDARs. Moreover, NMDAR regulation of $GABA_BRs$ may be important under conditions of neurological disease, such as epilepsy or ischemia.

Anchoring and Scaffold Proteins as Possible Mediators of GABA/Glutamate Receptor Cross-Talk

Both GABAergic and glutamatergic receptor complexes are regulated and orchestrated by anchoring and scaffold proteins, which are increasingly being implicated in the cross-talk between the two systems. Components of receptor signalosome are typically localized together via scaffold proteins, which co-assemble receptors with regulatory proteins such as protein kinases and phosphatases. AKAPs are typical examples of this class of scaffold proteins (Wong and Scott, 2004). For example, AKAP5 (or AKAP79/150) is thought to localize PKA, protein kinase C (PKC) and the calmodulin-activated protein phosphatase calcineurin (CaN) at specific synaptic sites to regulate excitatory synaptic strength (Gomez et al., 2002; Smith et al., 2006; Robertson et al., 2009; Jurado et al., 2010). AKAP5 is linked to NMDARs via PSD-95 (Colledge et al., 2000). AKAP5 is known to be a master scaffolding protein that links many proteins including kinases, phosphatases, cadherins, F-actin, MAGUKs and PIP_2 together with ion channels and receptors to regulate activity dependent signaling processes at synapses (Tunquist et al., 2008; Sanderson and Dell'Acqua, 2011). Many of the proteins binding to AKAP5 (such as PKA, PP2B) also regulate $GABA_BRs$ and perhaps there is possibility that AKAP5 scaffolding function may be required for glutamate/GABA receptors cross-talk.

Yotiao is another AKAP protein derived from alternative splicing of AKAP9 (also known as AKAP350/450) and plays a major role in regulating NMDARs. Yotiao was first identified as a binding partner of the GluN1 subunit and later found to be an AKAP via its ability to bind PKA-RII subunits *in vitro* (Lin et al., 1998; Westphal et al., 1999). Yotiao binds both protein phosphatase 1 (PP1) and PKA to form a phosphatase-kinase signaling complex with the GluN1A receptor splice variant. The Yotiao-PP1-PKA complex functions as dual switch, in that activation of anchored PKA enhances NMDAR currents while activation of PP1 exerts an inhibitory effect on NMDAR activity (Westphal et al., 1999; Colledge et al., 2000).

$GABA_{B1}Rs$ were previously shown to interact with a scaffold protein, GISP that enhances cell surface expression of heteromeric complex $GABA_{B1}/GABA_{B2}$ (Kantamneni et al., 2007). GISP is an AKAP9 C-terminal splice variant with more than 90% similarity to AKAP9 but lacking any RII domain, which are PKA binding sites (Kantamneni et al., 2007). As mentioned previously, the NMDAR binding protein Yotiao is also an AKAP9 splice variant, but within the N-terminal region. Therefore, theoretically, AKAP9 could interact simultaneously with NMDARs and $GABA_BRs$ as well as regulatory protein kinases and phosphatases. Thus, while speculative, it is tempting to suggest that AKAP9 functions to assemble the signaling complex responsible for mediating the observed cross-talk between the NMDARs and $GABA_BRs$. From expression studies it is known that AKAP9 is expressed in the brain and localized to synapses (Collado-Hilly and Coquil, 2009). In similarity to the AKAP5-CaN_PP2B-PKA complex, the AKAP9-PKA-PP1 complex might exist as one large macromolecular complex held together with receptor proteins such as $GABA_BRs$ and NMDARs. At least in yeast-two hybrid assay it has been confirmed that GISP does not interact with NMDAR sub-type 1 (Kantamneni et al., 2007). GISP binding to other subtypes of NMDARs or Yotiao binding to $GABA_BRs$ has not been tested, and that this warrants further work. Another protein that may potentially mediate direct crosstalk between $GABA_BR$ signaling and glutamate receptor signaling is CaMKII. CaMKII is a Ca^{2+} calmodulin dependent protein kinase, previously been shown to interact with both $GABA_B$ and NMDA receptors and regulate NMDAR mediated plasticity (Bayer et al., 2001; Guetg et al., 2010; El Gaamouch et al., 2012). Unlike the earlier examples of indirect receptor modulation, AKAPs and other signaling molecules like CaMKII potentially function as direct links between glutamate

and $GABA_B$ receptors. If further characterized these complexes may eventually serve as potential drug targets.

Conclusions

Taken together we can conclude that there is very tight regulation between glutamate and $GABA_B$ receptors. Regulation of NMDAR-mediated synaptic signals by $GABA_B$Rs comprises a powerful mechanism for controlling the major excitatory systems in brain. Conversely, NMDAR-mediated control of $GABA_B$Rs is clearly an important emerging concept in dictating the balance of excitability in the brain. Studying the trafficking and signaling pathways utilized by these excitatory and inhibitory receptors in an integrated manner will undoubtedly provide more understanding of these critical regulatory mechanisms and will ultimately shed light on how the balance between excitatory and inhibitory neurotransmission is dictated in the brain. While many examples of interactions between glutamate and $GABA_B$ receptors have been discovered, importantly, the molecular players involved in mediating this cross-talk are only just beginning to be discovered. With this in mind, investigation of the potential players in these processes, such as the AKAPs, is an exciting future avenue of study. Ultimately, targeting these specific regulatory pathways may form the basis of new therapies to treat a number of neurological disorders that are characterized by aberrant balance between excitatory and inhibitory neurotransmitter systems in the brain.

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Nicotinic α7 receptor activation selectively potentiates the function of NMDA receptors in glutamatergic terminals of the nucleus accumbens

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We here provide functional and immunocytochemical evidence supporting the co-localization and functional interaction between nicotinic acetylcholine receptors (nAChRs) and N-methyl-D-aspartic acid receptors (NMDARs) in glutamatergic terminals of the nucleus accumbens (NAc). Immunocytochemical studies showed that a significant percentage of NAc terminals were glutamatergic and possessed GluN1 and α7-containing nAChR. A short-term pre-exposure of synaptosomes to nicotine (30 μM) or choline (1 mM) caused a significant potentiation of the 100 μM NMDA-evoked [^{3}H]D-aspartate ([^{3}H]D-Asp) outflow, which was prevented by α-bungarotoxin (100 nM). The pre-exposure to nicotine (100 μM) or choline (1 mM) also enhanced the NMDA-induced cytosolic free calcium levels, as measured by FURA-2 fluorescence imaging in individual NAc terminals, an effect also prevented by α-bungarotoxin. Pre-exposure to the α4-nAChR agonists 5IA85380 (10 nM) or RJR2429 (1 μM) did not modify NMDA-evoked ([^{3}H]D-Asp) outflow and calcium transients. The NMDA-evoked ([^{3}H]D-Asp) overflow was partially antagonized by the NMDAR antagonists MK801, D-AP5, 5,7-DCKA and R(-)CPP and unaffected by the GluN2B-NMDAR antagonists Ro256981 and ifenprodil. Notably, pre-treatment with choline increased GluN2A biotin-tagged proteins. In conclusion, our results show that the GluN2A-NMDA receptor function can be positively regulated in NAc terminals in response to a brief incubation with α7 but not α4 nAChRs agonists. This might be a general feature in different brain areas since a similar nAChR-mediated bolstering of NMDA-induced ([^{3}H]D-Asp) overflow was also observed in hippocampal synaptosomes.

Keywords: nicotinic receptors, NMDA receptors, nicotine treatment, neurotransmitters release, synaptosomes, nucleus accumbens

INTRODUCTION

Adaptive changes in the glutamatergic inputs triggering information processing in the nucleus accumbens (NAc) are increasingly recognized as key features underlying mood dysfunction and addiction (Carlezon and Thomas, 2009; Reissner and Kalivas, 2010). In particular N-methyl-D-aspartic acid receptors (NMDARs) play a critical role in these adaptive changes (Ma et al., 2009), which are modulated by the cholinergic system, namely through nicotinic acetylcholine receptors (nAChRs; Giocomo and Hasselmo, 2007; Timofeeva and Levin, 2011). These two signaling systems are intertwined as heralded by the ability of nicotine to modulate both the subunit composition (Delibas et al., 2005; Levin et al., 2005; Wang et al., 2007) and several functions of NMDAR (Yamazaki et al., 2006; Liechti and Markou, 2008; Lin et al., 2010; Li et al., 2013; Ávila-Ruiz et al., 2014; Callahan et al., 2014; Salamone et al., 2014). This interaction between nAChR and NMDAR seems most evident

Abbreviations: ECL, enhanced chemiluminescence; NAc, nucleus accumbens; nAChR, nicotinic acetylcholine receptors; NMDAR, N-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; *t*-TBS, Tris-buffered saline-Tween; 5IA85380, 5-iodo-A-85380; FURA-2AM, Fura-2-acetoxymethyl ester; DHβE, dihydro-β-erythroidine; R(-) CPP, 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid; MK801, (5*R*,10*S*)-(-)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cylcohepten-5,10-imine maleate; 5,7-DCKA, 5,7-Dichloro-4-hydroxyquinoline-2-carboxylic acid; D-AP5, D-(-)-2-Amino-5-phosphonopentanoic acid; Ro256981, (α*R*,β*S*)-α -(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate; RJR2403, (*E*)-*N*-Methyl-4-(3-pyridinyl)-3-buten-1-amine oxalate.

in nerve terminals (Lin et al., 2010; Salamone et al., 2014): this is of particular interest in view of the increasingly recognized role of presynaptic NMDARs in the control of synaptic plastic changes in different brain areas (Sjöström et al., 2003; Corlew et al., 2008; Bidoret et al., 2009). Thus, we now combined immunological, pharmacological and neurochemical approaches applied to purified nerve terminals to study NMDAR function in glutamatergic terminals in the NAc and we tested whether these presynaptic NMDARs were controlled by nAChRs.

MATERIALS AND METHODS

ANIMALS AND BRAIN TISSUE PREPARATION

Adult male rats (Sprague–Dawley, 200–250 g) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (light 7.00 a.m.–7.00 p.m.) with food and water freely available. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section (University of Genoa) (protocol number 124/2003-A), in accordance with the Italian and European legislation on animal experimentation (2010/63/EU). All efforts were made to minimize animal suffering and to use the minimal number of animals required to produce reliable results.

PREPARATION OF SYNAPTOSOMES

Synaptosomes were prepared essentially as previously described (Grilli et al., 2008, 2009). Rats were killed by decapitation, their brains were rapidly removed at 0–4°C and dissected to collect the NAc (sections between Bregma 0.7–2.2 mm), according to the atlas of Paxinos and Watson (1986), or the hippocampus. The tissue was homogenized in 40 volumes of 0.32 M sucrose, buffered to pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was centrifuged at 1000 *g* for 5 min, to remove nuclei and cellular debris, and crude synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in Krebs medium with the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, glucose 10, pH 7.2–7.4. The purification of nerve terminals for calcium imaging and immunocytochemical assays was carried out using a sucrose/Percoll fractionation, as previously described (Rodrigues et al., 2005).

NEUROTRANSMITTER RELEASE

The release of glutamate was gauged using the non-metabolizable tracer [^{3}H]D-aspartate ([^{3}H]D-Asp), which was loaded by incubation of the synaptosomes for 20 min at 37°C with 0.08 μM [^{3}H]D-Asp. Identical samples of the synaptosomal suspension were then layered over microporous filters at the bottom of parallel superfusion chambers thermostated at 37°C and the synaptosomes were superfused with a flow rate of 0.5 mL/min with Krebs medium. After 36 min (t = 36 min), four consecutive 3-min fractions of the eluent were collected. Synaptosomes were then exposed to NMDAR agonists (100 μM NMDA and 10 μM glycine) or to depolarizing agent (4-aminopyridine, 4AP, 10 μM) from t = 39 min onwards, while antagonists were present from 8 min before addition of the agonists onwards. Exposure to nAChR agonists was done at t = 29 min for 10 min in absence or in presence of nAChR antagonists. The superfusate samples and the synaptosomes were then counted for radioactivity. Agonist effects were expressed as percent of the induced outflow over basal outflow, upon subtraction of the radioactivity released in the four fractions collected under basal condition (no drug added) from that released in presence of the stimulus.

CALCIUM IMAGING

Purified nerve terminals (500 μg of protein) were resuspended in 1 mL of HEPES-buffered medium (HBM with 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH2PO4, 5 mM NaHCO3, 1.2 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, pH 7.4). They were loaded with FURA-2 through incubation with HBM supplemented with 5 μM FURA-2-AM, 0.02% pluronic acid F-127, 0.1% bovine serum albumin (BSA, fatty-acid free) and 1.33 mM $CaCl_2$ for 1 h at 25°C and then allowed to attach onto poly-D-lysine-coated coverslips. The terminals were washed with HBM containing 1.33 mM $CaCl_2$ and mounted in a small superfusion chamber (RC-20; Warner Instruments, Harvard, UK) on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss, Jena, Germany).

Nerve terminals were alternately excited with UV light centered at 340 and 380 nm using an optical splitter (Lambda DG4; Sutter Instruments, Novato, CA, USA), with an exposure time of 2360 ms, and the emitted fluorescence images were captured through a 40× oil objective and a 510 nm band-pass filter (Carl Zeiss) connected to a digital camera (Cool SNAP; Roper Scientific, Trenton, NJ, USA). Results were expressed by plotting the time course of the ratio, R, of the average fluorescence light intensity emitted by a small elliptical region inside each terminal upon alternated excitation at 340 and 380 nm (R = F340/F380).

Increases in R correspond to increases of the levels of cytosolic free calcium, [Ca^{2+}] (Lev-Ram et al., 1992; Castro et al., 1995). The basal ratio was measured during 60 s (i.e., 12 cycles) before stimulating the nerve terminals by superfusion with NMDA (100 μM) + glycine (10 μM) for 60 s. To measure the effects of the pretreatment with different agonists and antagonists, nicotine (100 μM), 5IA85380 (10 nM), choline (1 mM) and α-bungarotoxin (10 nM) were added 1 min before the stimulus. A 30 s pulse of KCl (25 mM) was applied at the end of each experiment to confirm the viability of the studied nerve terminals. Changes in Calcium response were measured as ΔR, subtracting the baseline (before the drug stimulation) to the peak (after the drug stimulation). All tested compounds were prepared in HBM medium lacking Mg^{2+} ions to disclose the NMDA receptor-mediated effect, and they were added to the superfused nerve terminals, through a pressurized fast-exchange solution delivery system (AutoMate Scientific, Berkeley, CA, USA), with constant gassing of all superfusion solution with 95% O_2/5% CO_2.

IMMUNOCYTOCHEMICAL ASSAYS

Nerve terminals (500 μg of protein) were resuspended in 1 mL of phosphate-buffered saline (PBS, composed of 137 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and allowed to attach onto poly-D-lysine-coated coverslips. The follow-up immuno-characterization of the nerve terminals used in FURA-2 fluorescence imaging experiments required the use

of grid-etched glass coverslips. The platted nerve terminals were fixed with 4% (w/v) paraformaldehyde for 15 min, washed twice with PBS, permeabilized in PBS with 0.2% Triton X-100 for 10 min, and then blocked for 1 h in PBS with 3% BSA and 5% normal horse serum and washed twice with PBS. Triplicate coverslips from each sample were incubated at 25°C for 1 h and the primary antibodies were diluted in PBS with 3% BSA and 5% normal horse serum: mouse anti-GluN1 (1:500), guinea pig anti-vGLUT (1:1000), rabbit anti-α7 nAChR (1:500), rabbit anti-α4 nAChR (1:500). After three washes with PBS containing 3% BSA and 3% normal horse serum, the nerve terminals were incubated for 1 h at room temperature with AlexaFluor-594 (red)-labeled goat anti-rat IgG secondary antibodies (1:200) together with Alexa Fluor-488 (green)-labeled donkey anti-rabbit and with Alexa Fluor-350 (blue)-labeled donkey anti-mouse IgG secondary antibodies (1:200). We confirmed that the secondary antibodies only yielded a signal in the presence of the adequate primary antibodies and that the individual signals obtained in double-labeled fields were not enhanced over the signals obtained under single-labeling conditions. After washing and mounting onto slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analyzed with AxioVision software (version 4.6). Each coverslip was analyzed by counting three different fields containing a minimum of 500 elements each.

BIOTINYLATION AND IMMUNOBLOTTING

Synaptosomes from the NAc of two rats were re-suspended in HBM at 4°C. The cell surface density of GluN2A was evaluated by performing surface biotinylation followed by immunoblots analysis, as previously described (Ciruela et al., 2006), with minimal modications. The synaptosomes were divided into two aliquots (500 μg protein each) and both were incubated for 10 min at 37°C under mild shaking; one aliquot was then treated for 10 min with 1 mM choline (T) while the other was kept as control (C). Choline exposure was terminated by dilution in cold washing buffer composed of 150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6. After washing twice in ice-cold washing buffer, the synaptosomes were labeled with 2 mg/ml of sulfo-NHS-SS-biotin in PBS with 1.5 mM $MgCl_2$ and 0.2 mM $CaCl_2$, pH 7.4 (PBS/Ca-Mg) for 1 h at 4°C. The biotinylation reaction was stopped by incubating with 1 M NH_4Cl for 15 min at 4°C, followed by two washes with ice cold 100 mM NH_4Cl in PBS/Ca-Mg, to quench biotin. Subsequently, biotinylated synaptosomes were lysed in RIPA buffer (500 μL) composed of 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM orthovanadate, protease inhibitor cocktail and 10 mM Tris, pH 7.4. The lysate was centrifuged at 20,000 × *g* for 10 min at 4°C, and samples (100 μg) were incubated with streptavidin magnetic beads (40 μL) for 1 h at room temperature under shaking. Biotinylated proteins, linked to streptavidin magnetic beads, were then added to pulled-down by exposure of the mixture to a magnetic field. After extensive washes, 1 × SDS-PAGE buffer was added and samples were boiled for 5 min at 95°C. Proteins were then loaded and electrophoretically separated on a 10% sodium dodecyl sulfate-PAGE gel and then transferred to PVDF membranes and probed for the proteins of interest by incubation with rabbit anti-GluN2A (1:2,000) or mouse anti-β -actin (1:10,000) primary antibodies for 1 h at room temperature with Tween 20-containing Tris-buffered saline (t-TBS), composed of 150 mM NaCl, 0.1% Tween 20, 5% non-fat dried milk and 20 mM Tris, pH 7.4. After washing, membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody (1:20,000), and immunoblots were visualized with an ECL (enhanced chemiluminescence) Plus Western blotting detection system. GluN2A subunit density was determined in the total synaptosomal lysate (Syn) and in the streptavidin-pulled-down fraction of control and choline-pretreated biotinylated synaptosomes (Ctr and Ch, respectively).

DATA ANALYSIS

Statistical comparison of the results was carried out using a Student's *t*-test for independent means (for single pairs comparison); multiple comparisons were performed with one- or two-way ANOVA followed by Tukey-Kramer *post hoc* test. Values are expressed as means ± SEM and are considered significant for $p < 0.05$.

MATERIALS

[2,3-^{3}H]D-aspartate (specific activity 11.3 Ci/mmol) was from Perkin Elmer (Boston, MA, USA); nicotine hydrogen tartrate salt, 4-aminopyridine (4-AP), N-methyl-D-aspartate (NMDA), fatty-acid free BSA, anti-β-actin monoclonal mouse IgG1, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies and the protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO, USA); 5-iodo-A-85380, ifenprodil, Ro256981, 5,7-dicholoro-kynuremic acid (DCKA), D-AP5, MK-801, (R)-CPP and RJR-2403 oxalate were from Tocris (Bristol, UK); FURA-2 AM, pluronic acid F-127 were performed by Molecular Probes, Leiden, Netherlands. β-actin monoclonal mouse IgG1, horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sulfo-NHS-SS-biotin and Streptavidin 14. Magnetic Beads were purchased from Pierce Thermo Scientific (Rockford, IL, USA), Western blotting detection system was purchased from GeHealthcare (Italy). Guinea pig anti-vGLUT, mouse anti-GluN1, AlexaFluor-594 (red)-labeled goat guinea pig IgG, Alexa Fluor-488 (green)-labeled donkey anti-rabbit, Alexa Fluor-350 (blue)-labeled donkey anti-mouse, secondary antibodies were from Invitrogen. Rabbit anti-α4 nAChR (1:500), rabbit anti-α7 nAChR and anti-rabbit polyclonal GluN1 antibody was from Chemicon International (Millipore, Billerica, MA, USA).

RESULTS

CO-LOCALIZATION AND FUNCTIONAL INTERACTION OF nAChR AND NMDAR IN GLUTAMATERGIC TERMINALS OF THE RAT NUCLEUS ACCUMBENS

Figure 1 shows that NMDA (100 μM, plus 1 μM glycine) triggered the release of [^{3}H]D-Asp from pre-labeled NAc synaptosomes. A 10 min pre-exposure of the synaptosomes to nicotine (100 μM) or choline (1 mM) significantly potentiated the NMDA-induced [^{3}H]D-Asp outflow (+58%, and

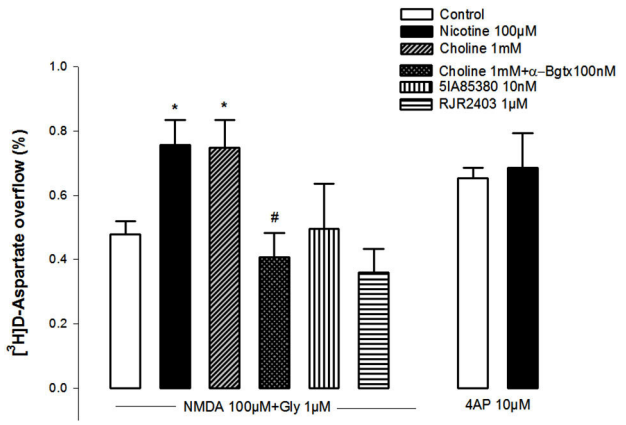

FIGURE 1 | Impact of the pre-treatment during 10 min with different nAChR agonists on the ability of NMDAR agonists (100 μM NMDA and 10 μM glycine) and of 4-AP to trigger [^{3}H]D-Asp from rat NAc nerve terminals. Data are means ± SEM of at least five experiments run in triplicate. $^*p < 0.05$ vs. control; $^\#p < 0.05$ vs. pretreatment with choline.

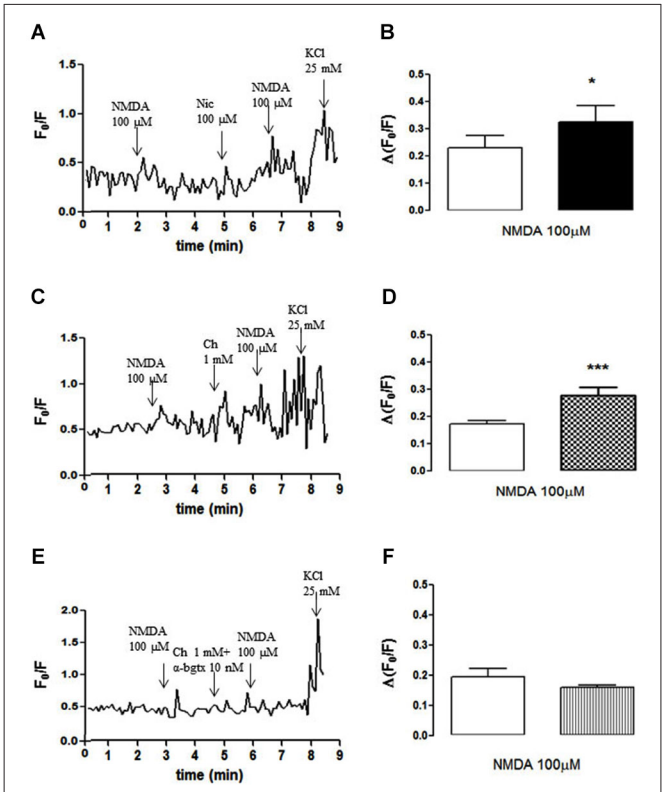

FIGURE 2 | (A, C, E) Time course of FURA-2 fluorescence emission in individual nerve terminals from the rat NAc, which were challenged twice with NMDAR agonists (100 μM NMDA and 10 μM glycine), before and 60 s after pre-treatment with either 100 μM nicotine **(A)**, 1 mM choline **(C)** or 1 mM choline together with 10 nM α-bungarotoxin **(E)**. **(B, D, F)** Comparison of the average modification of calcium transients caused by NMDA agonists before (open bars) and 60 s after (filled bars) the exposure to 100 μM nicotine **(B)**, 1 mM choline **(D)** or 1 mM choline together with 10 nM α-bungarotoxin **(F)**. Drugs were applied for 60 s, at the end of the wash out of the previous application and the arrows identify the peaks. Values are mean ± SEM of at least four experiments $^*p < 0.05$ and $^{***}p < 0.001$, using a paired Student's *t* test.

+56%, respectively). This potentiation was abolished in synaptosomes pretreated with the selective α7 nAChR antagonist α-bungarotoxin (100 nM; **Figure 1**). In contrast, the pre-exposure of the synaptosomes to the selective α4-nAChR agonist 5IA85380 (10 nM) or RJR2403 (1 μM) did not modify the NMDA-induced [^{3}H]D-Asp outflow. It should be noted that the pre-treatment of NAc synaptosomes with nicotine failed to modify the 4-AP-induced [^{3}H]D-Asp outflow (**Figure 1**).

The amplitude of the NMDA (100 μM, plus 10 μM glycine)-induced increase in cytosolic free calcium in individual NAc terminals (**Figures 2A,B**) was also potentiated by pre-exposure to nicotine (100 μM; **Figures 2A,B**) or choline (1 mM; **Figures 2C,D**), an effect that was blunted by α-bungarotoxin (10 nM; **Figures 2E,F**). These observations provide further evidence that the activation of α7-containing nAChR bolsters NMDAR-mediated functions in NAc synaptosomes.

We next carried out an immunocytochemical characterization of NAc nerve endings to gauge the extent of the co-localization between α7 nAChR and NMDAR in glutamatergic nerve terminals. As shown in **Figure 3**, we identified individual nerve terminals (e.g., terminal 1) that were glutamatergic (vGluT1-positive) and endowed with both GluN1 and α7 subunits (**Figures 3B,C**), where the pre-treatment with choline (1 mM) potentiated the NMDA (100 μM)-induced calcium transient (**Figure 3A**). In fact, this analysis revealed that more than 40% of glutamatergic nerve terminals (vGluT1-positive) possessed GluN1 and α7 subunits (**Figure 3D**), thus confirming that the co-localization of NMDAR and α7 nAChR on the same glutamatergic terminal is a generalized feature in the NAc. The analysis of individual NAc terminals further revealed non-glutamatergic (vGluT1-negative) NAc terminals (e.g., terminal 2) containing both GluN1 and α7 subunits (**Figures 3B,C**), where choline (1 mM) failed to modify the NMDA (100 μM)-induced calcium transient (**Figure 3A**). We also found terminals that responded only to the α7 nAChR agonist (e.g., terminal 3 in **Figure 3A**) or to NMDA (e.g., terminal 4 in **Figure 3A**).

We also identified individual glutamatergic nerve terminals (vGluT1-positive) containing both GluN1 and α4 subunits (terminal 2; **Figures 4B,C**), where the pre-treatment with 5IA85380 (10 nM) did not modify the NMDA (100 μM)-induced calcium transient (**Figure 4A**). Interestingly, we found other terminals (e.g., terminal 1) also containing both GluN1 and α4 subunits, where the pre-treatment with 5IA85380 (10 nM) actually reduced the NMDA (100 μM)-induced calcium transients (**Figure 4A**), a phenomenon previously observed in dopaminergic NAc terminals (Salamone et al., 2014). Additionally, we also observed nerve terminals responding only to NMDA (e.g., terminal 3) or to an α4 nAChR agonist (e.g., terminal 4 in **Figure 4A**). The average co-localization betweenGluN1 and α4 subunits (**Figure 4D**) showed that only 3–4% of the NAc glutamatergic nerve endings were endowed with both subunits, in contrast to the frequent co-localization of GluN1 and α7 subunits (**Figure 3D**).

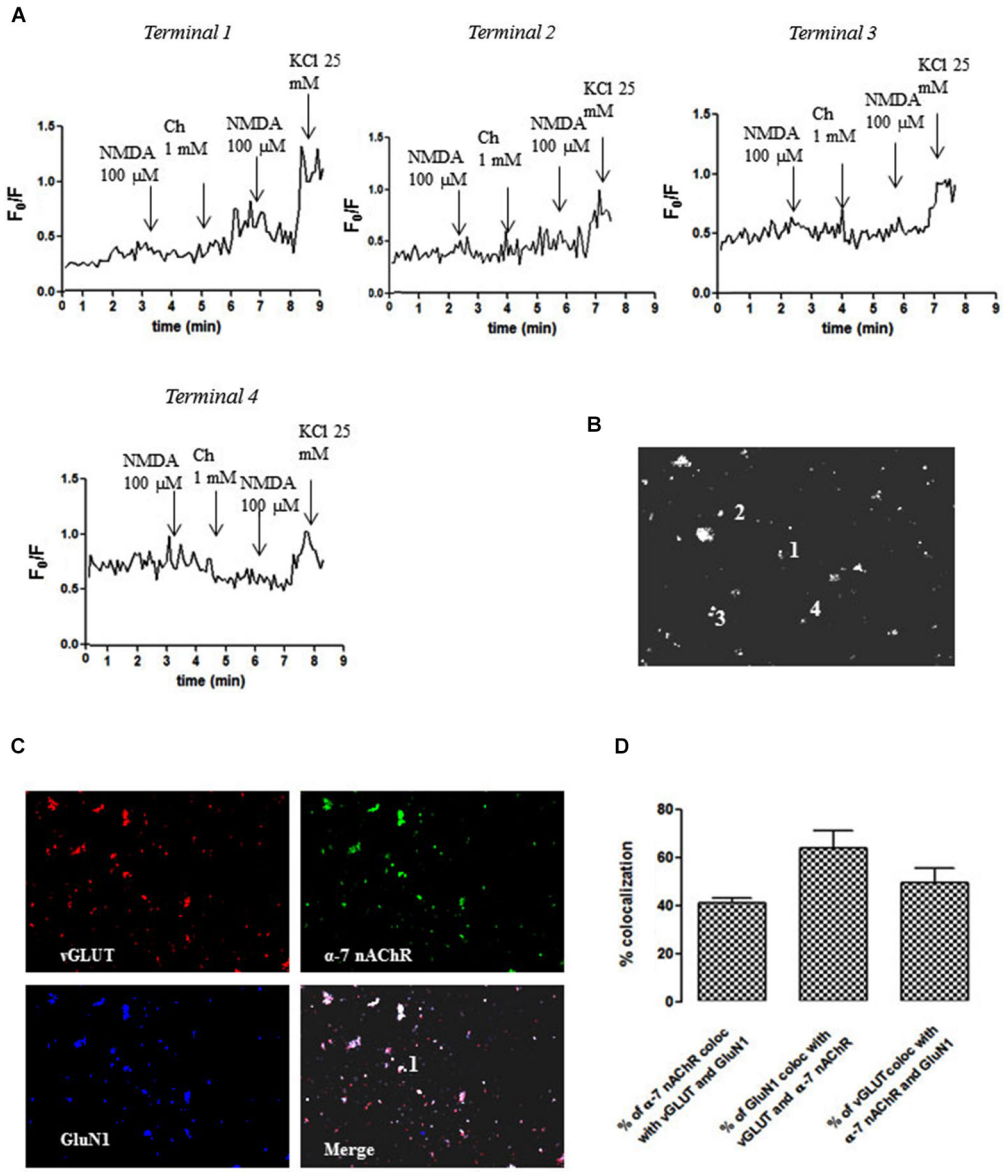

FIGURE 3 | Different impact of choline (1 mM) pre-treatment on the ability of NMDAR agonists to trigger calcium transients in different individual terminals from the rat NAc. **(A)** Time course of FURA-2 fluorescence emission in different individual nerve terminals (terminal 1–terminal 4), which were challenged twice with NMDAR agonists (100 μM NMDA and 10 μM glycine), before and 60 s after pre-treatment with 1 mM choline. Drugs were applied for 60 s, at the end of the wash out of the previous application and the arrows identify the peaks. **(B)** Fluorescence image of a field of FURA- 2-labelled synaptosomes including terminals 1–4. **(C)** Immunocytochemical co-localization of α7-nAChR, vGLUT and GluN1 in terminal 1. **(D)** Average co-localization of α7-nAChR, vGLUT and GluN1 in nerve terminals from the rat NAc. Values are mean ± S.E.M of at least four experiments.

PHARMACOLOGICAL CHARACTERIZATION OF NMDAR PRESENT IN NAc GLUTAMATERGIC TERMINALS

The pharmacological characterization of the NMDAR involved in the NMDA (100 μM)-evoked [^{3}H]D-Asp outflow from NAc synaptosomes is presented in **Figure 5**. The NMDA (100 μM)-evoked [^{3}H-]D-Asp outflow was antagonized by MK801 (10 μM) and by D-AP5 (1 μM), as well as by the selective GluN1 antagonist 5,7-DCKA (1 μM). Furthermore, the GluN2A-preferring antagonist (R)-CPP (1 μM) also attenuated the NMDA (100 μM)-evoked [^{3}H]D-Asp outflow (−48%), while the GluN2B-selective antagonists Ro256981 (1 μM) and ifenprodil (1 μM) were ineffective.

nAChR ACTIVATION DRIVES GluN2A TRAFFICKING TO THE PLASMA MEMBRANE

We next tested whether nicotine pre-treatment selectively impacts this NR2A-mediated component of the NMDA-evoked

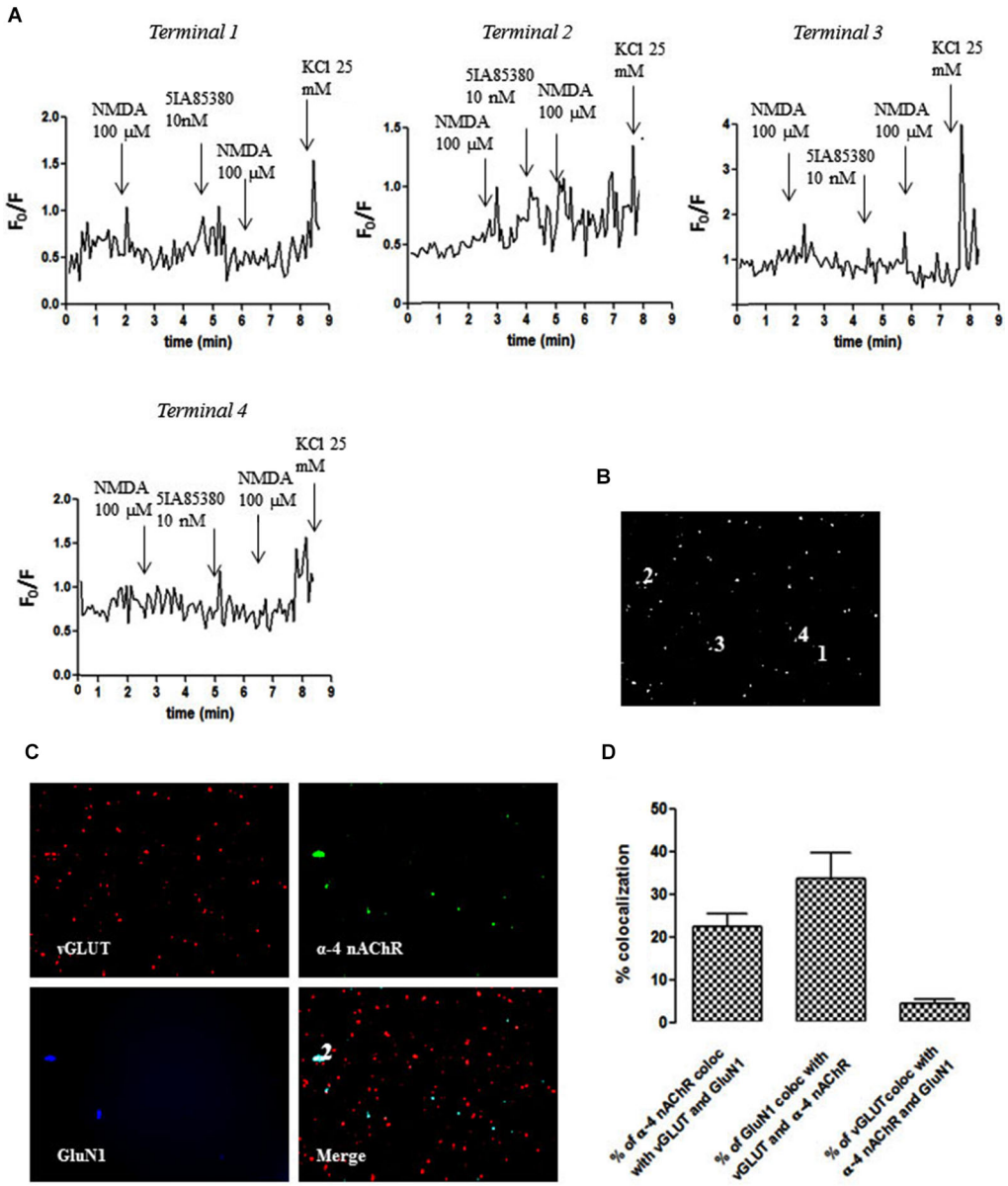

FIGURE 4 | Different impact of 5IA85380 (10 nM) pre-treatment on the ability of NMDAR agonists to trigger calcium transients in different individual terminals from the rat NAc. **(A)** Time course of FURA-2 fluorescence emission in different individual nerve terminals (terminal 1–terminal 4), which were challenged twice with NMDAR agonists (100 µM NMDA and 10 µM glycine), before and 60 s after pre-treatment with 5IA85380 (10 nM). Drugs were applied for 60 s, at the end of the wash out of the previous application and the arrows identify the peaks. **(B)** Fluorescence image of a field of FURA-2-labelled synaptosomes including terminals 1–4. **(C)** Immunocytochemical co-localization of α4-nAChR, vGLUT and GluN1 in terminal 1. **(D)** Average co-localization of α4-nAChR, vGLUT and GluN1 in nerve terminals from the rat NAc. Values are mean ± S.E.M of at least four experiments.

[^{3}H]D-Asp outflow. As shown in **Figure 6A**, after (Choline 1 mM) pre-treatment, the inhibitory effect of the NR2A-preferring antagonist (R)-CPP (1 µM) was significantly increased (−78%) compared to the effects on control (non-pre-treated) synaptosomes (−48%; **Figure 5**). By contrast, nicotine pre-treatment did not enhance the inhibition caused by the NR2B-selective antagonist Ro256981 (1 µM), which was still non-significant (**Figure 6A**).

Since we have previously shown that nAChR can control the responses of presynaptic ionotropic glutamate receptors through the regulation of their trafficking in and out of the plasma membrane (Grilli et al., 2012; Salamone et al., 2014), we posited that the nicotine-induced increase of the NMDA response in NAc glutamatergic terminals would also rely on a control of the trafficking of GluN2A-containing NMDAR. Indeed, the quantification of the density of biotin-tagged

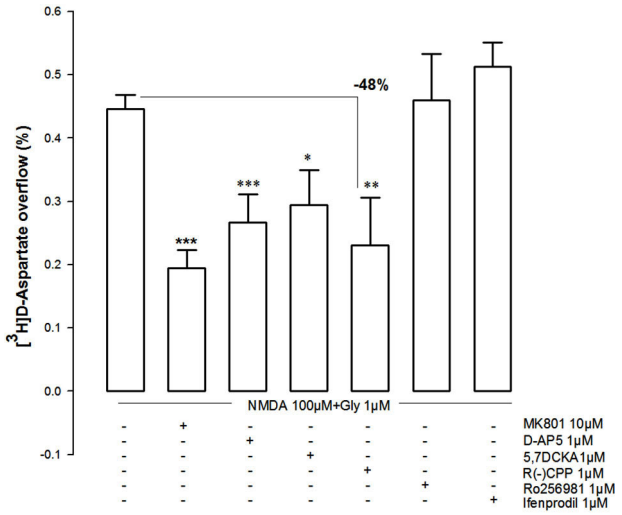

FIGURE 5 | Effect of different NMDAR antagonists on the evoked [^{3}H]D-Asp release from rat NAc synaptosomes triggered by 100 μM NMDA and 1 μM glycine and lack of effect of GluN2B-NMDAR antagonists (Ro256981 and ifenprodil). Data are mean ± SEM of at least five experiments run in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. 100 μM NMDA using a one-way ANOVA followed by Tukey-Kramer *post hoc* test.

GluN2A subunit proteins in NAc synaptosomes before and after choline pre-treatment (**Figures 6B,C**) showed that choline (1 mM) pre-treatment for 10 min increased (+15%, **Figure 6C**) the density of GluN2A at the plasma membrane (**Figure 6B**, lane Ch) respect to control (**Figure 6B**, lane Ctr).

CHOLINE POTENTIATES THE NMDA-INDUCED D-Asp RELEASE FROM HIPPOCAMPAL NERVE TERMINALS

N-methyl-D-aspartic acid (100 μM, plus 10 μM glycine) caused a marked outflow of [^{3}H]D-Asp from pre-labeled hippocampal synaptosomes (**Figure 7**), which was quantitatively higher than that observed in NAc synaptosomes (cf. **Figures 1**, **7**). The pre-exposure of hippocampal synaptosomes to choline (1 mM) for 10 min significantly potentiated the NMDA-induced [^{3}H]D-Asp outflow while the pre-incubation with nicotine (100 μM) was ineffective. As observed in NAc synaptosomes, the pre-exposure of hippocampal synaptosomes to the α4β2 nAChR agonists 5IA85380 (10 nM) or cytisine (100 μM) for 10 min did not modify the NMDA-induced [^{3}H]D-Asp outflow.

DISCUSSION

The present study shows that the activation of nAChR enhances the ability of NMDAR to trigger neurotransmitter release from glutamatergic terminals of the NAc. Our combined pharmacological and immunocytochemical characterization at the individual nerve terminal level revealed that this involved the ability of α 7-containing nAChR to selectively bolster GluN2A-containing NMDA receptor function. Further biochemical studies showed that nAChR activation enhanced the plasma membrane levels of

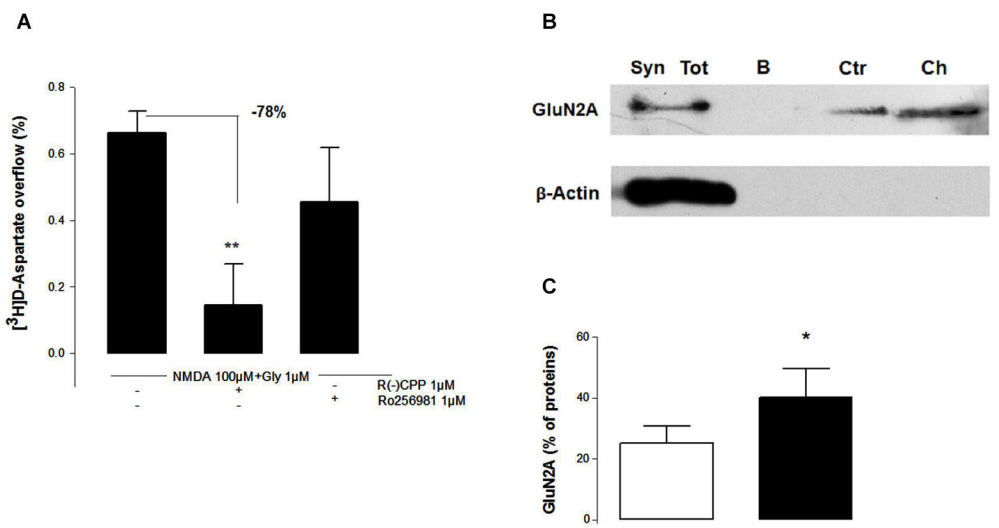

FIGURE 6 | Nicotinic acetylcholine receptors activation selectively bolsters GluN2A-dependent [^{3}H]D-Asp release (A) and GluN2A membrane insertion (B, C) in NAc terminals. **(A)** The selective GluN2A-NMDAR antagonist R(-)CPP, but not the GluN2B-NMDAR antagonist Ro256981, attenuated the potentiating effect resulting from the pre-treatment for 10 min with (1 mM Choline) of the evoked [^{3}H]D-Asp release from rat NAc synaptosomes triggered by 100 μM NMDA and 10 μM glycine. Values are mean ± SEM of six experiments run in triplicate. **$p < 0.01$ vs. control using a one-way ANOVA followed by Tukey-Kramer *post hoc* test. **(B)** Representative Western blot of GluN2A subunit surface density in NAc terminals. The Western blots compares total synaptosomal membranes before adding biotin (Syn Tot), synaptosomal membranes that are not treated with biotin and are subject to a streptavidin pull-down **(B)**, synaptosomal membranes incubated with biotin and subject to a streptavidin pull-down (Ctr) and membranes from synaptosomes that were pre-treated for 1 mM choline before incubation with biotin and pull-down with streptavidin (Ch). The blots are representative of four different experiments carried out with synaptosomal preparations from different rats. **(C)** Comparison of the average density of biotin-labelled GluN2A proteins in NAc synaptosomal membranes without (open bars) and after (filled bars) a 10 min exposure to 100 μM nicotine. Values are mean ± SEM of four experiments. *$p < 0.05$ using a paired Student's *t* test.

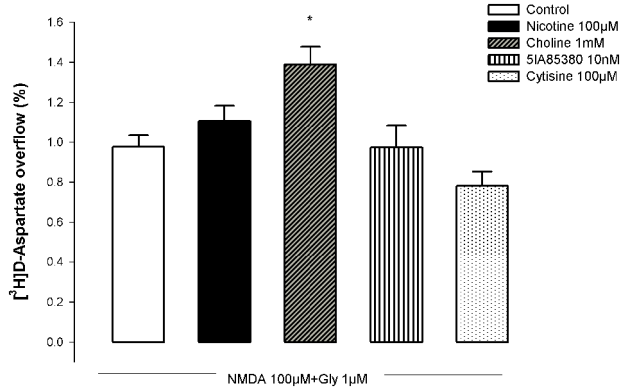

FIGURE 7 | Impact of the pre-treatment with different nAChR agonists on the ability of NMDA to trigger [^{3}H]D-Asp outflow from nerve terminals of the rat hippocampus. Each nAChR agonist was added 10 min before challenging with NMDA. Values are mean ± SEM of five experiments run in triplicate. * $p < 0.05$ vs. control.

GluN2A subunits in NAc terminals, allowing to argue that the nAChR-mediated control of GluN2A trafficking into the plasma membrane underlies the potentiation of presynaptic NMDAR-mediated actions by nAChR activation in NAc glutamatergic terminals.

Although ionotropic receptors are traditionally recognized as supporting fast synaptic transmission by acting as postsynaptic sensors of released neurotransmitters, evidence accumulated over the last decades also supports a parallel fine-tuning neuromodulation role for ionotropic receptors as controllers of the release of different neurotransmitters (MacDermott et al., 1999; Dorostkar and Boehm, 2008), with critical impact on adaptive changes of synaptic efficiency (Sjöström et al., 2003; Corlew et al., 2008; Bidoret et al., 2009). Accordingly, it has been shown that different nAChR and NMDAR subtypes are present in glutamatergic nerve terminals in different brain areas, where they efficiently modulate the release of glutamate (McGehee et al., 1995; Marchi et al., 2002; Bardoni et al., 2004; Dickinson et al., 2008; Musante et al., 2011; Gomez-Varela and Berg, 2013). The present study provides an additional layer of complexity in the presynaptic signaling by ionotropic receptors, dwelling on the interaction between presynaptic ionotropic receptors. In fact, building on the observation that different ionotropic receptors are co-localized in nerve terminals, we explored the nature of their interactions to grasp the fine-tuning of neurotransmitter release. Thus, our immunocytochemical findings showed that both α7 and α4 nAChR were co-localized with GluN1 subunits of NMDAR in NAc nerve terminals, namely in glutamatergic nerve endings. This led to the key observation that the two modulation systems are actually engaged in a cross-talk, since the pre-treatment of NAc synaptosomes with nicotine caused a significant increase of the NMDA-evoked intra-terminal cytosolic free calcium transient and [^{3}H]D-Asp outflow.

It has been previously described that glutamate exocytosis is controlled by α7-nAChR and by α4β2-nAChR subtypes (Dickinson et al., 2008; Zappettini et al., 2010). However, our pharmacological characterization showed a primary involvement of α7-nAChR controlling presynaptic NMDA responses, based on the effects of the α7-nAChR-selective agonist choline and α7-nAChR-selective antagonist α-bungarotoxin. This is further confirmed by the lack of effect of 5IA85380, indicating the inability α4β2-nAChR to modify the functional response of presynaptic NMDAR. This contention is further strengthened by our observation that nicotine or choline triggered an increase of the NMDA-induced intra-terminal calcium transients selectively in glutamatergic nerve endings (see **Figure 3**), which were also endowed with α7-nAChR. Notably, the impact of nAChR activation was qualitatively similar and displayed a similar pharmacology when measuring the NMDA-induced intra-terminal calcium transients or the release of [^{3}H]D-Asp. This strongly suggests that the increased NMDA-evoked outflow of glutamate probably results from the modulation of the calcium transient. Furthermore, it should be noted that α4-nAChR are also present in glutamatergic terminals (see **Figure 4**) and can trigger calcium entry into nerve terminals (Dickinson et al., 2007; Zappettini et al., 2010). However, α7-nAChR triggers a direct calcium entry, whereas the α4-nAChR-mediated increase of intra-terminal free calcium levels involves a depolarization of the terminal and the subsequent activation of voltage-sensitive calcium channels (Dickinson et al., 2007). This prompts the hypothesis that the different mechanisms of nAChR-induced raise of intra-terminal free calcium may be linked to their different ability to control presynaptic NMDAR function, a question that remains to be solved.

The pharmacological characterization of the nAChR-mediated control of presynaptic NMDAR responses also allowed establishing the selective involvement of GluN2A-containing NMDAR, in spite of the known presence of both GluN2A and GluN2B subunits in NMDA autoreceptors located in hippocampal glutamatergic nerve endings (Luccini et al., 2007). In fact, the NMDA-induced outflow of [^{3}H]D-Asp was selectively attenuated by selective antagonists of GluN2A-containing NMDAR, whereas selective GluN2B antagonists were devoid of effects. Additionally, the pre-activation of nAChR selectively bolstered the amplitude of the inhibitory effect of GluN2A antagonists, rather than that of GluN2B antagonists, further indicating the selective nAChR modulation of presynaptic GluN2A-containing NMDAR. This was further re-enforced by the biochemical identification of an increased density of GluN2A subunits in the plasma membrane of NAc terminals after pre-activation of nAChR. This poses the control of the trafficking of NMDAR subunits as the likely mechanism operated by nAChR to bolster the effects of presynaptic NMDAR, whereas a possible impact on the exocytotic machinery is made unlikely by the lack of effect of α7-nAChR activation on the 4AP-evoked [^{3}H]D-Asp outflow. Although the intracellular pathway operated by nAChR to control GluN2A trafficking remains to be defined, this might involve a nAChR-mediated control of kinase activity, since NMDAR trafficking is regulated by phosphorylation (Lan et al., 2001; Chen and Roche, 2007; Lau and Zukin, 2007).

We have previously reported that nAChR also controlled NMDAR-mediated responses in NAc dopaminergic terminals, but we found that nAChR activation depressed presynaptic NMDAR-mediated responses (Salamone et al., 2014), in contrast

to the potentiation observed in NAc glutamatergic terminals and described above. Remarkable, in NAc dopaminergic nerve terminals, we observed that it was the activation of α4β2-nAChR that depressed GluN2B containing NMDAR (Salamone et al., 2014), instead of α7-nAChR potentiating GluN2A containing NMDAR in NAc glutamatergic terminals. Taken together, these findings indicate a striking difference between the interplay of nAChR and NMDAR in different nerve terminals, which seems to depend on the types of nAChR and of NMDAR playing the prime role in each different type of nerve terminal within the NAc. This prompted us to test if there were also differences between brain areas and we found that nAChR activation also triggered a potentiation of NMDAR-induced release of [^{3}H]D-Asp from hippocampal nerve terminals, as occurred in the NAc glutamatergic terminals. It still remains to understand the signaling mechanisms responsible for the different setup of nAChRs and NMDARs in different types of nerve terminals in the brain.

There is increasing recognition of the importance of presynaptic NMDAR on the control of synaptic plasticity (Sjöström et al., 2003; Corlew et al., 2008; Bidoret et al., 2009), together with the role that adaptive changes in the efficiency of glutamatergic synapses may have in the addictive behavior (Ma et al., 2009; Kalivas and Volkow, 2011; Grueter et al., 2012). We characterized the ability of nAChRs to bolster presynaptic NMDAR-mediated responses in NAc glutamatergic terminals. This nAChRs-mediated control of NMDAR function in glutamatergic terminals of the NAc could help to understand the parallel effects of cholinergic and glutamatergic systems on higher brain functions involving information processing in NAc circuits such as mood, memory or addiction (Carlezon and Thomas, 2009; Reissner and Kalivas, 2010).

AUTHOR CONTRIBUTIONS

Stefania Zappettini, performed calcium imaging analysis, immunocytochemical experiments and release experiments, revised critically the paper and approved the final version; Massimo Grilli contributed to the design of the work, coordinated and performed the release experiments, revised critically the paper and approved the final version, Guendalina Olivero, Jiayang Chen and Cristina Padolecchia performed the release experiments and revised critically the paper and approved the final version; Anna Pittaluga contributed to the design of the work, revised critically the paper and approved the final version; Angelo R. Tomé and Rodrigo A. Cunha contributed to the design of the work and coordinated the calcium imaging analysis and immunocytochemical experiments, revised critically the paper and approved the final version, Mario Marchi provided a substantial contributions to the design of the work and to the interpretation of data and wrote the paper.

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