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Regulation of NMDA glutamate receptor functions by the GluN2 subunits

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Abstract

The *N*-methyl-*D*-aspartate receptors (NMDARs) are ionotropic glutamate receptors that mediate the flux of calcium (Ca^{2+}) into the postsynaptic compartment. Calcium influx subsequently triggers the activation of various intracellular signaling cascades that underpin multiple forms of synaptic plasticity. Functional NMDARs are assembled as heterotetramers composed of two obligatory GluN1 subunits and two GluN2 or GluN3 subunits. Four different GluN2 subunits (GluN2A-D) are present throughout the central nervous system; however, they are differentially expressed, both developmentally and spatially, in a cell- and synapse-specific manner. Each GluN2 subunit confers NMDARs with distinct ion channel properties and intracellular trafficking pathways. Regulated membrane trafficking of NMDARs is a dynamic process that ultimately determines the number of NMDARs at synapses, and is controlled by subunit-specific interactions with various intracellular regulatory proteins. Here we review recent progress made towards understanding the molecular mechanisms that regulate the trafficking of GluN2-containing NMDARs, focusing on the roles of several key synaptic proteins that interact with NMDARs via their carboxyl termini.

Graphical Abstract

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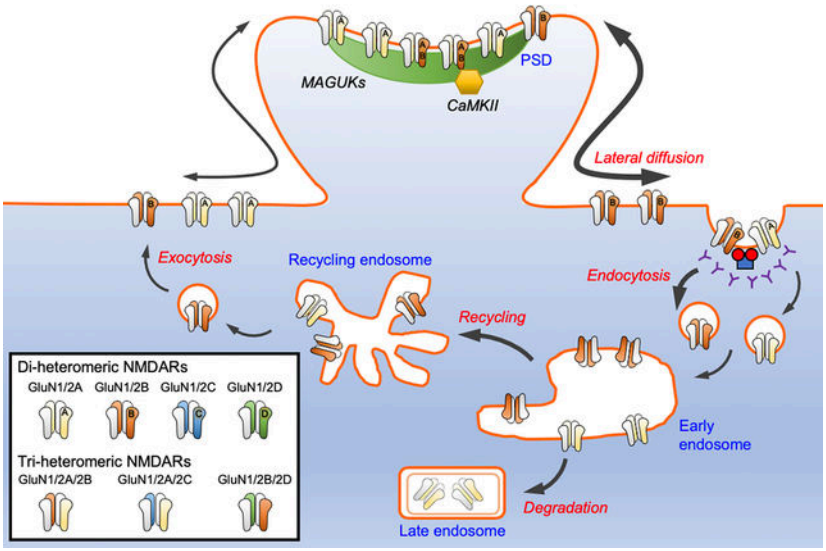

The *N*-methyl-*D*-aspartate glutamate receptors (NMDARs) mediate calcium-dependent signaling that underpins multiple forms of synaptic plasticity. Different GluN2 (GluN2A-D) subunits confer NMDARs with distinct ion channel properties and intracellular trafficking pathways. This review article summarizes the current knowledge of the molecular mechanisms that regulate the trafficking of GluN2-containing NMDARs, focusing on the roles of several key binding partners.

Keywords

NMDA receptors; synaptic plasticity; endosomal recycling; endocytosis; exocytosis; receptor trafficking; protein-protein interactions

Glutamate is the major excitatory neurotransmitter in the brain. At most excitatory synapses, the postsynaptic specialisation that receives input from a presynaptic nerve terminal exists as a small membrane protrusion from the dendrite, known as the dendritic spine. Each spine contains glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-*D*-aspartate (NMDA) receptors. In response to glutamate binding, the AMPA receptors (AMPARs) open and allow sodium (Na^+) ions to enter the postsynaptic compartment, thereby mediating fast excitatory postsynaptic currents (EPSCs) that result in the depolarisation of the postsynaptic membrane. Under resting membrane potential, most NMDARs are inactive due to the voltage-dependent block by extracellular magnesium (Mg^{2+}) ions. Upon heightened neuronal activity, such as during long-term potentiation (LTP), the Mg^{2+} blockade is relieved, leading to the opening of NMDARs that permit Ca^{2+} influx into the postsynaptic compartment (Nicoll & Roche 2013). The rise in intracellular Ca^{2+} subsequently triggers various signaling cascades, including the activation of the protein kinase Ca^{2+}/calmodulin-dependent kinase II (CaMKII), leading to profound reorganisation of the molecular composition of the postsynaptic density (Lisman *et al.* 2012). This causes subsequent enlargement of dendritic spines and an increase in the number of AMPARs on the plasma membrane, producing a long-lasting increase in synaptic efficacy. Given the major function of NMDARs as “coincidence detectors” of postsynaptic

depolarisation (which relieves the Mg^{2+} block) and the presynaptic release of glutamate (which binds to GluN2 subunits) during synaptic plasticity, pharmacological and genetic manipulations that interfere with NMDAR functions usually lead to impairments in synaptic plasticity and deficits in cognition, learning and memory in mice. Elucidating the molecular mechanisms that control NMDAR trafficking and function is therefore critical for understanding the cellular basis of neuronal plasticity and experience-dependent changes in adaptive behaviour.

NMDA Receptor Structure and Subunit Composition

Like other ionotropic glutamate receptors, NMDARs are assembled as tetrameric complexes of subunits that are permeable to Na^+, potassium (K^+) and Ca^{2+} (Traynelis *et al.* 2010). NMDAR subunits can be categorized into three different classes according to their sequence homology, including the glycine/D-serine binding GluN1 and GluN3 (GluN3A and GluN3B) subunits, as well as the glutamate binding GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D) (Fig. 1a) (Traynelis *et al.* 2010; Paoletti *et al.* 2013). All NMDAR subunits consist of a highly homologous extracellular amino-terminal domain (ATD), a bi-lobed ligand-binding domain (LBD), and three transmembrane regions and a re-entrant ion pore-lining loop; as well as a more divergent intracellular carboxyl-terminal domain (CTD) (Fig. 1b). Each of these subunits are encoded by a distinct gene in the mammalian genome. Functional NMDARs are assembled as di-heteromers composed of two obligatory GluN1 subunits and two GluN2 or GluN3 subunits (Fig. 1c). However, a variety of studies have also reported the presence and prevalence of tri-heteromeric NMDARs containing GluN1 and two different GluN2 subunits in many brain regions and neuronal types, such as GluN1/2A/2B, GluN1/2A/2C and GluN1/2B/2D complexes (Fig. 1c) (Rauner & Kohr 2011; Tovar *et al.* 2013; Luo *et al.* 1997; Brickley *et al.* 2003; Pina-Crespo & Gibb 2002; Jones & Gibb 2005; Chazot *et al.* 1994; Swanger *et al.* 2015; Sheng *et al.* 1994).

Activation of NMDARs requires the simultaneous binding of glutamate and glycine/D-serine to the GluN2 and GluN1 subunits respectively. Glutamate binds to GluN2 subunits in a pocket located in the extracellular ligand-binding domain. Interestingly, all four GluN2 subunits display striking differences in their spatiotemporal expression patterns in the brain, as well as unique pharmacological and functional properties (Monyer *et al.* 1994; Sheng *et al.* 1994; Hansen *et al.* 2017; Paoletti *et al.* 2013). These distinctions essentially create a repertoire of functional NMDARs with stark biophysical and signaling properties that underpin synaptic plasticity, dendritic integration and information processing in various regions of the brain.

Biophysical properties of GluN2-containing NMDARs

NMDARs possess several unique features that distinguish them from the AMPA- or kainate-type of ionotropic receptors, including: strong voltage-dependent block by extracellular Mg^{2+}, the requirement for simultaneous binding of a co-agonist (glycine or D-serine) for activation, high Ca^{2+} permeability and slow deactivation kinetics (Hansen *et al.* 2018; Glasgow *et al.* 2015; Wyllie *et al.* 2013). These gating and ion channel properties differ among NMDAR subtypes and are determined by their subunit compositions (Fig. 1d). For

instance, di-heteromeric GluN1/2A and GluN1/2B receptors have higher single-channel conductance (γ ~ 40–50 pS), Ca^{2+} permeability (P_{Ca}/P_{Cs} ~ 7.5), and Mg^{2+} sensitivity (IC_{50} ~ 15 μM at −100 mV) compared to GluN1/2C and GluN1/2D receptors (γ ~ 18–35 pS; P_{Ca}/P_{Cs} ~ 4.5; IC_{50} ~ 80 μM at −100 mV). On the other hand, GluN1/2D receptors have the highest affinity for glutamate (EC_{50} ~ 0.4 μM) compared to other GluN2-containing di-heteromeric receptors (GluN2C, EC_{50} ~ 1 μM > GluN2B, EC_{50} ~ 2 μM > GluN2A, EC_{50} ~ 4 μM). In addition, the channel open probability when agonists are fully bound to the receptors is the highest for the GluN1/2A receptors (P_o ~ 0.5), followed by the GluN1/2B receptors (P_o ~ 0.1) and the GluN1/2C and GluN1/2D receptors (P_o ~ 0.05). Importantly, each of the GluN1/2 di-heteromeric receptor subtypes display a wide-range of deactivation time constants. GluN2A-containing receptors have the fastest deactivation kinetic (τ_{decay} ~ 50 ms), which is more than 20-fold higher than GluN2D-containing ones (τ_{decay} > 1 s), whereas GluN1/2B (τ_{decay} ~ 400 ms) and GluN1/2C (τ_{decay} ~ 290 ms) show intermediate decay times. These GluN2 subunits also impart NMDAR subtypes with differences in sensitivity to extracellular Zn^{2+}, protons, spermine and other pharmacological agonists, antagonists and allosteric modulators, which are described in detail in several excellent reviews (Hansen *et al.* 2018; Traynelis *et al.* 2010; Zhu & Paoletti 2015; Hackos & Hanson 2017). It is interesting to note that the tri-heteromeric GluN1/GluN2A/GluN2B receptors, possibly the most abundant NMDARs in the adult forebrain, display intermediate levels of agonist sensitivity, channel open probability, and deactivation kinetics, as well as in their sensitivity to ifenprodil (GluN2B specific blocker), Zn^{2+} and protons (Stroebel *et al.* 2018; Yi *et al.* 2017; Hansen *et al.* 2014). These differences in the biophysical and pharmacological properties of various GluN2 receptor subtypes determine the time-course of NMDAR-mediated EPSCs that affect synaptic integration and plasticity in various brain regions.

Spatiotemporal expression and localisation of GluN2-containing NMDARs

The expression of GluN2 subunits is differentially regulated during development across multiple brain regions. In rodents, both GluN2B and GluN2D subunits are widely expressed in the embryonic brain (Monyer *et al.* 1994; Ishii *et al.* 1993; Akazawa *et al.* 1994). The expression of GluN2B remains high, but becomes restricted to the forebrain, in the adult. By contrast, the expression of GluN2D is significantly decreased in the adult and can be found mostly in the mid-brain structures, including the diencephalon and mesencephalon. GluN2A expression starts at birth, increases progressively and becomes abundant throughout the central nervous system. Thus, in higher brain structures such as the cortex and hippocampus, NMDARs change from predominantly GluN2B-containing to GluN2A-containing during development (Sheng *et al.* 1994). Finally, GluN2C expression starts in the second postnatal week, but is confined to the cerebellum and the olfactory bulb. A similar developmental switch from GluN2B- to GluN2C-containing NMDARs also occurs in the cerebellar granule cells where GluN2B expression drops dramatically in the adult (Monyer *et al.* 1994).

In addition to their regional expression patterns in the brain, many different GluN2-containing NMDAR subtypes can also be found in distinct neuronal populations. For example, while the overall expression level of GluN2C and GluN2D is low in the cortex and hippocampus, they are specifically expressed in interneurons and glial cells (Monyer *et al.*

1994; Perszyk *et al.* 2016). Similarly, GluN2B and GluN2D are present in cerebellar Golgi cells despite their low expression in the cerebellum (Brickley *et al.* 2003). Remarkably, heterogeneity of synaptic NMDARs within individual neurons that are segregated in an input-specific manner also occurs. In the adult hippocampal CA3 region, GluN2B subunits are present in the dendrites of pyramidal neurons that receive inputs from the entorhinal cortex, but not from the dentate gyrus (Fritschy *et al.* 1998). In the layer 5 pyramidal neurons, GluN2A and GluN2B are differentially enriched at synapses of the callosal and the intracortical pathways, respectively (Kumar & Huguenard 2003).

Furthermore, the localization of different GluN2-containing NMDARs also varies at the subcellular level. In the adult forebrain, GluN2A-containing receptors are predominantly localized at synaptic sites, whereas GluN2B receptors are also found at the peri- or extra-synaptic sites (Bellone & Nicoll 2007; Hardingham & Bading 2010; Gladding & Raymond 2011). However, GluN2 subunits other than GluN2A can also mediate synaptic NMDAR currents in specific brain regions, such as in the amygdala, striatum and substantia nigra pars compacta (Lopez de Armentia & Sah 2003; Brothwell *et al.* 2008; Logan *et al.* 2007). Importantly, the subunit composition of synaptic NMDARs can be dynamically altered by activity and experience, allowing for fine regulation of receptor subtypes during plasticity. Interestingly, GluN2A and GluN2B-containing receptors were recently shown to organize in distinct nanodomains in the synapse and that this organization is also developmentally regulated (Kellermayer *et al.* 2018).

Activity-dependent switches of NMDAR subunit composition

During development, synapses possess GluN2B-containing NMDARs (Aizenman & Cline 2007; Barria & Malinow 2002) but no functional AMPARs, rendering them silent (Isaac *et al.* 1995; Isaac *et al.* 1997; Liao *et al.* 1995). For synapse maturation to occur, concomitant insertion of AMPARs (Leinekugel *et al.* 1997) and a switch in NMDAR subunit composition from GluN2B to GluN2A are necessary (Barria & Malinow 2002). This developmental switch is evolutionarily conserved and occurs in many brain areas, including cortex, hippocampus, amygdala and cerebellum. Due to the profound difference in their ion channel and gating properties, increased contribution of GluN2A subunits markedly changes the magnitude and timing of NMDAR-mediated Ca^{2+} influx, resulting in subsequent changes in dendritic integration and signaling underlying synaptic plasticity (Yashiro & Philpot 2008). The switch in NMDAR subunit composition is driven in part by developmental changes in the mRNA and protein expression of GluN2A and GluN2B, but also by sensory experience, indicating a direct contribution of neuronal activity in the process. In the developing visual cortex, the change from synaptic GluN2B- to GluN2A-containing NMDARs occurs during the critical period and is not observed in dark-reared animals, until they are exposed to light, and is reversible (Quinlan *et al.* 1999; Philpot *et al.* 2001). This process coincides with synaptic maturation, which is critical for circuit refinement, synaptic- and meta-plasticity (Yashiro & Philpot 2008; Sanz-Clemente *et al.* 2013b; Kirkwood *et al.* 1996).

A rapid switching (in minutes) of GluN2B- to GluN2A-containing NMDARs can be induced by an LTP inducing protocol in acute hippocampal slices from young animals (Bellone & Nicoll 2007). This type of plasticity is reversible and is dependent on group 1 metabotropic

glutamate receptor (mGluR) signaling, intracellular Ca^{2+} release, protein kinase C (PKC) and phospholipase C (PLC) activation (Mameli *et al.* 2011; Matta *et al.* 2011). This process is also facilitated by the phosphorylation of the GluN2B subunit by casein kinase 2 (CK2), which promotes the internalization and removal of GluN2B receptors from the neuronal plasma membrane (Sanz-Clemente *et al.* 2010). Selective rearrangement of the relative ratio of GluN2A- and GluN2B-containing NMDARs within synaptic nanodomains bidirectionally tunes neuronal plasticity (Kellermayer *et al.* 2018). These data indicate the requirement of regulated receptor trafficking as a mechanism that determines the number and subunit composition of NMDARs at synapses.

Routes of NMDA Receptor Trafficking

The number and subunit composition of NMDARs at synapses are tightly controlled by the delicate balance between the biosynthesis, dendritic transport, exocytosis, lateral diffusion, endocytosis, recycling and degradation of the receptors (Fig. 2). The cytoplasmic carboxyl termini of AMPA- and kainate receptors have been shown to play critical roles in orchestrating receptor trafficking to and stabilization at synapses (Anggono & Huganir 2012; Evans *et al.* 2019). Similarly, the mechanisms associated with NMDAR trafficking also involve the CTD, through which the receptors establish protein-protein interactions with intracellular trafficking, scaffolding and signaling molecules (Sanz-Clemente *et al.* 2013b). In addition, the CTD of GluN2 subunits undergoes a variety of post-translational modifications that function to regulate receptor trafficking and stabilization of NMDARs at synaptic sites (Lussier *et al.* 2015). The diversity of CTDs among different GluN2 subunits is therefore crucial in determining the subunit-specific trafficking of GluN2-containing NMDARs in mammalian central neurons.

Receptor biogenesis

Tetrameric NMDARs are assembled in the endoplasmic reticulum (ER) and mature through a series of glycosylation steps in the Golgi prior to their trafficking in vesicles to the plasma membrane (Horak *et al.* 2014). Cells have evolved strict mechanisms to ensure that no unassembled or misfolded NMDARs are transported out to the cell surface. Transfection of individual GluN2 and some GluN1 splice variants results in retention in the ER unless assembled as GluN1/GluN2 tetramers (McIlhinney *et al.* 1998). Importantly, genetic deletion of GluN1 leads to an accumulation of GluN2 subunits in the ER in the hippocampus (Fukaya *et al.* 2003). One of the underlying mechanisms is the presence of ER retention signals in different regions of GluN subunits, which are masked by complementary subunits when assembled into functional tetramers (Horak *et al.* 2008). For instance, the obligatory GluN1 subunit contains well characterized ER retention motifs (KKK and RRR) in the C-terminal C1 cassette (Standley *et al.* 2000; Scott *et al.* 2001; Horak & Wenthold 2009). Overexpression of GluN2A and GluN2B in the cerebellar granule neurons significantly promote the number and synaptic targeting of NMDARs (Prybylowski *et al.* 2002). The proximal C-terminal region of the GluN2B subunit has been proposed to also contain an ER retention motif (HLFY) (Hawkins *et al.* 2004). However, subsequent mutagenesis and deletion studies suggest that the HLFY motif is playing a structural role in orienting the C-terminal domain of GluN2B during ER processing, but it is not sufficient to act as a

retention signal (Yang *et al.* 2007). The ER retention signals in GluN2 subunits are generally not well-defined to date.

An important step in the NMDAR secretory pathway lies in the interaction between GluN2 subunits with members of the membrane-associated guanylate kinase (MAGUK) family of proteins, particularly synapse-associated protein-102 kDa (SAP102) and −97 kDa (SAP97) (Sans *et al.* 2003; Sans *et al.* 2005). SAP102 is highly expressed in the hippocampus as early as postnatal day 2 and binds to both GluN2A and GluN2B subunits of NMDARs through their C-terminal postsynaptic density-95/discs-large/zona-occludens-1 (PDZ) binding motifs and a PDZ domain of SAP102 (Sans *et al.* 2000; Muller *et al.* 1996). Interestingly, SAP102 is localized not only at synapses, but also in the cytoplasm and ER (Sans *et al.* 2000). As a multi-PDZ domain containing protein, SAP102 acts as a scaffold for a complex containing the exocyst protein Sec8 and GluN2B (Sans *et al.* 2003). Furthermore, through its Src-homology 3 (SH3)/guanylate kinase (GK) domains, SAP102 can interact with mPins to stabilize the SAP102-exocyst-NMDAR complex in the ER, a process that is crucial for promoting the forward trafficking and membrane targeting of NMDARs in neurons (Sans *et al.* 2003; Sans *et al.* 2005).

NMDARs have also been proposed to bypass the conventional somatic Golgi network that is commonly associated with AMPAR trafficking. These receptors are instead sorted via an ER sub-compartment that directly merges with dendritic Golgi outposts (Jeyifous *et al.* 2009). Such an alternative strategy could signify a more efficient path to promote insertion of NMDARs at the postsynaptic density (PSD). Vesicles generated from this alternative pathway are highly mobile (0.76 μm/sec) as they contain large protein complexes containing the scaffolding molecules SAP97, CASK (mLin2), X11/Mint1 (mLin10) and mLin7 (MALS/Velis) that couple GluN2B to the motor protein KIF17 and facilitate the long-range transport of NMDAR-containing vesicles on microtubules along dendrites (Jeyifous *et al.* 2009; Setou *et al.* 2000; Guillaud *et al.* 2003). KIF17-mediated transport of NMDARs is essential for LTP, LTD, learning and memory (Yin *et al.* 2011). Genetic deletion of *kif17* causes a significant, but not complete, loss of synaptic GluN2A and GluN2B receptors due to enhanced ubiquitin-mediated degradation of NMDARs. This suggests that other kinesin motor proteins could be involved in mediating the microtubule-based anterograde transport of NMDARs along dendrites. Indeed, KIF1B has been reported to interact directly with SAP97 and SAP102; however, its role in regulating the transport of NMDARs in the secretory pathway has not been determined (Mok *et al.* 2002). It is worth noting that the binding of CASK induces SAP97 into an extended confirmation that preferentially binds NMDARs relative to AMPARs (Lin *et al.* 2013). The function of SAP97 is tightly regulated by the phosphorylation of CaMKII on two key sites, Ser-39 (within the L27 domain) and Ser-232 (within the PDZ1 domain) (Gardoni *et al.* 2003; Mauceri *et al.* 2007). SAP97 phosphorylation at Ser-39 drives the translocation of SAP97 from the ER to the postsynaptic compartment, whereas its phosphorylation at Ser-232 disrupts SAP97 interaction with GluN2A. In addition, CaMKII-dependent phosphorylation of KIF17 has also been shown to promote the unloading of cargoes from microtubules (Yin *et al.* 2012). Thus, sequential phosphorylation of SAP97 and KIF17 may conceivably provide the driving force to release NMDAR complexes from the ER and facilitate the insertion of receptors at synapses.

Palmitoylation of NMDARs has recently emerged as an important regulator of post-Golgi trafficking of NMDARs (Thomas & Huganir 2013; Lussier *et al.* 2015). Both GluN2A and GluN2B subunits undergo activity-dependent palmitoylation at two distinct clusters in their CTDs (Hayashi *et al.* 2009). Palmitoylation of GluN2A and GluN2B in the second cluster of cysteines in the middle of their CTDs retains and accumulates NMDARs in the Golgi apparatus, and therefore reduces receptor surface expression (Hayashi *et al.* 2009). Consequently, mutations of cysteines in the second cluster leads to an increase in surface expression of NMDARs. However, these mutations do not increase the levels of synaptic NMDARs (Mattison *et al.* 2012). Importantly, in the YAC128 striatal neurons, aggregated mutant huntingtin proteins disrupt the interaction between two palmitoyl acetyltransferases, zDHHC13 and zDHHC17, and GluN2B, leading to a reduction in the level of GluN2B palmitoylation on cluster II (Kang *et al.* 2019). This subsequently causes an increase in the levels of extrasynaptic GluN2B-containing NMDARs, which may contribute to the cell death-signaling pathways underlying Huntington's disease.

Exocytosis

Dendritic exocytosis of many neurotransmitter receptors, including NMDARs, is a tightly regulated process (Kennedy & Ehlers 2011). Exocytosis of NMDARs takes place mainly at extrasynaptic sites along the somatodendritic compartment (Gu & Huganir 2016), with the receptors then relocating to the synaptic membrane via lateral diffusion (Ladepeche *et al.* 2014). In general, vesicular fusion to the plasma membrane is mediated by the assembly soluble N-ethylmaleimide (NEM)-sensitive factor attachment protein receptors (SNAREs) protein family, including the target SNARE (t-SNARE) syntaxin, synaptosome-associated protein (SNAP) and the vesicular SNARE (v-SNARE) vesicle-associated membrane protein (VAMP) (Sudhof & Rothman 2009). Although the requirement of the SNARE complex in NMDAR exocytosis is well established, the precise contribution of specific members within this protein family remains controversial.

Earlier studies identified a critical role for SNAP25 in mediating the exocytosis of NMDARs, first in *Xenopus* oocytes and later in CA3 pyramidal neurons (Lan *et al.* 2001a; Lan *et al.* 2001b). In these studies, the authors demonstrated that mGluR1- and PKC-dependent exocytosis of NMDARs can be blocked by botulinum toxin A, a neurotoxin that specifically cleaves and inactivates SNAP25. Subsequently, SNAP25 was found to be a substrate of PKC and mutation of a single SNAP25 phosphorylation site (Ser-187) could abolish PKC-induced insertion of NMDARs (Lau *et al.* 2010). Consistent with this, shRNA-mediated knockdown of SNAP25 reduces NMDAR-mediated transmission in the hippocampus (Jurado *et al.* 2013).

In contrast, other studies have provided compelling evidence for another SNAP family member that is enriched at the excitatory synapses on dendritic spines, SNAP23, in regulating the forward trafficking of endogenous NMDARs (Washbourne *et al.* 2004; Suh *et al.* 2010). Genetic deletion of SNAP23 ($SNAP23^{+/-}$) significantly reduces surface expression of endogenous GluN1, GluN2A and GluN2B subunits of NMDARs without affecting AMPAR levels (Suh *et al.* 2010). Furthermore, shRNA-mediated knockdown of SNAP23, but not of SNAP25, causes a similar decrease in the levels of surface NMDARs.

The apparent discrepancies among these studies may arise from differences in the age and neuronal subtypes used, as well as in the choice of reagents and methodologies employed (knockout vs knockdown, endogenous vs overexpression). It is plausible that SNAP23 and SNAP25 may differentially regulate the exocytosis of NMDARs originating from separate intracellular pools (*de novo* insertion vs recycling) or during basal vs activity-dependent conditions.

In a more recent study, exocytosis of overexpressed super-ecliptic pH-sensitive green fluorescent protein (SEP)-tagged NMDARs was visualized directly in neurons using a total internal reflection florescence microscopy (Gu & Huganir 2016). This approach identified the SNAP25–VAMP1–syntaxin4 as the core SNARE complex essential for constitutive exocytosis of NMDARs. Consistent with this study, SNAP25 and syntaxin4 have previously been shown to mediate the group II mGluR-dependent increase in surface NMDARs in cortical pyramidal neurons (Cheng *et al.* 2013). Furthermore, genetic deletion of syntaxin4 leads to reduced surface expression of NMDARs and a deficit in LTP (Bin *et al.* 2018). Given the role of syntaxin4 as the t-SNARE for AMPARs (Kennedy *et al.* 2010; Bin *et al.* 2018), it raises the question whether both AMPARs and NMDARs are co-delivered to the plasma membrane on the same vesicles.

Lateral diffusion

NMDARs diffuse laterally on the plasma membrane between synaptic and extrasynaptic sites (Tovar & Westbrook 2002), an observation that upended the early dogma that NMDARs are static at synapses. This study employed electrophysiological recordings on cultured hippocampal neurons in the presence of NMDAR open-channel blockers, MK-801 or ketamine, and reported a rapid recovery of synaptic NMDAR currents, indicating the lateral movement and replacement of “unblocked” receptors from the extrasynaptic pools. In fact, advances in super-resolution microscopy and single-molecule tracking techniques have since provided direct evidence for the dynamic nature of NMDAR mobility on neuronal plasma membrane (Dupuis *et al.* 2014; Groc *et al.* 2004; Ladepeche *et al.* 2014). Lateral mobilities of synaptic AMPA- and NMDA-Rs are comparable, while extrasynaptic AMPARs are more mobile than NMDARs (Groc *et al.* 2004).

Remarkably, surface trafficking of NMDARs is regulated in a subunit-dependent manner that eventually determines the number and size of NMDAR nanoclusters within the synapse. There are several lines of evidence supporting the notion that extrasynaptically located GluN2B-containing NMDARs are more mobile than GluN2A, which remains more synaptically localized (Groc *et al.* 2006; Dupuis *et al.* 2014; Ferreira *et al.* 2017; Kellermayer *et al.* 2018). These two subtypes of NMDARs display substantial differences in their nanoscale organization, which changes during development in hippocampal neurons (Kellermayer *et al.* 2018). In mature synapses, there are considerably more GluN2A-NMDAR nanoclusters, which are also relatively larger, compared to those containing the GluN2B subunit. Synaptic retention of GluN2A requires its CTD, likely through its stable interaction with PDZ domain-containing scaffolding molecules such as PSD-95 (Kellermayer *et al.* 2018; Bard *et al.* 2010). Acute application of GluN2A-derived C-terminal peptides results in an increase in GluN2A diffusion rates and a reduction in the

number of synaptic GluN2A-NMDARs. Remarkably, a similar cell-permeable peptide designed based on the GluN2B C-terminal PDZ binding motif has little effect on the synaptic retention of GluN2B-NMDARs, despite an increase in their diffusion rates that can be readily observed. These data suggest that subunit-specific nanoscale organization of NMDARs is differentially regulated by distinct sets of PDZ scaffolds at the synapse.

Furthermore, surface mobility of NMDARs can also be regulated by neuronal activity. During chemically-induced LTP (cLTP) in immature neurons, the diffusion rate of GluN2B-NMDARs increases, which results in the redistribution of GluN2B away from glutamatergic synapses, a process that requires the activities of CaMKII and CKII (Dupuis *et al.* 2014). In contrast, cLTP does not alter the surface diffusion of GluN2A-NMDARs and does not exert any effects on GluN2B-NMDARs in immature neurons. Interestingly, the binding of co-agonists, glycine and D-serine, also differentially affects the surface mobility of NMDARs in a subunit-specific manner (Ferreira *et al.* 2017; Papouin *et al.* 2012).

In addition to the interaction with PDZ scaffolds, surface trafficking of NMDARs is also regulated by protein phosphorylation on their CTDs. One such mechanism involves the phosphorylation of GluN2B at Ser-1480, which controls its interaction with PDZ-domain containing proteins (Bard *et al.* 2010; Chiu *et al.* 2019). Moreover, modulation of GluN2A phosphorylation at Ser-900 during development impacts NMDAR currents in retinocollicular synapses, in a calcineurin-dependent mechanism (Shi *et al.* 2000; Townsend *et al.* 2004), suggesting a role of this phosphatase in the regulation of the subunit switch. Other factors that are known to modulate NMDAR diffusion include the extracellular matrix (Michaluk *et al.* 2009), secreted proteases (Lesept *et al.* 2016), growth factors (De Rossi *et al.* 2016), cell surface receptors (Ladepeche *et al.* 2013; Mikasova *et al.* 2012) and hormones (Potier *et al.* 2016; Mikasova *et al.* 2017).

Endocytosis and post-endocytic sorting

Receptor internalization is a major mechanism by which neurons regulate the abundance and function of receptors at the plasma membrane. NMDAR endocytosis is developmentally and activity regulated, with younger neurons displaying a high rate of internalization, which decreases as neurons mature (Roche *et al.* 2001). The regulation of trafficking events like receptor endocytosis and recycling has been mostly studied in GluN2A- and GluN2B-containing NMDARs and several modulators and regulators of such events have been identified. This mechanism generally involves clathrin-coated pits, which occur laterally relative to the PSD (Petralia *et al.* 2003; Blanpied *et al.* 2002), and require the adaptor protein-2 (AP-2) complex (Racz *et al.* 2004; Lavezzari *et al.* 2004; Roche *et al.* 2001). Notably, glycine conditioning promotes receptor endocytosis by increasing the interaction with the endocytic AP-2 complex, thereby priming NMDARs for internalization upon receptor activation (Nong *et al.* 2003).

Both GluN2A and GluN2B subunits contain endocytic motifs in their CTD, but GluN2B subunits display a higher rate of endocytosis than GluN2A in mature neurons (Lavezzari *et al.* 2004). Clathrin-dependent internalization of GluN2B-containing NMDARs is mediated by the $YEKL_{1472–1475}$ motif in the CTD, a few residues upstream of the PDZ-binding domain, which regulates MAGUK binding to the subunit (Lavezzari *et al.* 2003; Roche *et al.*

2001). This endocytic motif mediates the binding to the AP-2 complex, and this interaction is modulated by the phosphorylation state of Tyr-1472 by Fyn kinase. This phosphorylation leads to increased synaptic delivery of GluN2B-containing receptors and thereby enhanced NMDAR-mediated currents (Prybylowski *et al.* 2005). Notably, the analogous tyrosine in GluN2A ($YKKM_{1454–1457}$) does not mediate endocytosis of the subunit. Instead, GluN2A contains a di-leucine motif ($LL_{1319–1320}$) for regulating receptor internalization (Lavezzari *et al.* 2004). Such distinction in subunit endocytic regulation likely underlies the differential post-endocytic sorting of these two subunits.

GluN1 subunits also contribute to receptor internalization, as demonstrated by the endocytosis of heteromeric receptors containing GluN1/GluN2B-ΔCTD (Scott *et al.* 2004). In fact, two independent internalization motifs in the C0 cassette in GluN1 subunits are sufficient to mediate dynamin-dependent endocytosis of NMDARs: $YKRH_{838–841}$ and $VWRK_{858–861}$. Notably, both GluN2A ($YWKL_{841–844}$) and GluN2B ($YWQF_{842–845}$) contain similar motifs at the membrane proximal region of their CTDs, which have been shown to mediate dynamin-dependent endocytosis of the subunits. These membrane-proximal motifs were shown to target the receptors to the late endosome and lead to lysosomal degradation in all three subunits. Interestingly, while endocytosis can be driven by either GluN1 or GluN2 subunits, recycling of the receptors requires the functional GluN2 CTD.

A well-established mechanism of regulation of receptor surface expression is the phosphorylation/dephosphorylation of specific residues in the CTD of GluN2A and GluN2B subunits. Indeed, tyrosine phosphorylation of GluN2B at the endocytic motif YEKL was shown to contribute to receptor stabilization at synaptic sites by abrogating the interaction with the AP-2 complex subunits. Interestingly, palmitoylation of GluN2B within the first cluster of cysteines on the membrane proximal region exerts a synergistic effect in stabilizing surface NMDARs at synapses by increasing the phosphorylation of GluN2B at Tyr-1472 (Hayashi *et al.* 2009; Mattison *et al.* 2012). On the contrary, phosphorylation of Ser-1480, which lies on the PDZ-binding domain of GluN2B has the opposite effect, contributing to increased internalization of the receptor (Lavezzari *et al.* 2003; Prybylowski *et al.* 2005), due to impaired binding to MAGUK proteins (Chung *et al.* 2004). Phosphorylation of NMDARs on the YEKL motif can be decreased by the striatal-enriched protein tyrosine phosphatase (STEP), which directly interacts with NMDAR subunits and PSD-95 and whose downregulation leads to enhanced levels of GluN1, GluN2A and GluN2B subunits (Braithwaite *et al.* 2006; Won *et al.* 2016).

In addition to the modulatory role of post-translational modifications in surface expression (Chen & Roche 2007; Lussier *et al.* 2015), several scaffolding proteins have been identified as NMDAR regulators. The classical example of this is PSD-95, a member of the MAGUK family of scaffold proteins. Interaction with PSD-95 contributes to the stabilization of both GluN2 subunits at the cell surface, thereby reducing their rate of endocytosis (Lavezzari *et al.* 2004; Lavezzari *et al.* 2003; Roche *et al.* 2001). Another example is the protein Scribble1 (Scrib1), which was identified as an interactor of GluN2A and GluN2B subunits, via their PDZ-binding domains (Piguel *et al.* 2014). Scrib1 regulates surface expression of GluN2A/B

subunits in an activity-dependent manner, by playing a modulatory role in endocytosis via interaction with the AP-2 complex, and recycling of these subunits.

Notably, GluN2A and GluN2B seem to display differential post-endocytic sorting. Although both subunits are initially present in early endosomes, the majority of GluN2A is sorted into late endosomes, as demonstrated by increased co-localization with the late endosomal marker Rab-9, whereas GluN2B is sorted into recycling endosomes, where it co-localizes with Rab-11 (Lavezzari *et al.* 2004; Scott *et al.* 2004). Interestingly, GluN2B seems to exert a dominant effect, as seen by preferential sorting into recycling endosomes of GluN2A/GluN2B heterodimers (Tang *et al.* 2010). This sorting into recycling endosomes could constitute a readily available pool of NMDARs to be inserted at the neuronal surface upon changes in synaptic activity and reflect the dynamic nature of NMDAR cycling before synaptic development when GluN2B is expressed, but not GluN2A.

As with endocytosis, neurons can respond to changes in synaptic activity by modulating the rate of vesicle recycling, which may involve specialized protein complexes, distinct from the *de novo* trafficking of receptors to the membrane. These mechanisms have been thoroughly studied in AMPARs but new light is now being shed on the specific regulation of NMDAR recycling. A few proteins have been proposed to participate in this process. Among these is the protein RIM1, via its interaction with Rab11, a marker of recycling endosomes. Knockdown of this protein specifically affected NMDAR transmission but had no impact on AMPAR currents (Wang *et al.* 2018). Furthermore, a recent report identified neurobeachin and KIF21B as regulators of GluN2B subunit recycling (Gromova *et al.* 2018). It should be noted that although mechanisms involving the retromer complex have not been extensively addressed in NMDAR regulation, recent evidence points to a role of this protein complex in modulation of surface expression of these receptors (Wang *et al.* 2013; Choy *et al.* 2014; Farias *et al.* 2014), by promoting receptor recycling to the membrane. Binding to this complex is proposed to be regulated by phosphorylation of specific residues in the PDZ-binding motif of GluN2 subunits (Clairfeuille *et al.* 2016).

GluN2 C-terminal Interacting Proteins

Genetic deletion of the C-termini (ΔC) of GluN2A, GluN2B and GluN2C in mice reveals an indispensable role of GluN2 CTDs for NMDAR-mediated intracellular signaling and synaptic physiology (Sprengel *et al.* 1998). Among the three GluN2$^{\Delta C/\Delta C}$ lines, GluN2B$^{\Delta C/\Delta C}$ mice (lacking the entire or two thirds of the CTD) exhibit the most severe phenotype and die shortly after birth (Sprengel *et al.* 1998; Mori *et al.* 1998). Deletion of GluN2B CTD also reduces surface expression and synaptic retention of GluN2B receptors (Mori *et al.* 1998; Sprengel *et al.* 1998; Barria & Malinow 2002; Mohrmann *et al.* 2002). GluN2A$^{\Delta C/\Delta C}$ mice show severe reductions in synaptic GluN2A expression and NMDAR-mediated EPSCs, as well as impairments in LTP and contextual memory (Sprengel *et al.* 1998; Steigerwald *et al.* 2000). GluN2C$^{\Delta C/\Delta C}$ mice display impaired motor coordination and the GluN2A$^{\Delta C/\Delta C}$/GluN2C$^{\Delta C/\Delta C}$ double mutant mice show defective cerebellar synaptic plasticity (Rossi *et al.* 2002; Sprengel *et al.* 1998). Importantly, studies using both organotypic hippocampal slices (Foster *et al.* 2010) or chimeric knock-in mice (GluN2A with GluN2B CTD and GluN2B with GluN2A CTD) (Ryan *et al.* 2013) have revealed the

contribution of subunit-specific CTD of NMDARs to the regulation of synaptic signalling, plasticity and complex behaviour. Altogether, these studies provide strong evidence for the crucial roles that the CTD of GluN2 subunits and their C-terminal interacting proteins play in controlling the precise subcellular localisation, synaptic targeting and intracellular Ca^{2+} signaling of GluN2-containing NMDARs. In this review, we will focus only on a subset of key NMDAR-interacting proteins. Other proteins that have been reported to interact with NMDAR CTD are summarized in Table 1.

MAGUKs

PSD-95 was the first member of the MAGUK family identified as the main scaffold protein that anchors and stabilizes surface NMDARs at glutamatergic synapses (Kornau *et al.* 1995; Niethammer *et al.* 1996). Other MAGUK family members, including SAP102, SAP97 and PSD-93 were subsequently shown to interact with the C-terminal type I PDZ binding motif (-ES[D/E]V) in the GluN2 subunits of NMDARs (Muller *et al.* 1996; Niethammer *et al.* 1996; Kim *et al.* 1996). These MAGUKs are defined by the presence of three conserved PDZ domains, one SH3 domain followed by a catalytically inactive GK modular domain (Long *et al.* 2003; McGee *et al.* 2001; Doyle *et al.* 1996). Although they share similar structural features, these proteins are vastly different in terms of their protein interaction and distribution profiles during development, and in different regions of the brain or subcellular compartments in neurons. For instance, both PSD-93 and PSD-95 are highly expressed at later stages of postnatal development, whereas SAP102 is expressed earlier in development (Sans *et al.* 2000). PSD-93 and PSD-95, as their names suggest, are found on the PSD; however, SAP97 and SAP102 are distributed along axons and dendrites as well as in cytoplasm and at synapses (Valtschanoff *et al.* 2000; Rumbaugh *et al.* 2003). With respect to protein-protein interactions, these MAGUK proteins demonstrate similar protein specificity *in vitro*; however, they exhibit a different specificity profile *in vivo*, such that PSD-95 has a preference for GluN2A, whereas SAP102 has a stronger affinity for GluN2B (Sans *et al.* 2000). In immature synapses, SAP102-GluN2B complexes are highly expressed, but as the synapses reach maturity, PSD-95/PSD-93-GluN2A are the predominant complexes (Sans *et al.* 2000; Elias *et al.* 2008).

MAGUKs play a critical role in the formation and maintenance of the PSD (Won *et al.* 2017). At the ultrastructural level, PSD-95 appears as vertically oriented filaments that anchor both AMPA- and NMDARs at the PSD (Chen *et al.* 2008). Genetic deletion or shRNA-mediated knockdown of PSD-95 results in the fragmentation of the PSD and a patchy loss of synaptic AMPARs, but not NMDARs (Chen *et al.* 2011b; Beique *et al.* 2006; Elias *et al.* 2006). Moreover, downregulation of the expression of individual MAGUK family members does not significantly affect NMDAR-mediated transmission (Elias *et al.* 2006; Howard *et al.* 2010). These data suggest that, at least for the maintenance of the number of NMDARs at synapses, these MAGUKs display some levels of functional redundancy and are able to compensate for the loss of function of an individual family member. However, simultaneous downregulation of two (PSD-95 and PSD-93) or three (PSD-95, PSD-93 and SAP102) MAGUKs causes a profound loss of functional synaptic glutamate receptors due to disintegration of the PSD structure (Elias *et al.* 2006; Chen *et al.* 2015; Levy *et al.* 2015). Indeed, overexpression of either SAP97 or SAP102 fully restores glutamatergic transmission

in neurons derived from PSD-93/PSD-95 double-knockout mice (Elias *et al.* 2006; Howard *et al.* 2010).

SAP102 and PSD-95 both function to traffic and localise NMDARs in a subunit-dependent manner (Sans *et al.* 2000; Elias *et al.* 2008). SAP102 is expressed early in development and binds to GluN2B to mediate synaptogenesis. Following synaptogenesis, PSD-95 replaces the function of SAP102 to regulate the maturational switch of GluN2B to GluN2A. Knockdown of SAP102 during early development reduces both AMPAR- and NMDAR-mediated postsynaptic currents, whereas mice lacking PSD-95/PSD-93 fail to undergo the subunit switch from GluN2B to GluN2A (Elias *et al.* 2008), highlighting the role of PSD-95 and SAP102 at distinct developmental stages of excitatory synapses. Apart from the canonical PDZ binding of SAP102 to GluN2B, a secondary PDZ independent interaction to GluN2B was identified on the N-terminus of SAP102 (Chen *et al.* 2011a). The PDZ independent interaction is thought to mediate the removal of synaptic GluN2B containing NMDARs to the extra synaptic membrane (Chen *et al.* 2012a).

The clustering of NMDARs at synapses is mediated largely by an interaction between GluN2B with the PDZ domains of PSD-95. In accordance, the deletion of the C-terminal PDZ binding motif of GluN2B heightens its internalization rate (Roche *et al.* 2001) and alters the expression of synaptic GluN2A and GluN2B receptors (Sanz-Clemente *et al.* 2010). Moreover, overexpression of a C-terminally truncated form of the GluN2A and GluN2B subunits leads to impaired localization of synaptic NMDARs *in vivo* (Steigerwald *et al.* 2000). Phosphorylation of GluN2B at Ser-1480 by CK2 disrupts PSD-95 binding, leading to a significant reduction of surface and synaptic NMDARs, an important mechanism that plays a role in the GluN2B to GluN2A switch during development (Sanz-Clemente *et al.* 2010). More recently, PSD-95 has been shown to interact with the tyrosine phosphatase STEP61 and promotes its degradation via the ubiquitin-proteasome system (Won *et al.* 2016). STEP61 is known to dephosphorylate GluN2B at Tyr-1472, which is part of the YEKL endocytic motif, and induces the internalization of GluN2B (Snyder *et al.* 2005; Kurup *et al.* 2010). These data highlight multiple mechanisms exerted by PSD-95 in controlling the trafficking and stabilization of surface NMDARs.

Although binary interactions between individual GluN2 subunits with MAGUKs *in vitro* relies on these C-terminal PDZ binding motifs, a recent study has revealed that they are dispensable for the formation of the 1.5 mega-Dalton NMDAR-MAGUK supercomplexes *in vivo* (Frank et al. 2016; Frank & Grant 2017). The assembly of NMDAR-MAGUK supercomplexes follows the “tripartite rule” that describes the genetic requirement of PSD-95, PSD-93 and GluN2B. Interestingly, although the entire CTD of GluN2A is dispensable for the assembly of these supercomplexes, the C-terminal tail of GluN2B (~600 amino acids without the PDZ binding motif) is absolutely essential. Sequences upstream of the PDZ motifs are most variable among different GluN2 subunits, and they may possess different regulatory signals that confer distinct binding affinities towards different MAGUK or other PDZ domain-containing proteins (Bard *et al.* 2010; Clairfeuille *et al.* 2016; Frank *et al.* 2016; Chen *et al.* 2012b).

CaMKII

CaMKII is a multi-subunit protein kinase comprising 12 catalytically active subunits that is crucial for synaptic plasticity, learning and memory (Herring & Nicoll 2016; Lisman *et al.* 2012; Hell 2014). It is composed of mainly the α and β isoforms, is enriched in the PSD, and is one of the most abundant proteins in the brain (Chen *et al.* 2005). Each subunit present on the holoenzyme has a central organizing hub formed from its carboxy-terminal, a linker region, two rings of 6 catalytic kinase domains and a regulatory segment (S-site) that regulates the activity of the kinase domain (Hoelz *et al.* 2003). Under the autoinhibited state, the CaMKII dodecamer is inactive as the conformation of the enzyme is folded in a pseudosubstrate manner, in which the catalytic domain remains inaccessible for Ca^{2+}/calmodulin binding. Upon NMDAR activation, Ca^{2+} binds to calmodulin, which in turns binds to the catalytic domain of CaMKII, alleviating autoinhibition and inducing autophosphorylation on Thr-286. The same stimulation also drives the translocation of CaMKII from the cytosol into the PSD (Shen & Meyer 1999; Bayer *et al.* 2001). This autophosphorylation allows the activity of CaMKII to persist long after the removal of Ca^{2+} and calmodulin in the PSD (Colbran & Brown 2004; Bayer & Schulman 2019).

A large body of evidence has demonstrated that CaMKIIα is both necessary and sufficient for NMDAR-dependent LTP (Silva *et al.* 1992; Giese *et al.* 1998; Pettit *et al.* 1994; Incontro *et al.* 2018). In one model, CaMKII-induced synaptic potentiation is associated with the phosphorylation and insertion of AMPARs into the synapses (Hayashi *et al.* 2000; Lee *et al.* 2003), as well as through an enhancement in AMPAR single channel conductance (Derkach *et al.* 1999; Kristensen *et al.* 2011). On the other hand, CaMKII also plays a critical structural role through its interaction with F-actin to maintain activity-dependent enlargement of dendritic spines during LTP (Bosch *et al.* 2014; Kim *et al.* 2016; Incontro *et al.* 2018). Given the localization, abundance, and catalytic activity, CaMKII is a versatile central organizer of the PSD during LTP.

One of the major interacting partners for CaMKII is the GluN2B subunit of NMDARs (Bayer *et al.* 2001; Leonard *et al.* 1999; Leonard *et al.* 2002; Strack & Colbran 1998). Their interaction is enhanced by NMDAR activity and requires either Ca^{2+}/calmodulin or Thr-286 autophosphorylation. Genetic manipulations that disrupt the binding between CaMKII to NMDARs by either overexpression or the generation of knock-in mice containing point mutations on the CTD of GluN2B (R1300Q/S1303D or L1298A/R1300Q) or on the kinase domain of CaMKII (I205K) severely impair LTP (Barria & Malinow 2005; Halt *et al.* 2012; Incontro *et al.* 2018; Zhou *et al.* 2007). More recently, a role for CaMKIIα, but not CaMKIIβ, in controlling basal AMPAR and NMDAR neurotransmission has been reported (Incontro *et al.* 2018). Unlike AMPARs, NMDAR transmission does not require CaMKIIα kinase activity or its binding to GluN2B, suggesting that CaMKIIα exerts an important scaffolding role on NMDAR function.

CaMKIIα directly phosphorylates GluN2B on Ser-1303 *in vitro* (Omkumar *et al.* 1996), which is located within the CamKIIα binding site on the GluN2B CTD. Phosphomimetic GluN2B mutations on Ser-1303 reduce CaMKII/GluN2B binding (O'Leary *et al.* 2011; Strack *et al.* 2000) and subsequently result in an increase in surface GluN2B expression in neurons. Interestingly, activated CaMKII recruits CKII and GluN2B to form a tripartite

complex, which is required to maintain the phosphorylation of GluN2B at Ser-1480 by CKII (Sanz-Clemente *et al.* 2013a). Phosphorylation of GluN2B at Ser-1480 disrupts its interaction with MAGUKs, which results in lateral diffusion of GluN2B to extrasynaptic sites and the internalization of NMDARs through a coordinated dephosphorylation of Tyr-1472 within the endocytic motif (Sanz-Clemente *et al.* 2010; Sanz-Clemente *et al.* 2013a; Chung *et al.* 2004). This mechanism has become a widely accepted model that underlies the developmental- and activity-dependent switches of NMDAR subunit composition at synapses. However, this model has recently been challenged by a study utilizing two lines of knock-in mice in which mutations on the CaMKII binding site on the GluN2B CTD were introduced (L1298A/R1300N/S1303D) or the entire GluN2A CTD was replaced by that of GluN2B (McKay *et al.* 2018). The developmental switch of GluN2B- to GluN2A-containing NMDARs proceeds normally in these mice, arguing against the need of distinct GluN2 subunit CTDs in this process. However, the effects on activity-dependent switches have not been formally tested.

Sorting nexin 27

Sorting nexin 27, which contains an N-terminal PDZ domain, a lipid-binding PX domain and three C-terminal FERM (4.1N/ezrin/radixin/moesin) domains, is a highly conserved regulator of cargo recycling from endosomes to the plasma membrane (Teasdale & Collins 2012). It is the only member of the SNX family that contains a PDZ domain through which it directly interacts with the C-terminal PDZ binding motifs of cargo proteins, including NMDARs (Clairfeuille *et al.* 2016; Cai *et al.* 2011). Loss of SNX27 leads to a profound decrease in surface expression of AMPA- and NMDA-Rs; and, as a consequence, SNX27 knockout mice display deficits in LTP and behavioural abnormalities (Wang *et al.* 2013; Hussain *et al.* 2014; Loo *et al.* 2014). Despite the clear involvement of SNX27 in regulating the surface expression of NMDARs, the critical question of how the interaction between SNX27 and NMDAR subunits is regulated remains elusive. Given the strong interaction between SNX27 and the retromer complex (Burd & Cullen 2014), it is likely that SNX27 is an important regulator of NMDAR recycling from endosomes to the plasma membrane.

A unique feature of the SNX27 PDZ domain binding to its cargo molecules involves the formation of an ‘electrostatic clamps’ by acidic residues at the (−3) and (−5) positions upstream of the PDZ binding motif (Clairfeuille *et al.* 2016). Interestingly, the corresponding amino acids at these critical positions within the CTD of all GluN2 subunits consist of mainly serine residues, suggesting that the binding affinity of SNX27 and NMDAR subunits can be modulated by protein phosphorylation of these serine residues. Indeed, the affinity of GluN2B binding to SNX27 PDZ increases 10-fold when these sites are phosphorylated or mutated to acidic residues (Clairfeuille *et al.* 2016), raising the possibility of a phosphorylation-dependent regulation of NMDAR recycling by SNX27.

Death-associated protein kinase 1 (DAPK1)

The role of NMDAR-mediated excitotoxicity, primarily via excessive Ca^{2+} influx through the extrasynaptic GluN2B-containing receptors, in the context of both acute and chronic neurological disorders including ischemic stroke and Alzheimer’s disease is well-established (Hardingham *et al.* 2002; Martel *et al.* 2012; Parsons & Raymond 2014). In a cerebral

ischemia model, the death-associated protein kinase 1 (DAPK1) was discovered as a specific cell death signal when recruited to the extrasynaptic GluN2B-containing NMDARs (Tu *et al.* 2010). DAPK1 belongs to the CaMK family of protein kinases, which consists of an N-terminal kinase domain, a Ca^{2+}/calmodulin-binding regulatory domain, eight ankyrin repeats, a cytoskeletal binding domain, a death domain and a Ser-rich C-terminal tail (Shiloh *et al.* 2014). Both the kinase and death domain are required for its proapoptotic activity (Cohen *et al.* 1997; Cohen *et al.* 1999).

DAPK1 directly interacts with the GluN2B CTD in a region that overlaps with the CaMKII binding site (Tu *et al.* 2010). Activated DAPK1 phosphorylates GluN2B at Ser-1303, which enhances GluN1/2B channel conductance and allows detrimental amount of Ca^{2+} influx, consequentially inducing infarction in the cortex of ischemic stroke mice. Genetic deletion of DAPK1 provides neuroprotective effects, including reduced infarct size and improved neurological behaviour in a stroke model in mice. However, a more recent study using an independently generated DAPK1 knockout mouse line fails to produce a similar protection of neuronal death following excitotoxic and ischemic insults (McQueen *et al.* 2017). Furthermore, neither DAPK1 nor ischemic insults enhance GluN2B phosphorylation at Ser-1303. Notwithstanding these differences, the CTD of GluN2B remains an important factor that mediates excitotoxic signaling in neurons (Martel *et al.* 2012).

The role of DAPK1 is not only confined as a mediator of cell death in neuropathology, but has recently emerged as a critical regulator of synaptic plasticity (Goodell *et al.* 2017). In contrast to the CaMKII/GluN2B interaction, DAPK1 binding to GluN2B is negatively regulated by Ca^{2+}/calmodulin. As a consequence, CaMKII translocates into and accumulates in dendritic spines while DAPK1 is being removed by LTP stimuli. However, during LTD, DAPK1 is activated via a calcineurin-dependent dephosphorylation mechanism, accompanied by DAPK1-dependent GluN2B phosphorylation at Ser-1303. The phosphorylation of GluN2B strengthen its interaction with DAPK1 and prevents CaMKII binding and accumulation of in dendritic spines despite being auto-phosphorylated at Thr-286. Moreover, overexpression of DAPK1 prevents the accumulation CaMKII resulting in an inhibition of CaMKII-induced LTP. Therefore, the differential translocation of DAPK1 and CaMKII into dendritic spines provides a mechanism for bi-directional synaptic plasticity in the hippocampus.

NMDAR subunit-specific role in neurological disorders

NMDAR dysfunction has long been thought to have a paramount role in a variety of neurological disorders, ranging from acute (e.g. stroke and global cerebral ischemia) to chronic disorders such as Alzheimer's or Huntington's disease (Zhou & Sheng 2013; Ittner & Gotz 2011). These effects are mostly due to excitotoxicity, which results from excessive glutamate release from the presynaptic terminal. The term excitotoxicity was first used to describe neurodegeneration associated with the overactivation of excitatory amino acid receptors (Olney 1969). Indeed, a combination of excessive glutamate release from the presynaptic terminal and the failure of the mechanisms responsible for removing this neurotransmitter from the synapse leads to the accumulation of glutamate in the synaptic cleft. This subsequently leads to overactivation of iGluRs, particularly postsynaptic

NMDARs, which causes excessive Ca^{2+} influx (Choi 1987; Choi *et al.* 1987). This overload of Ca^{2+} contributes to the activation of damaging Ca^{2+}-dependent processes that trigger the initiation of cell death mechanisms and ensuing neurodegeneration in various brain regions (Arundine & Tymianski 2003; Choi 1988; Mehta *et al.* 2013; Tymianski & Tator 1996; Ong *et al.* 2013).

A long-standing debate in the field concerns the differential contribution of GluN2A- and GluN2B-containing NMDARs to excitotoxic neuronal death (Wyllie *et al.* 2013). GluN2B is generally thought to be a major mediator of neuronal degeneration (Aarts *et al.* 2002; Martel *et al.* 2012; Soriano *et al.* 2008). On the other hand, GluN2A is considered to have a pro-survival role (Liu *et al.* 2007; Terasaki *et al.* 2010). Notably, the CTD of GluN2 subunits is a major determinant for NMDAR-mediated toxicity (Martel *et al.* 2012; Vieira *et al.* 2016), as demonstrated by the enhanced neuronal demise in an excitotoxic context, elicited by the molecular swapping of the CTD of GluN2A for that of GluN2B. A complementary experiment involving the GluN2B chimera containing the CTD of GluN2A produces the opposite effect, promoting neuronal survival (Cepeda & Levine 2012; Martel *et al.* 2012; Vieira *et al.* 2016). Additional evidence supporting the concept of a detrimental role of the GluN2B subunit comes from several reports demonstrating a neuroprotective effect of interfering with activation of downstream signaling pathways, specifically the neuronal nitric oxide synthase (nNOS) pathway (Aarts *et al.* 2002; Cook *et al.* 2012; Cui *et al.* 2007). In fact, interfering peptides that disrupt the coupling of GluN2B to PSD-95 and nNOS (Aarts *et al.* 2002; Ittner *et al.* 2010), reducing nNOS activation and consequently nitrosative and oxidative stresses, have entered clinical trials and are efficacious in improving stroke patient outcome (Ballarin & Tymianski 2018; Hill *et al.* 2012).

Despite the data supporting a differential role of these two subunits in excitotoxic neuronal death, other reports also show evidence for a role of GluN2A in neuronal demise (Stanika *et al.* 2009; Zhou *et al.* 2013). This incongruence may be due to the contribution of triheteromeric receptors, as noted above. The most likely explanation, however, is that an interplay of factors beyond subunit composition, such as receptor localization, contributes to the decision between activation of pro-death versus pro-survival signaling pathways (Lai *et al.* 2011; Stanika *et al.* 2009; Zhou *et al.* 2013). In accordance with this notion, it has been observed that stimulation of extrasynaptic NMDARs contributes to activation of detrimental pathways. On the contrary, activation of synaptic NMDARs is considered to be neuroprotective (reviewed in (Hardingham & Bading 2010) and (Parsons & Raymond 2014)).

Mechanistically, synaptic NMDARs exert a neuroprotective effect via the activation of pro-survival transcription factors, such as CREB, and CREB-dependent expression of pro-survival genes. This effect is counteracted by activation of extrasynaptic NMDARs, which shuts-off CREB-dependent signaling (Dieterich *et al.* 2008; Hardingham & Bading 2002; Hardingham *et al.* 2002; Karpova *et al.* 2013). Extrasynaptic NMDARs also induce the activation of deleterious proteins, such as calpains (Xu *et al.* 2009). Overall, extrasynaptic NMDARs contribute to neuronal death by concomitantly suppressing pro-survival pathways and activating deleterious signaling cascades. Despite the plethora of evidence showing a pro-survival role of synaptic NMDARs, these have also been shown to be capable of

inducing excitotoxic neuronal death (Papouin *et al.* 2012), thereby highlighting the intricate regulation of NMDARs and the consequences of their activation to neuronal fate.

In addition to the well-established link between NMDAR activity and excitotoxicity in neurodegeneration, recent discoveries from genetic studies have identified strong associations between mutations in genes encoding NMDAR subunits and neurological disorders, including autism-spectrum disorders (ASDs), intellectual disability and epilepsy. One of the greatest challenges in the search for the causative genes of neurodevelopmental disorders is the fact that there is not a single gene that causes autism or epilepsy. Instead, these are highly complex multigenic disorders for which hundreds of putative causative genes have been identified (Krumm *et al.* 2014) and share a high degree of co-morbidity (Ravizza *et al.* 2017; Sundelin *et al.* 2016). In accordance, there is a significant overlap in genes associated with ASDs, intellectual disability, epilepsy and schizophrenia (Hardingham & Do 2016; Helbig *et al.* 2009; International Schizophrenia 2008; Miller *et al.* 2009; Sharp *et al.* 2008; Stefansson *et al.* 2008). Among these, a cluster of synapse-associated genes have been identified (Gilman *et al.* 2011; Glessner *et al.* 2009; Krumm *et al.* 2014), which includes genes that encode various NMDAR subunits (De Rubeis *et al.* 2014; Sanders *et al.* 2015). Indeed, a significant number of rare variants of NMDAR subunits have been identified in probands of different neurological disorders (Hamdan *et al.* 2011; Endele *et al.* 2010; Hu *et al.* 2016; Yuan *et al.* 2015), which include both inherited and *de novo* copy number variations and single nucleotide variants.

NMDAR subunits are remarkably intolerant to variation, in particular GluN1, GluN2A and GluN2B (Ogden *et al.* 2017). One interesting aspect of these two genes – *GRIN2A* and *GRIN2B* – is that they seem to be preferentially associated with distinct disorders. Indeed, *GRIN2A* variants are closely linked with epilepsy (Carvill *et al.* 2013; Lesca *et al.* 2019; Lesca *et al.* 2013), whereas those found in *GRIN2B* are more commonly associated with ASDs and intellectual disability (Kenny *et al.* 2014; Sanders *et al.* 2015; De Rubeis *et al.* 2014; Iossifov *et al.* 2014). Functional characterization of most of *GRIN2* variants is lacking, impeding our understanding on their impact on receptor function and activity. Despite this fact, it is evident that both hyper- and hypo-function of the subunits can be associated with disease (Swanger *et al.* 2016), including gene haploinsufficiency, which results from only one of the alleles being expressed in cells. A greater understanding of a wide range of functional outcomes resulting in mutations or variations in genes encoding NMDAR subunits may help explain the varying degrees of phenotypic severity observed in patients harboring mutations in these genes.

Such disparate observations in the functional impact of different variants can arise due to either evolutionary constraints to variation (Petrovski *et al.* 2013) or to the role exerted by the specific substitution in the amino acid residues. It is conceivable that any mutations in critical regions of NMDAR subunits that regulate ion channel properties or agonist binding are likely to be less tolerant to variation. Indeed, variants identified in the agonist binding domain (Swanger *et al.* 2016; Sceniak *et al.* 2019) or the channel forming regions (Ogden *et al.* 2017; Yuan *et al.* 2014; Fedele *et al.* 2018; Vyklicky *et al.* 2018) in both *GRIN2A* and *GRIN2B* are known to cause a wide spectrum of alterations in receptor function, including agonist potency, response time, channel gating, receptor biogenesis and trafficking.

Despite being generally considered less deleterious, mutations that reside in the CTD of GluN2 subunits have also been found to be damaging and potentially with disease-causing consequences. These variants are more likely to affect the molecular complex associated with the receptor, trafficking regulation and stability at synaptic sites. Therefore, mutations that affect these aspects of receptor function are likely to alter the surface expression of NMDARs, and consequently affects the downstream signaling pathways and neuronal plasticity. Accordingly, a recent study characterizing variants identified in human patients have demonstrated deficits in surface expression of NMDARs and in spine density (Liu *et al.* 2017). Many of these mutations have been reviewed in detail elsewhere (Burnashev & Szepetowski 2015; Hu *et al.* 2016), but new variants are still being identified. Accumulating evidence that supports the importance of NMDAR dysfunction in the etiology of neurodevelopmental and neuropsychiatric disorders makes them highly attractive targets for research into disease prevention and potential clinical intervention.

Closing remarks

The fact that NMDARs are critical for multiple forms of synaptic plasticity, learning and memory has continued to fascinate neuroscientists. Despite decades of research and progress in our understanding of the molecular mechanisms that underlie subunit-specific receptor assembly and trafficking into and out of synapses, many important questions remain unanswered. Research to date has focused heavily on the differential roles of GluN2A- and GluN2B-containing NMDARs, while subunit-specific regulation of GluN2C or GluN2D are not well studied. This is further complicated by the difficulty in studying the trafficking of tri-heteromeric NMDARs, which are thought to constitute a large proportion of native NMDARs in the brain. In addition, molecular mechanisms that regulate the trafficking of NMDARs in other neuronal subtypes, such as in interneurons or dopaminergic neurons are still not well understood. Future studies concerning subunit-specific interacting proteins and their roles in regulating the endosomal trafficking, supercomplex formation and neuronal plasticity-related signaling of NMDARs both *in vitro* and *in vivo* are crucial to better understand the regulation of NMDAR roles in higher brain functions, as well as the contribution of NMDAR dysfunction in neurological disorders.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPARs	AMPA receptors
ASDs	autism-spectrum disorders

ATD	amino terminal domain
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CK2	casein kinase 2
cLTP	chemically-induced LTP
CTD	carboxyl terminal domain
CREB	cAMP responsive element binding protein
DAPK1	death-associated protein kinase 1
EPSCs	excitatory postsynaptic currents
ER	endoplasmic reticulum
LBD	ligand binding domain
LTD	long-term depression
LTP	long-term potentiation
MAGUKs	membrane-associated guanylate kinases
mGluRs	metabotropic glutamate receptors
NEM	N-ethylmaleimide
NMDA	*N*-methyl-*D*-aspartate
NMDARs	: NMDA receptors
nNOS	neuronal nitric oxide synthase
PDZ	postsynaptic density-95/discs-large/zona-occludens-1
PKA	protein kinase A
PKC	protein kinase C
PSD	postsynaptic density
SAP	synapse-associated protein
SNARE	soluble NEM-sensitive factor attachment protein receptor

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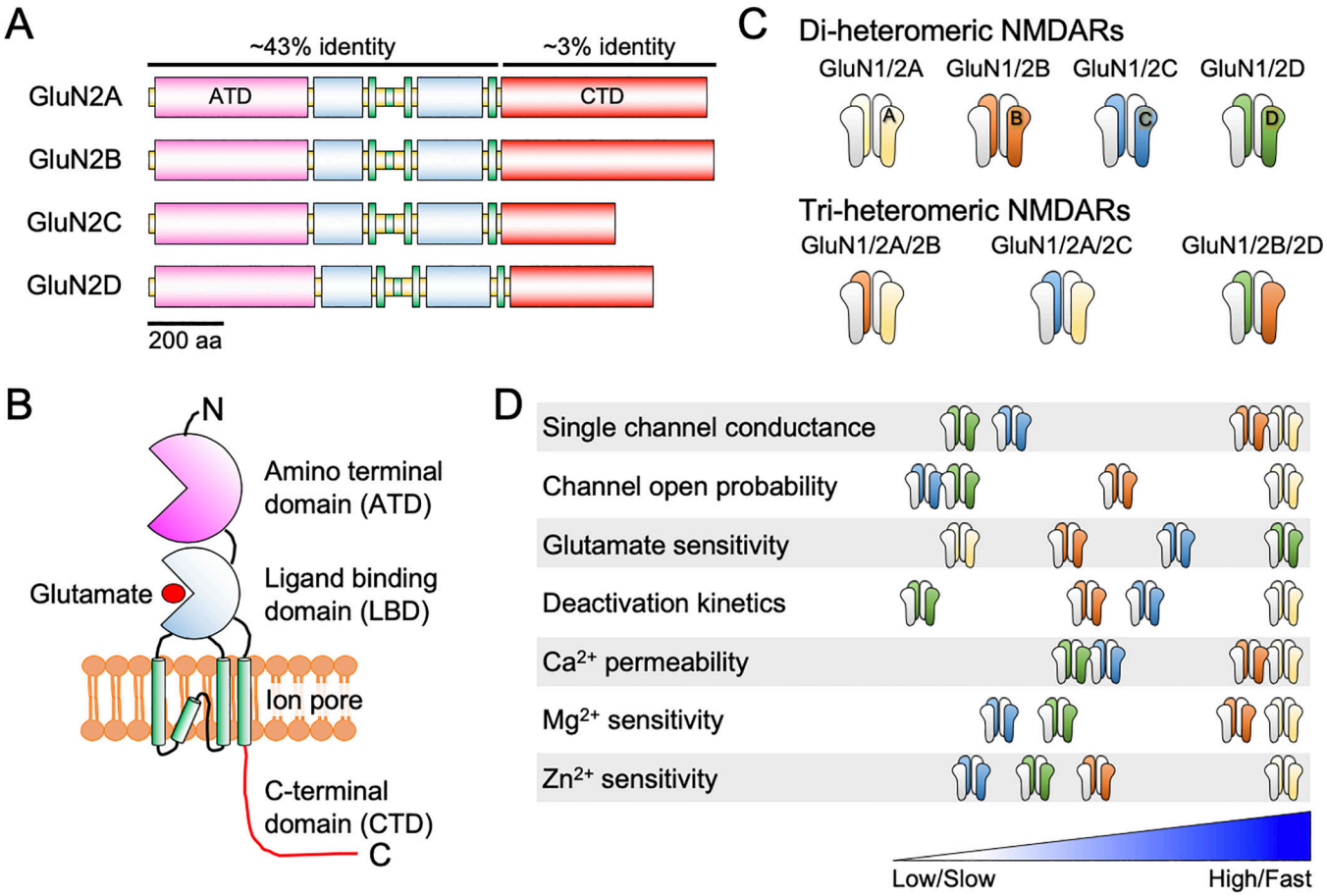

Figure 1. Diversity and biophysical properties of GluN2-containing NMDARs.

A. Schematic diagram of the domain structure of the four GluN2 subunits of NMDARs, GluN2A – GluN2D. B. The modular architecture of a single GluN2 subunit comprising four distinct domains: an amino terminal domain (ATD), the ligand binding domain (LBD) that binds to glutamate, three transmembrane regions and a re-entrant loop that form the ion channel pore, as well as a carboxy terminal domain, which is the most divergent among all GluN2 subunits. C. Representative assemblies of GluN2-containing di- or tri-heteromeric NMDARs known to exist in the mammalian central nervous system. D. Schematic diagram depicting relative differences in the properties of di-heteromeric GluN2-containing NMDAR subtypes.

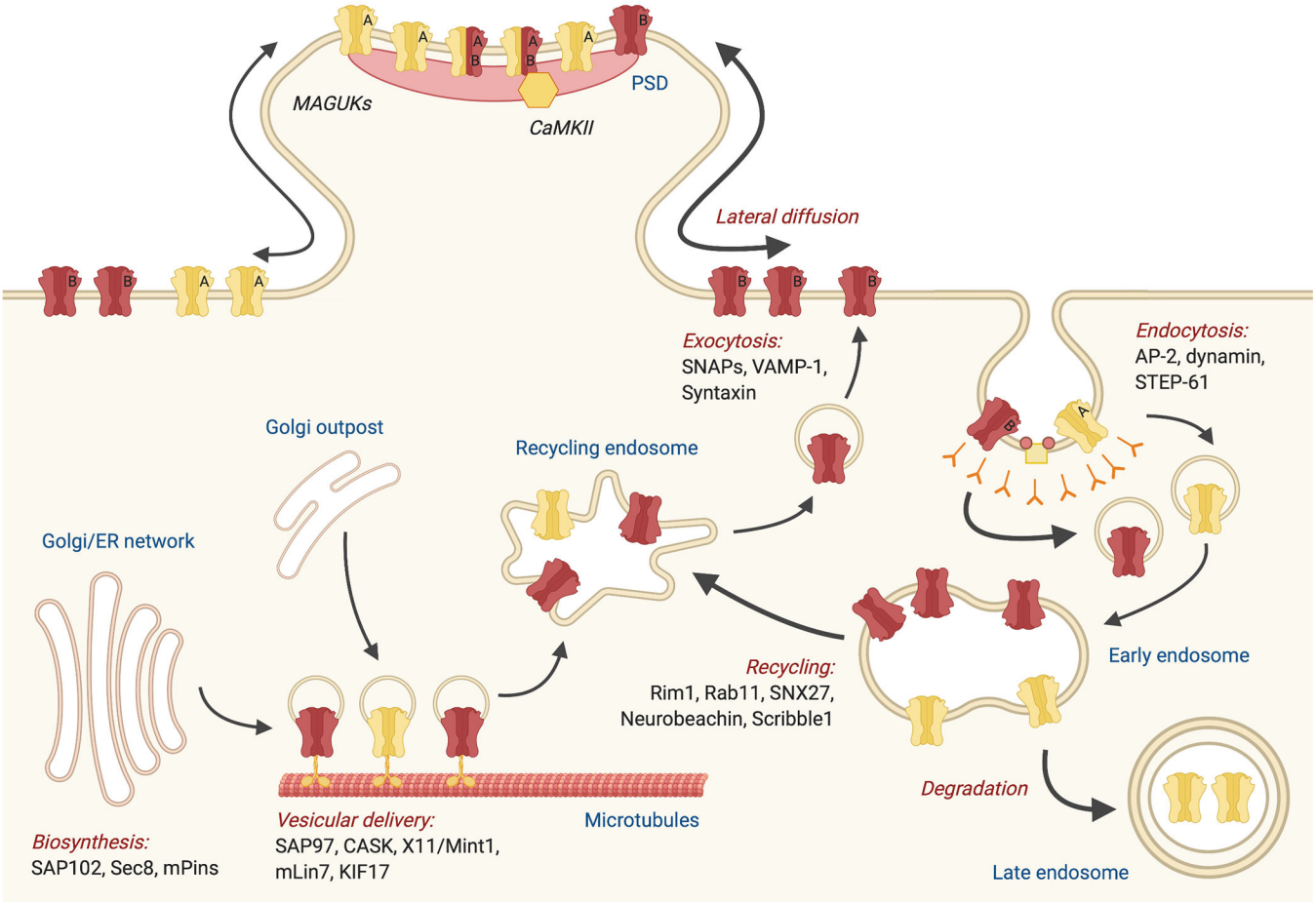

Figure 2. Routes of NMDAR trafficking.

NMDARs are assembled in the ER and Golgi apparatus in the soma or in dendritic Golgi outposts. They are subsequently transported along the dendrite via kinesin-dependent vesicular trafficking on microtubule networks prior to their insertion onto the plasma membrane. Via lateral diffusion, surface NMDARs are incorporated into synapses and stabilized by PSD scaffolding proteins. NMDARs are internalized from the plasma membrane by clathrin-mediated endocytosis and trafficked to early endosomes. From early endosomes, receptors can be recycled back to the plasma membrane or can enter the degradation pathway to late endosomes. The trafficking of distinct GluN2 subunits are differentially regulated at multiple levels, including the rate of lateral diffusion, internalization and post-endocytic sorting.

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Table 1

Other known NMDAR CTD interacting proteins

NMDAR CTD Interacting Proteins	Subunit Specificity				Molecular Functions	References
	2A	2B	2C	2D		
14–3–3	-	-	+ *y, h, b*	-	Various isoforms of 14–3–3 adaptor proteins interact with GluN2C when phosphorylated at Ser-1096 by Akt to promote the surface expression of GluN2C in neurons.	(Chen & Roche 2009; Chung *et al.* 2015)
α-actinin	-	+ *y, h, b*	n.d.	n.d.	The binding of α-actinin antagonizes the interaction of calmodulin to NMDARs (via GluN1) and is required for Ca^{2+}-dependent inactivation of the receptors. The exact role of α-actinin binding to GluN2B is unknown.	(Wyszynski *et al.* 1997; Zhang *et al.* 1998; Krupp *et al.* 1999)
AP2	+ *y, b*	+ *y, b*	n.d.	n.d.	AP2 binds to distinct endocytic motifs in the C-terminal tails of GluN2A and GluN2B to regulate the internalization of NMDARs	(Prybylowski *et al.* 2005; Nong *et al.* 2003; Lavezzari *et al.* 2003; Lavezzari *et al.* 2004)
BRAG1/2	+ *h*	+ *h*	n.d.	n.d.	The brefeldin A-resistant ADP-ribosylation factor (Arf) guanine nucleotide exchange factors (GEF), BRAG1 and 2, interact with GluN2A and GluN2B in heterologous system. GluN2B-BRAG1 signaling is required for the tonic activation of Arf6 in young neurons, whereas GluN2A-BRAG2 drives NMDA-induced Arf6 activation in mature neurons.	(Elagabani *et al.* 2016)
C-terminal binding protein-1 (CtBP1)	+ *y, h*	n.d.	n.d.	+ *y*	CtBP1 is a known transcriptional repressor. Co-expression of CtBP1 results in a reduction in surface GluN1/2A expression in heterologous cells.	(Cousins & Stephenson 2019)
Flotillin	+ *y, b*	+ *y, b*	n.d.	n.d.	Flotillin binds to the distal C-terminal tails of GluN2A and GluN2B, consistent with the presence of NMDARs within the lipid rafts. The functional roles of their interaction remain elusive.	(Swanwick *et al.* 2009; Delint-Ramirez *et al.* 2010)
FRMPD2	+ *h, b, i*	+ *h, b, i*	n.d.	n.d.	FRMDP2 is a multi-PDZ domain scaffold protein that interacts with GluN2A and GluN2B through its second PDZ domain. The function of FRMPD2 in regulating NMDAR functions is currently unknown.	(Lu *et al.* 2019)
GiPC	n.d.	+ *y, b*	n.d.	n.d.	GiPC is a single PDZ domain protein that is associated with extrasynaptic NMDARs. GiPC regulates the surface expression of both GluN2A and GluN2B in cultured hippocampal neurons.	(Yi *et al.* 2007)
Nedd4–1	+ *y*	-	-	+ *yh, b, i*	Nedd4–1 promotes poly-ubiquitination of GluN2D and reduces the number of functional cell surface GluN1/2D receptors in heterologous systems.	(Gautam *et al.* 2013)
Nck2	n.d.	+ *i, h*	n.d.	n.d.	Nck2 is an actin regulatory protein that interacts with phosphorylated GluN2B at Tyr-1252 to regulate NMDAR-mediated currents.	(Levy *et al.* 2018)
Rabphilin3A	+ *h, b*	-	n.d.	n.d.	Raphilin3A directly interacts with GluN2A and PSD-95, forming a ternary complex to stabilize GluN2A-containing NMDARs at synapses.	(Stanic *et al.* 2015)
RasGRF1	-	+ *h, b, i*	n.d.	n.d.	RasGRF1 is a Ca^{2+}/calmodulin-dependent Ras-guanine-nucleotide releasing factor that couples NMDARs to the downstream MAPK signaling pathway.	(Li *et al.* 2006; Krapivinsky *et al.* 2003)
Scribble1	+ *y, h, b, i*	+ *y, h, b, i*	n.d.	n.d.	Scribble1 interacts with AP2 and together, coordinate the internalization and recycling of NMDARs.	(Piguel *et al.* 2014)
X11 and X11-L	n.d.	+ *h, b*	n.d.	n.d.	X11 (Mint1) and X11-like (Mint2) are neuronal adaptor proteins that regulate kinesin-dependent vesicular trafficking and surface expression of extrasynaptic NMDARs.	(Motodate *et al.* 2019; Setou *et al.* 2000)

Interactions between GluN2 subunits and their CTDs were validated by ^{y}yeast two-hybrid system, ^{i}*in vitro* binding assays, or co-immunoprecipitation/pull-down assays in ^{h}heterologous systems, ^{n}primary neuronal cultures or ^{b}brain lysates. n.d. = not determined.