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Invited review

Protein-protein interactions at the NMDA receptor complex: From synaptic retention to synaptonuclear protein messengers

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels that support essential functions throughout the brain. NMDARs are tetramers composed of the GluN1 subunit in complex with GluN2- and GluN3-type regulatory subunits, resulting in the formation of various receptor subtypes throughout the central nervous system (CNS), characterised by different kinetics, biophysical and pharmacological properties, and the abilities to interact with specific partners at dendritic spines. NMDARs are expressed at high levels, are widely distributed throughout the brain, and are involved in several physiological and pathological conditions. Here, we will focus on the GluN2A- and GluN2B-containing NMDARs found at excitatory synapses and their interactions with plasticity-relevant proteins, such as the postsynaptic density family of membrane-associated guanylate kinases (PSD-MAGUKs), Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and synaptonuclear protein messengers.

The dynamic interactions between NMDAR subunits and various proteins regulating synaptic receptor retention and synaptonuclear signalling mediated by protein messengers suggest that the NMDAR serves as a key molecular player that coordinates synaptic activity and cell-wide events that require gene transcription. Importantly, protein-protein interactions at the NMDAR complex can also contribute to synaptic dysfunction in several brain disorders. Therefore, the modulation of the molecular composition of the NMDAR complex might represent a novel pharmacological approach for the treatment of certain disease states.

1. Introduction

In the central nervous system (CNS), excitatory neurotransmission is primarily mediated by glutamate and its ionotropic receptors: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs), which support rapid transmission (Malinow and Malenka, 2002; Diering and Huganir, 2018), and the N-methyl-D-aspartate (NMDA) receptors (NMDARs), which sustain the induction of long-term plasticity (Hunt and Castillo, 2012; Paoletti et al., 2013). The efficacy of the glutamatergic synapse is modulated by activity-dependent events (Paoletti et al., 2013). These processes, in turn, regulate the molecular and structural organisation of specific synaptic compartments, such as the postsynaptic density (PSD), in which the interactions of glutamate receptors with scaffolding proteins and signalling elements are dynamically modulated (Franchini et al., 2020).

NMDARs are tetrameric receptors composed of two obligatory GluN1 subunits that combine with two GluN2- or GluN3-type regulatory subunits. The identities of the regulatory subunits serve to fine-tune the biophysical and pharmacological properties of the receptor, including receptor kinetics, the opening probability, assembly, signalling, and synaptic localisation. The GluN2A and GluN2B subunits are widely accepted as the most abundant NMDAR regulatory subunits expressed in the CNS. Their expression levels change during brain development (Monyer et al., 1992, 1994) and after the induction of activity-dependent synaptic plasticity (Bellone and Nicoll, 2007; Paoletti et al., 2013). During development, NMDARs switch from tetramers being enriched in GluN1-GluN2B subunits to being enriched in GluN1-GluN2A or GluN1-GluN2A-GluN2B subunits (Bellone and Nicoll, 2007; Gray et al., 2011), a process that is regulated also by epigenetic mechanisms (Rodenas-Ruano et al., 2012). Importantly, the presence of the GluN2A subunit increases NMDAR stability at synapses. Conversely, GluN2B-containing NMDARs represent a much more mobile pool of NMDARs that are enriched at both synaptic and extrasynaptic sites (Groc et al., 2006; Hardingham and Bading, 2010; Franchini et al., 2020).

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2. Dynamic protein-protein interactions at GluN2 intracellular domains

The downstream intracellular signalling and synaptic retention of NMDAR pools are typically determined by the cytoplasmic C-terminal domains (CTDs) of their regulatory subunits. These CTDs are distinct and contain specific motifs that determine the binding of the NMDAR with synapse-associated proteins with a variety of neuronal functions, ranging from scaffolding proteins to enzymes involved in downstream signal transduction pathways (Sanz-Clemente et al., 2013; Sun et al., 2017). The most abundant regulatory subunits in the brain, GluN2A and GluN2B, share similar GluN2 CTD domains and are, therefore, capable of binding similar proteins. Molecular competition mechanisms have been demonstrated for different types of interacting proteins (Franchini et al., 2020). In particular, the dynamic regulation of these protein-protein interactions often depends on the activation state of the synapse and the induction of synaptic plasticity, and the physiological modulation of several protein–protein interactions at the NMDAR complex has been shown to play a key role in the induction of long-term potentiation (LTP). Therefore, identifying the GluN2-CTD interacting partners and determining the rules that govern their association/dissociation mechanisms can enhance our understanding of how GluN2-containing NMDAR function is regulated under basal conditions and following synaptic stimulation.

2.1. GluN2A/GluN2B interactions with PSD-MAGUKs and PSD-associated kinases

NMDARs at excitatory synapses have been described to serve as hubs for several protein families involved in synaptic plasticity, such as the scaffolding proteins of the postsynaptic density family of membrane-associated guanylate kinases (PSD-MAGUKs) and other key PSD-associated enzymes that regulate local signalling processes at the dendritic spine (Sanz-Clemente et al., 2013b; Paoletti et al., 2013).

The PSD-MAGUK family includes the prototypical scaffolding protein at excitatory synapses, the synapse-associated protein-90 (SAP-90)/post-synaptic density protein of 95 kDa (PSD-95) and other members such as PSD-93 (also known as Chapsyn-110), SAP102 and SAP97. All PSD-MAGUK family members are characterized by a very similar structural organization with three PDZ domains at the N-terminus, a Src-homology 3 (SH3) domain and a C-terminal inactive guanylate kinase domain and share a key role in the regulation of the physiological synaptic localisation and function of NMDARs (Elias and Nicoll, 2007).

Several observations support the existence of a differential interaction of NMDAR GluN2-subtypes with the different members of PSD-MAGUK family. A systematic study performed in a heterologous expression system found that PSD-95 and PSD-93 can increase surface levels of GluN1/GluN2A and GluN1/GluN2B NMDARs whereas SAP97 and SAP102 had no effect on cell surface expression of these NMDAR subtypes (Cousins et al., 2008). However, protein-protein interaction studies in rat neurons showed a preferential relationship of GluN2A with PSD-93, PSD-95 and SAP97 while GluN2B mainly forms complexes with SAP102 and PSD-95 (Sans et al., 2000; Gardoni et al., 2009).

Evaluation of the synaptic enrichment of PSD-MAGUK proteins during postnatal development demonstrated a very early expression of SAP102 at most synapses, whereas PSD-95 and PSD-93 increase slowly through 6 months (Sans et al., 2000). Accordingly, SAP102 and PSD-95 regulate the synaptic trafficking of distinct NMDAR subtypes at different developmental stages. SAP102 regulates synaptic trafficking of NMDARs during early development and after synaptogenesis PSD-95 assumes the functions of SAP102 playing also a major role in the developmental switch from GluN2B- to GluN2A-containing NMDARs that takes place during synapse maturation (Elias et al., 2008).

The interaction of PSD-MAGUKs with GluN2A and GluN2B C-terminal domains represents a key step also for the trafficking of NMDAR from the endoplasmic reticulum (ER) to the synapse. GluN2A and GluN2B interact early in the secretory pathway with SAP97 and SAP102 and only later at the trans-Golgi Network with PSD-95 (Mauceri et al., 2007; Standley et al., 2012). In particular, NMDARs are trafficked via a SAP97 dependent pathway from somatic ER to a dendritic ER sub-compartment and to Golgi outposts (Jeyifous et al., 2009). SAP97 overexpression in primary immature neurons leads to enhanced NMDAR-mediated synaptic transmission when overexpressed in immature neurons (Jeyifous et al., 2009). In addition, *in vivo* SAP97 overexpression throughout developmental stages increases NMDAR currents and modifies NMDAR kinetics in mature neurons suggesting SAP97 involvement in NMDAR trafficking particularly in early development (Howard et al., 2010).

Several studies have demonstrated interactions between NMDARs not only with scaffolding protein but also with PSD-enriched protein kinases. Our group and others have contributed to the identification of the various molecular mechanisms involved in the interaction between the NMDAR regulatory subunits GluN2A and GluN2B and α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), which is the most abundant kinase located at glutamatergic dendritic spines (Omkumar et al., 1996; Kennedy, 1997; Strack and Colbran, 1998; Gardoni et al., 1998, 1999, 2001a, 2001b; Leonard et al., 1999; Strack et al., 2000).

Two interaction domains for αCaMKII have been identified in the GluN2B CTD (aa839-1120 and aa1289-1310), and binding at these sites is known to be regulated by the activation of αCaMKII and by kinase autophosphorylation at Thr286; however, the presence of Ca^{2+}/Calmodulin (CaM) alone is sufficient to induce αCaMKII binding with the GluN2B CTD (Strack and Colbran, 1998; Bayer et al., 2006). Our group characterised the regulatory mechanism that governs the interaction between αCaMKII and the GluN2A CTD (aa1389–1464) (Gardoni et al., 1998, 1999, 2001a, 2001b). Similar to the mechanism described for GluN2B, the binding between αCaMKII and GluN2A is strengthened by αCaMKII autophosphorylation at Thr286 (Fig. 1) (Gardoni et al., 2001b).

Different groups have theorised that the molecular complex formed by αCaMKII and NMDAR serves as a form of molecular memory at the synapse and that the association between active αCaMKII and synaptic NMDARs may be necessary for synaptic plasticity (Barria and Malinow, 2005; Sanhueza et al., 2011; Gardoni et al., 2009). CaMKII inhibition in hippocampal neurons results in the decreased formation of the αCaMKII/GluN2B complex, which is associated with a significant reduction in the synaptic enrichment of GluN2B-containing NMDARs and a concomitant reduction in LTP induction without affecting long-term depression (LTD) (Gardoni et al., 2009). Similarly, Lisman's lab demonstrated that the use of peptides to inhibit the binding of αCaMKII with the GluN2B subunit produces a persistent reduction in basal synaptic transmission and can reverse saturated LTP (Sanhueza et al., 2011). Importantly, these peptides reduce synaptic strength only when applied at concentrations sufficient to disrupt the formation of the αCaMKII/NMDAR complex but not at lower concentrations that inhibited only the kinase enzymatic activity of αCaMKII (Sanhueza et al., 2011). Moreover, interfering with the GluN2B/αCaMKII interaction was found to disrupt extracellular signal-regulated kinase (ERK)-dependent increases in synaptic AMPARs and dendritic spine size (El Gaamouch et al., 2012).

Similar effects on NMDAR localisation and synaptic plasticity can be obtained by disrupting the formation of the GluN2B/PSD-95 complex through the use of cell-permeable peptides, indicating that both αCaMKII and PSD-95 play fundamental roles in the physiological synaptic retention of the pool of GluN2B-containing NMDARs, which are necessary for the induction of LTP (Gardoni et al., 2009). In agreement with all of these observations, more recent studies have demonstrated structural and scaffolding roles for the CaMKII protein at the NMDAR complex that are independent of the kinase enzymatic activity (Kim et al., 2016; Incontro et al., 2017). The use of super-resolution imaging and single-particle tracking has indicated that the interaction with αCaMKII is essential for the nanoscale organisation of

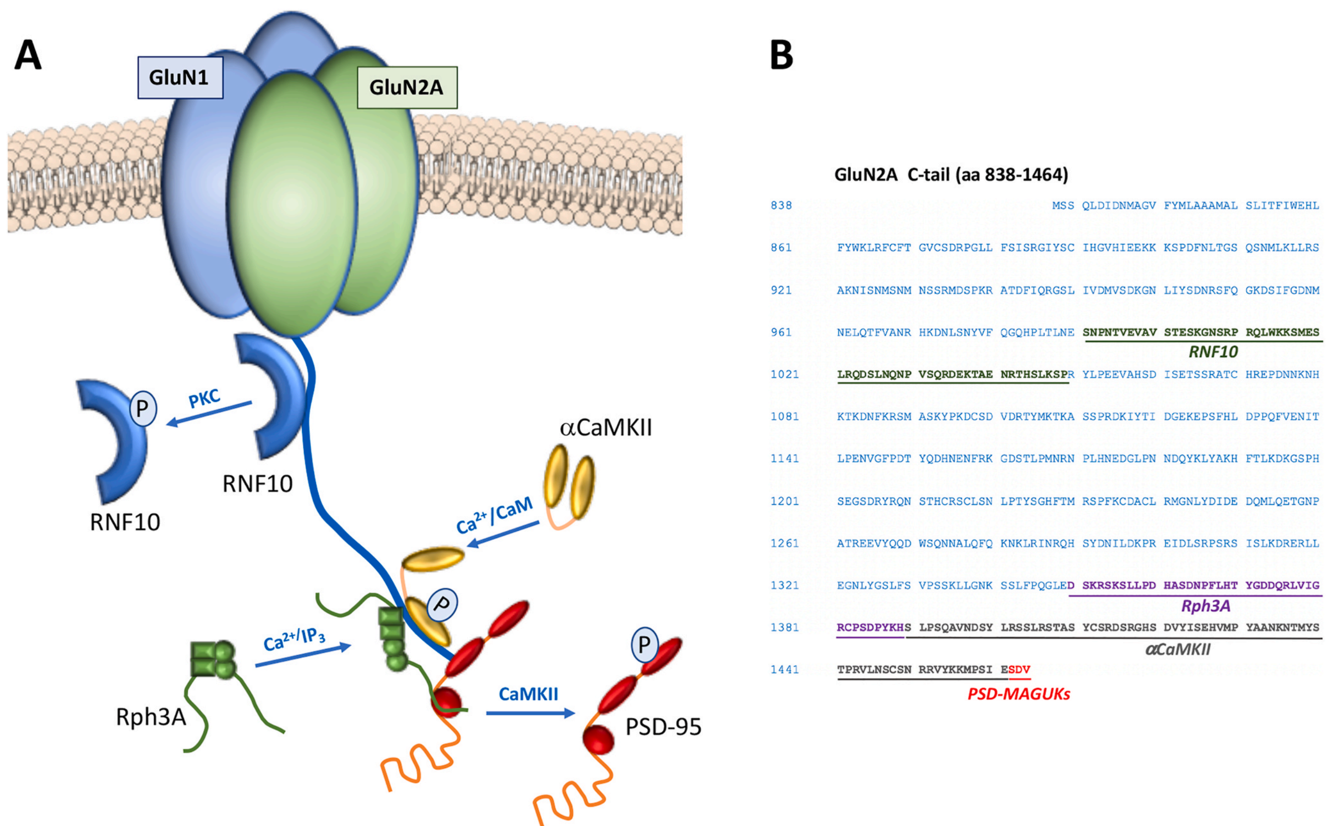

Fig. 1. Graphical representation **(A)** and amino acid sequences **(B)** of GluN2A interactions at the C-terminal domains (CTD) that are described in the review. At rest, GluN2A primarily interacts with RNF10 and PSD-MAGUK proteins, such as PSD-95. NMDAR-dependent calcium influx promotes the phosphorylation of RNF10 by PKC, resulting in RNF10 detachment. αCaMKII binds to activated calmodulin (CaM), resulting in αCaMKII autophosphorylation and leading to the increased interaction with the GluN2A subunit. Rph3A is activated by calcium/IP_3, leading to increased interaction with the GluN2A subunit.

GluN2B-containing NMDARs at proximal dendritic segments (Ferreira et al., 2020).

The modulation of protein-protein interactions within the NMDAR/αCaMKII/PSD-95 complex is quite dynamic and can be regulated by both synaptic plasticity and phosphorylation events. The association between αCaMKII and GluN2A can be increased by NMDAR activation, such as through the use of pharmacological tools or by the induction of LTP, which can lead to the significant detachment of PSD-95 from the GluN2A subunit (Gardoni et al., 2001b). The PSD-95 PDZ domains compete with both native and recombinant αCaMKII for binding with the GluN2A CTD. Moreover, the interaction between either αCaMKII or PSD-95 and GluN2A does not appear to be correlated with the CaMKII-dependent phosphorylation of the receptor subunit (Ser1289) (Gardoni et al., 2001b).

CaMKII-dependent phosphorylation can modulate with different mechanisms GluN2A and GluN2B interaction with both scaffolding proteins and proteins involved in receptor trafficking/endocytosis. Firstly, the protein-protein interactions within the GluN2A/αCaMKII/PSD-95 complex are regulated by the CaMKII-dependent phosphorylation of PSD-95 (Gardoni et al., 2006a; Steiner et al., 2008). In particular, NMDAR activation, the consequent αCaMKII autophosphorylation, and the CaMKII-dependent phosphorylation of PSD-95 Ser73 result in the binding between the autophosphorylated αCaMKII and the GluN2A subunit and the detachment of PSD-95 (Fig. 1) (Gardoni et al., 2006a). This mechanism can occur during synaptic events involving CaMKII activation. In contrast, αCaMKII autophosphorylation and the CaMKII-dependent phosphorylation of PSD-95 does not interfere with the ability of PSD-95 to bind with GluN2B (Gardoni et al., 2006a). In addition, the phosphorylation of PSD-95 can trigger the activity-dependent trafficking of PSD-95, which negatively regulates spine growth and the potentiation of synaptic currents (Steiner et al., 2008).

Consensus sites for CaMKII-mediated phosphorylation have been described both for PSD-95 and for other PSD-MAGUKs. CaMKII-mediated phosphorylation of SAP97 Ser39 is a key event to force the release of the SAP97/GluN2A complex from the ER and, consequently, to induce its trafficking to the postsynaptic compartment in hippocampal neurons (Mauceri et al., 2007). Conversely, CaMKII-dependent phosphorylation of SAP97 Ser232 within the PDZ1 domain disrupts SAP97 interaction with GluN2A suggesting that CaMKII activation in the postsynaptic compartment can serve as a molecular mechanism influencing GluN2A/SAP97 complex thereby regulating synaptic targeting of GluN2A-containing NMDARs (Gardoni et al., 2003).

Finally, CaMKII-dependent phosphorylation is also responsible for modulating the interaction of other scaffolding proteins with the NMDAR complex. Sorting nexin-27 (SNX27), a PDZ protein member of the large family of sorting nexins, has been shown to play an important role in the regulation of NMDAR endocytosis (Clairfeuille et al., 2016; Wang et al., 2013). Interestingly, CaMKII-dependent phosphorylation of GluN2A Ser1459, in proximity of the PDZ-binding domain, leads to a preferential binding of GluN2A-containing NMDARs to SNX27 and a concomitant decrease in PSD-95 binding, thus inducing a modification of NMDAR trafficking (Mota Vieira et al., 2020). Notably, the relevance of this CaMKII-dependent phosphorylation site has been recently addressed by the identification of the rare GluN2A Ser1459Gly mutation in an epileptic patient (Bowling et al., 2017; Mota Vieira et al., 2020). Furthermore, it is important to mention that a corresponding phosphorylation site with an analogue function has been described also within the GluN2B C-tail, close to the PDZ-binding motif (Clairfeuille et al., 2016).

Modulation of NMDAR complex by CaMKII can envisage the involvement of other PSD-associated protein kinases, such as casein kinase 2 (CK2). CK2 is a constitutively active kinase that drives the removal of GluN2B-containing NMDARs from the synapse. In particular, CK2-dependent phosphorylation of GluN2B at Ser1480 within the PDZ ligand interferes with the interaction of GluN2B with scaffolding proteins (Chung et al., 2004; Sanz-Clemente et al., 2010). Interestingly, CK2 can bind to GluN2B upon CaMKII activation and association with the NMDAR to form a trimolecular complex. Consequently, CaMKII activation leads to the CK2-mediated phosphorylation of GluN2B Ser1480 thus regulating the synaptic expression of NMDARs (Sanz-Clemente et al., 2013a).

Protein kinase C (PKC) can interact with and potentiate synaptic NMDARs (Hall and Soderling, 1997; Leonard and Hell, 1997) although the effects of PKC phosphorylation on NMDAR complex function have not been completely addressed (Zheng et al., 1999; Lu et al., 2000). Interestingly, the PKC-dependent phosphorylation of GluN2A at Ser 1416 can modulate the affinity of this NMDAR subunit for αCaMKII, and PKC stimulation in hippocampal slices has been shown to reduce the interaction between αCaMKII and GluN2A-containing NMDARs (Gardoni et al., 2001a).

Cyclin dependent kinase-5 (Cdk5), a proline-directed serine/threonine kinase mainly involved in brain development (Dhavan and Tsai, 2001), has a complex role in the modulation of NMDAR function through protein-protein interaction and kinase activity. Cdk5 interacts with GluN2A and phosphorylates the C-tail of the NMDAR subunit at Ser1232 (Li et al., 2001; Wang et al., 2003). Interestingly, selective Cdk5 inhibition blocks the induction of LTP in hippocampal neurons (Li et al., 2001). Furthermore, Cdk5 can phosphorylate PSD-95 at the N-terminus in a region overlapping the binding site of the tyrosine kinase src (Morabito et al., 2004). Accordingly, Cdk5-dependent phosphorylation of PSD-95 probably regulate the interaction of the tyrosine kinase with the NMDAR complex and, consequently, src-dependent phosphorylation of GluN2B at tyr1472 (Morabito et al., 2004; Zhang et al., 2008).

2.2. Role of interaction with other scaffolding elements in the modulation of NMDAR synaptic retention

The PDZ domains of PSD-95 and other members of the PSD-MAGUK family bind to the last 3 amino acids of the CTD of both the GluN2A and GluN2B sequences, which promotes the synaptic enrichment and stabilisation of the two subunits in the PSD. The binding of GluN2 subunits to distinct PSD-MAGUK proteins plays a key role in NMDAR localisation (Sanz-Clemente et al., 2013b). Current data has revealed that the disruption of the GluN2B PDZ-binding domain results in the loss of synaptic GluN2B (Chung et al., 2004; Prybylowski et al., 2005; Gardoni et al., 2009). The mechanisms responsible for the increased stability and synaptic retention of GluN2A-containing NMDARs are more complex than those for GluN2B-containing NMDARs and have been evaluated in minute details. Mice lacking the C-terminal domain of GluN2A show reduced synaptic GluN2A expression (Sprengel et al., 1998; Steigerwald et al., 2000), and our group and others have shown that peptides that disrupt GluN2A/PSD-MAGUK interactions reduce the synaptic retention of GluN2A-containing NMDARs (Bard et al., 2010; Gardoni et al., 2012; Paillè et al., 2010). In this framework, our lab recently described the role played by Rabphilin-3A (Rph3A), a GluN2A-binding protein that is specifically associated with NMDARs retained at the synapse (Stanic et al., 2015; Franchini et al., 2019).

Rph3A, which was previously recognised as a Rab3A binding partner in presynaptic vesicles (Burns et al., 1998), directly binds to several proteins, including the MAGUK protein Ca^{2+}/CaM-dependent serine protein kinase (CASK) (Zhang et al., 2001), synaptotagmin-1 (Giullen et al., 2013), synaptosomal-associated protein, 25 kDa (SNAP-25) (Ferrer-Orta et al., 2107), ADP ribosylation factor 6 (Arf6) (Ren et al., 2020), and myosin Va (MyoVa) (Brozzi et al., 2012), which are localised in both the pre- and postsynaptic compartments. At dendritic spines, Rph3A forms a ternary complex with GluN2A and PSD-95, which is necessary for NMDAR synaptic retention (Stanic et al., 2015). Notably, the Rph3A interaction with NMDARs is positively regulated by Ca^{2+} influx and inositol triphosphate (IP_3) levels at synapses (Fig. 1) (Coudevylle et al., 2008; Stanic et al., 2015; Franchini et al., 2019). Interestingly, through its C2A and C2B domains, Rph3A binds IP_3 in a Ca^{2+}-dependent manner. Ca^{2+} induces a conformational rearrangement of the CBL3 Rph3A loop, which is involved in IP_3 binding, dramatically increasing the binding of Rph3A with GluN2A and other interactors (Coudevylle et al., 2008; Stanic et al., 2015).

The disruption of the Rph3A interaction with either GluN2A or PSD-95 results in reduced NMDAR synaptic retention, associated with increased receptor endocytosis (Stanic et al., 2015; Franchini et al., 2019). Careful confocal and electron microscopy analyses demonstrated that under resting conditions, Rph3A is not evenly distributed in all dendritic spines; instead, Rph3A is localised to approximately 50% of hippocampal spines (Franchini et al., 2019). Rph3A-positive spines feature an increased spine head area and an enhanced PSD length and thickness compared with Rph3a-negative spines, suggesting that neuronal transmission through these Rph3A-enriched connections may be more stable (Franchini et al., 2019). LTP induction leads to an increased number of Rph3A-positive spines and improved NMDAR synaptic retention, indicating that activity-dependent plasticity promotes the postsynaptic localisation of Rph3A (Franchini et al., 2019). Importantly, Rph3A silencing or the disruption of the Rph3A/NMDAR complex through the use of interfering peptides not only prevents GluN2A accumulation at the postsynaptic membrane but also prevents the induction of LTP and the formation of new spines. The treatment of animals with either Rph3A silencing vectors or peptides that disrupt the Rph3A/NMDAR complex impairs the ability to acquire spatial memories (Franchini et al., 2019). Interestingly, Rph3A was recently indicated as a novel target for the maintenance of cognition in old age (Yu et al., 2020).

Several other proteins can modulate the levels of NMDAR subunits at the postsynaptic membrane by interacting either with GluN2A and/or GluN2B C-tail. Scribble 1, a large PDZ protein involved in synaptogenesis and synaptic plasticity, regulates the levels of NMDARs at the plasma membrane (Moreau et al., 2010; Piguel et al., 2014). In particular, following NMDAR activation Scribble1 prevents the lysosomal trafficking and degradation of GluN2A by increasing its recycling to the plasma membrane through a direct interaction with the AP2 adaptor proteins (Piguel et al., 2014).

2.3. Interaction with synaptonuclear protein messengers

NMDARs play a multifaceted role in functional and structural plasticity. NMDARs have also been implicated in transcriptionally dependent forms of plasticity because NMDAR activation regulates gene transcription, affecting global protein synthesis (Fig. 2) (Hardingham et al., 2002; Hardingham and Bading, 2010; Dieterich et al., 2008; Karpova et al., 2013). NMDARs have also been proposed to serve as key elementsat synapses by triggering modifications in gene transcription and conveying these changes to specific synapses to determine lasting changes in synaptic efficacy (Morris, 2006; Nonaka et al., 2013). This potential role for NMDARs supports another important theory of synaptic plasticity, known as the synaptic tagging and capture hypothesis, which provides a theoretical framework regarding how the products of activity-dependent genes might interact with potentiated synapses to facilitate and maintain long-lasting synaptic plasticity (Frey and Morris, 1997; Redondo and Morris, 2011).

Several studies have identified and characterised NMDAR-associated protein messengers that are able to travel from the synapse to the soma, where they enter the nucleus following specific stimulation patterns (Herbst and Martin, 2017; Lim et al., 2017; Panayotis et al., 2015). At the nucleus, these synaptonuclear messengers control various transcriptional pathways involved in the modulation of synaptic transmission and structural plasticity (Karpova et al., 2013; Dinamarca et al.,

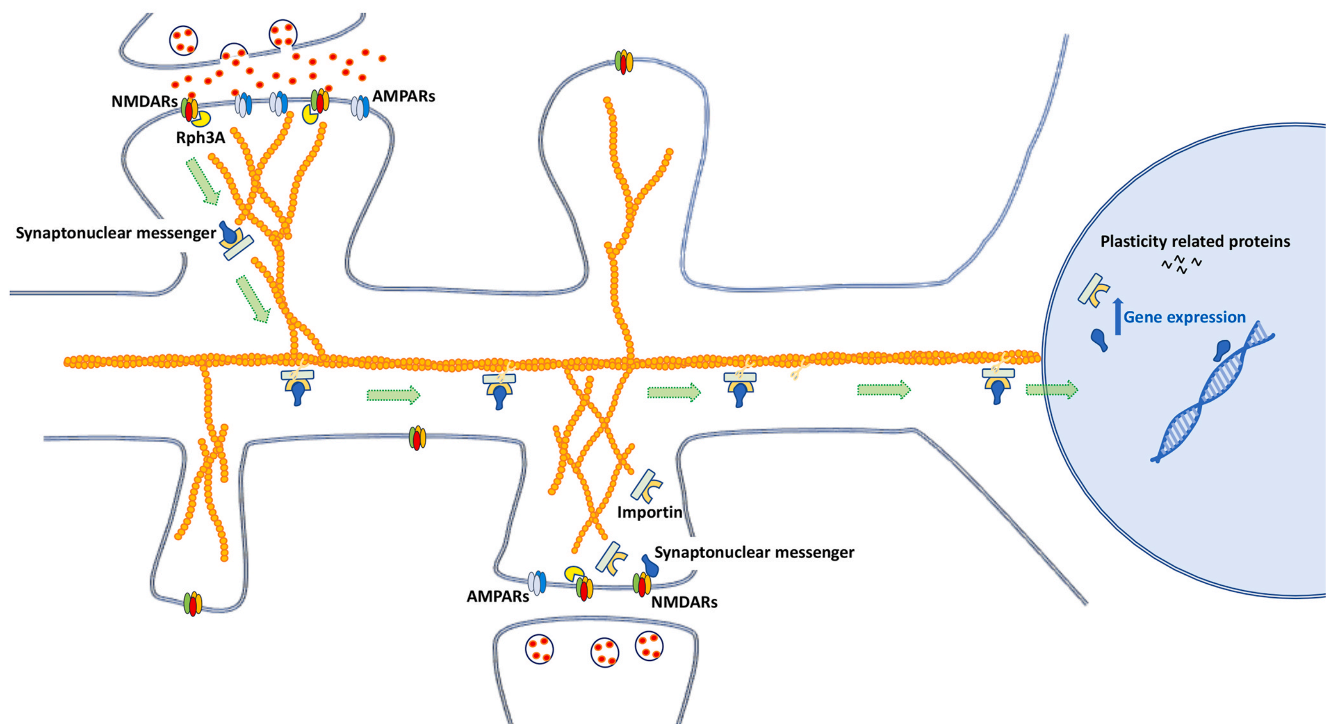

Fig. 2. Graphical representation of the NMDAR interaction with Rph3A at a potentiated synapse and the synapse-to-nucleus trafficking of synaptonuclear messengers following synaptic stimulation.

2016). A consistent pool of synaptonuclear messengers, including RING finger 10 (RNF10), Jacob, amyloid precursor protein intracellular domain associated-1 (AIDA-1), activating transcription factor 4 (ATF4), Abelson interactor 1 (Abi1), Cysteine-rich C-terminal 1 (CRCT1), and proline-rich 7 (PRR7), share common molecular and functional features (Herbst and Martin, 2017; Lim et al., 2017; Panayotis et al., 2015; Marcello et al., 2018): they are all located at dendritic spines, where they directly or indirectly bind the NMDAR complex, and their nuclear trafficking is induced by activity-dependent plasticity or NMDAR stimulation (Fig. 2). Our group identified and characterised the neuronal function of RNF10, a RING finger protein family member that acts as a synaptonuclear messenger. RNF10 is highly enriched at synapses, where it binds with synapse-retained GluN2A-containing NMDARs (Dinamarca et al., 2016; Carrano et al., 2019). The selective binding of RNF10 with synaptic NMDARs allows this synaptonuclear messenger to immediately sense Ca^{2+} flux through NMDAR channels. At rest, RNF10 interacts with the aa991–1049 domain of GluN2A (Fig. 1). The activation of synaptic NMDARs stimulates the PKC-dependent release of RNF10 from the synapse, and RNF10 is translocated to the nucleus through an interaction with the transporter protein importin-α (Dinamarca et al., 2016). Interestingly, RNF10 and CaM share the same GluN2A binding domain (Bajaj et al., 2014). At rest, GluN2A preferentially forms a complex with RNF10, which is disrupted by synaptic activity-dependent Ca^{2+} influx, allowing the formation of the CaM/GluN2A complex. The binding of RNF10 to GluN2A plays a key role in anchoring RNF10 to the excitatory synapse and interferes with the formation of the CaM/NMDAR complex. A PKC-dependent phosphorylation mechanism has been carefully analysed, resulting in the detachment of RNF10 from NMDARs (Fig. 1) and promoting the binding of RNF10 to the importin-mediated motor system, facilitating the trafficking of RNF10 from the spine to the nucleus (Dinamarca et al., 2016; Carrano et al., 2019). The translocation of RNF10 to the nucleus triggers various signalling pathways, allowing the translation of NMDAR activation at synapses into long-term changes in gene expression (Dinamarca et al., 2016; Carrano et al., 2019).

Several reports have indicated that the modification of synaptonuclear activity among synaptonuclear messengers might be strictly correlated with synaptic dysfunction, such as that described in synaptopathies (Marcello et al., 2018). RNF10 silencing in hippocampal neurons prevents LTP induction and results in the significant reduction of AMPAR and NMDAR localisation at synapses (Dinamarca et al., 2016). Moreover, RNF10 silencing triggers alterations in the expression of genes associated with excitatory synaptic transmission and results in the significant reduction of dendritic spine density and alterations in the neuronal architecture (Dinamarca et al., 2016; Carrano et al., 2019). Similarly, Jacob knockout mice are characterised by LTP impairment at CA1 synapses and deficits in hippocampal learning tasks (Spilker et al., 2016). AIDA-1 knockout mice show modifications of LTP and LTD at CA1 hippocampal synapses, which have been associated with molecular modifications of NMDARs (Tindi et al., 2015). CRTC1 serves as a coincidence detector between Ca^{2+} and cAMP signals, which are both involved in the late-phase of LTP maintenance at the Schaffer collateral–CA1 synapses (Kovacs et al., 2007). Finally, a putative role for synaptonuclear messengers in the development of CNS disorders has been hypothesised (Marcello et al., 2018). Studies have linked the AIDA-1 gene to neuropsychiatric disorders, including schizophrenia (McClay et al., 2011; Fromer et al., 2014) and autism spectrum disorders (Uddin et al., 2014), and the expression of synaptonuclear messengers has been correlated with memory deficits in Alzheimer's disease (Parra-Damas et al., 2014, 2017). These results confirm that i) the correct functioning of NMDAR-associated synaptonuclear messengers is fundamental for the regulation of synaptic plasticity; and ii) alterations in synaptonuclear messenger activity might be involved in the pathogenesis of brain disorders (Marcello et al., 2018).

3. Roles played by NMDAR protein-protein interaction in CNS disorders

Molecular and functional alterations in the NMDAR complex have been documented in several pathological conditions, including Parkinson's disease (PD), ischemia, neuropathic pain, schizophrenia, and other neurodegenerative disorders (Gardoni and Di Luca, 2006; Paoletti et al., 2013). Taking into account the high number of preclinical and clinical

studies published in the last twenty years, here we will focus on the role of NMDAR protein-protein interaction in PD and ischemia.

3.1. Parkinson's disease

In the striatum, spiny projection neurons (SPNs) represent more than 95% of the neuronal population (Chang et al., 1982; Graveland and DiFiglia, 1985) and they exclusively express GluN2A- and GluN2B-containing NMDARs (Chapman et al., 2003; Dunah and Standaert, 2003; Mellone et al., 2019). Modifications of the GluN2A and GluN2B interactions with PSD-MAGUKs proteins and PSD-associated kinases, such as αCaMKII, play key roles in triggering the diverse alterations in the composition of NMDAR subunits and synaptic NMDAR enrichment that can be observed in animal models of PD, levodopa (L-DOPA)-induced dyskinesia, and post-mortem tissue obtained from PD patients (Mellone et al., 2015; Mellone and Gardoni, 2018). In particular, studies performed in the classical 6-hydroxydopamine (6-OHDA)-neurotoxic rat model of PD have indicated various alterations in the interaction patterns for both PSD-95 and αCaMKII with GluN2A and GluN2B (Picconi et al., 2004; Gardoni et al., 2006b; Paillé et al., 2010). An important observation was that different degrees of dopamine degeneration, associated with varying degrees of disease severity, were characterised by diverse alterations in the NMDAR complex (Paillé et al., 2010). PD animals featuring a full dopaminergic lesion, which resembles advanced disease stages, show increased αCaMKII binding to GluN2A–GluN2B, accompanied by a reduced association between PSD-95 and NMDARs (Picconi et al., 2004). This effect was accompanied by increased levels of CaMKII-dependent phosphorylation for the two NMDAR regulatory subunits. In contrast, PD animals that present only 75% dopamine denervation, resembling the early disease stage, show an aberrant formation only of the GluN2A/PSD-95 complex, resulting in the increased synaptic localisation of GluN2A at the dendritic spines of striatal SPNs (Paillé et al., 2010). The use of a cell-permeable peptide to reduce the GluN2A/PSD-95 interaction results in the complete rescue of physiological GluN2A levels at synapses, which is associated with a concomitant improvement in motor behaviour (Paillé et al., 2010).

In parkinsonian rats that present dyskinetic behaviours following chronic treatment with L-DOPA, several alterations have been described in the compositions of NMDAR complexes, including changes in the binding of GluN2A and GluN2B with members of the PSD-MAGUK family (Gardoni et al., 2006b; Bastide et al., 2015) and in PSD-MAGUK protein levels (Nash et al., 2005; Porras et al., 2012). These modifications lead to the redistribution of GluN2B to the extrasynaptic membrane, whereas the synaptic levels of GluN2A become aberrantly increased in the striatum (Gardoni et al., 2006b). Interestingly, the treatment of non-dyskinetic animals with a peptide that disrupts GluN2B/PSD-MAGUK binding, forcing the extrasynaptic localisation of GluN2B, was sufficient to induce a shift in the treated rats toward dyskinetic motor behaviours (Gardoni et al., 2006b). The molecular alterations in NMDAR complexes that have been observed in rodent models of dyskinesia have also been identified in nonhuman primate models and in post-mortem tissue samples obtained from dyskinetic PD patients (Mellone et al., 2015), indicating that these modifications reflect a cross-species trait of PD. Treatment with a peptide to disrupt the GluN2A/PSD-MAGUKs interaction rescues the physiological composition of synaptic NMDARs, which induced a significant reduction in the percentage of animals displaying dyskinetic behaviours and reduced the severity of dyskinesia (Gardoni et al., 2012; Mellone et al., 2015). Similarly, and in support of the role played by Rph3A in the stabilisation of the GluN2A/PSD-95 complex (Stanic et al., 2015), the treatment of dyskinetic animals with a peptide that decreased the Rph3A/GluN2A association also significantly reduced their abnormal motor behaviours (Stanic et al., 2017).

3.2. Ischemia

Several papers have described protein-protein interactions involving the C-terminal domains NMDAR subunits in experimental models of ischemia, some of them coupling PSD-95 or PSD-associated kinase activity to the induction of excitotoxic signalling. Martel et al. (2012) nicely demonstrated by using chimeric subunits that the C-tails of GluN2A and GluN2B play distinct roles in driving the response of NMDAR-mediated excitotoxicity indicating that the identity the GluN2 C-tail strictly regulate the toxicity dose-response to activation of NMDARs. In particular, GluN2B C-tail induces NMDAR-dependent toxicity and neuronal cell death more efficiently than GluN2A C-tail.

Different molecular mechanisms have been put forward to explain these events. PSD-95 can act as a molecular bridge, linking the CTD of GluN2B with pro-death signalling pathways through NMDARs. For example, PSD-95 brings NMDAR in close proximity to neuronal nitric oxide synthase (nNOS) and provides the mechanism through which NMDAR stimulation can induce NO production (Ballarin and Tymianski, 2018). The formation of a GluN2B/PSD-95/nNOS complex plays a fundamental role in the induction of pathological mechanisms associated with ischemic stroke at excitatory synapses. Cerebral ischemia increases the interaction of nNOS with PSD-95 and disruption of nNOS/PSD-95 complex by overexpression of the nNOS domain involved in the binding with PSD-95 is sufficient to prevent glutamate-induced excitotoxicity and cerebral ischemic damage (Zhou et al., 2010). Importantly, the formation of the pathological nNOS/PSD-95 complex can be counteracted also by treatment with a selective small-molecular inhibitor with a potent neuroprotective activity *in vitro* and able to ameliorate focal cerebral ischemic damage in rodents (Zhou et al., 2010). Notably, this pharmacological approach would allow to preserve NMDAR function and catalytic activity of nNOS.

Furthermore, cell-permeable peptides that interfere with the interaction between PSD-MAGUK and GluN2B-containing NMDARs have been widely studied as potential therapeutic approaches to stroke (Ballarin and Tymianski, 2018). Among these compounds, the NA1 peptide, which consists of a peptide sequence of the GluN2B CTD (KLSSIESDV), has demonstrated high efficacy for the disruption of downstream neurotoxic signalling, without interfering with the physiological synaptic activation of NMDARs. Both *In vitro* and *in vivo* treatment with NA-1 was shown to protect cortical neurons under various experimental settings without affecting NMDAR-mediated pro-survival pathways, resulting in significant reductions in the infarct volumes observed in both rodent and macaque models of ischemia (Aarts et al., 2002; Sun et al., 2008; Cook et al., 2012; Ballarin and Tymianski, 2018). A Phase 2 clinical trial evaluated the safety and efficacy of NA-1 for reducing embolic stroke in patients who underwent an endovascular procedure against brain aneurysms (Hill et al., 2012). More recently, a clinical assessment of the efficacy and safety of this pharmacological approach to prevent against ischemia-reperfusion injury associated with rapid endovascular thrombectomy in patients who suffered from acute ischemic stroke did not show any improvements in the proportion of patients who achieved good clinical outcomes compared with placebo (Hill et al., 2020).

CaMKII/NMDAR complex plays also a key role in driving excitotoxic signals in ischemia. In hippocampal neurons, activation of GluN2B-NMDARs/CaMKII cascade in global ischemia increases CaMKII-dependent phosphorylation of hippocampal acid-sensing ion channels (ASICs) composed of ASIC1a subunit and characterized by a high Ca^{2+} permeability (Gao et al., 2005). This phosphorylation is responsible for the neuronal enhancement of ASIC currents thus resulting in an elevated intracellular Ca^{2+} and leading to acidotoxic neuronal death in neuronal cultures (Gao et al., 2005).

Moreover, the use of GluN2B mutants for the binding site with αCaMKII suggests a specific role for GluN2B-NMDARs/CaMKII interaction in neuronal death induction in oxygen and glucose deprivation

(OGD) conditions (Vieira et al., 2016). Accordingly, CaMKII inhibition or knock-down can lead to a neuroprotective effect against OGD-induced death, whereas kinase overexpression has a detrimental effect. Notably, overexpression of a mutant form of the kinase unable to bind GluN2B induces a partial protection towards OGD-induced damage, thus confirming the pathological role of GluN2B-NMDARs/CaMKII complex (Vieira et al., 2016).

Recent studies have shown that also death-associated protein kinase 1 (DAPK1) can be considered as a potential pharmacological target in stroke (Pei et al., 2014, 2015; Wang et al., 2017), mainly correlated to its interaction with NMDARs (Tu et al., 2010; Buonarati et al., 2020). DAPK1 is a $Ca^{2+/}$CaM-dependent serine/threonine protein kinase that can interact directly with GluN2B(1292–1304) C-terminal domain and active DAPK1 phosphorylates GluN2B at Ser1303 leading to an enhanced NMDAR channel conductance (Tu et al., 2010). Cerebral ischemia promotes the formation of the GluN2B/DAPK1 complex and DAPK1-dependent phosphorylation of GluN2B-Ser1303 that contribute to neuronal death (Tu et al., 2010; Buonarati et al., 2020). Importantly, a peptide able to uncouple activated DAPK1 from the NMDAR complex protects against brain damage in stroke in mice without affecting the physiological activation of the NMDAR (Tu et al., 2010, 2018). Interestingly, disruption of DAPK1/GluN2B complex has been shown to exert also anti-depressant like effects (Li et al., 2018) and could represent a novel target in Huntington disease (Schmidt et al., 2020) thus suggesting the role of this protein-protein interaction in different brain disorders.

All above mentioned studies addressed the key role of GluN2B-containing NMDARs in ischemia. GluN2B-selective antagonists have been widely evaluated both in preclinical models and clinical trials (Lai et al., 2014). Unfortunately, even if GluN2B agents are effective against ischemic neuronal death both *in vitro* and *in vivo* and they have been shown to cause less neurological side effects compared to non-selective antagonists, they demonstrated limited clinical efficacy and other clinical limitations (Lai et al., 2014; Yuan et al., 2015). Accordingly, the identification of more recent pH-dependent GluN2B antagonists, characterized by a higher activity at acidic pH 6.9 associated with ischemic tissue compared to pH 7.6, could open new perspectives for a more specific neuroprotection in ischemic tissue while reducing side-effects associated with NMDAR inhibition in non-injured brain areas (Yuan et al., 2015).

4. Conclusions

Several studies during the last two decades have clearly demonstrated that the modulation of protein-protein interactions between NMDAR regulatory subunits and a variety of scaffolding and signalling proteins represent fundamental events that encode glutamatergic signalling and induce plasticity events. In agreement with these observations, the altered binding of proteins, such as PSD-MAGUK members, with the NMDARs has been identified in several disease states, associated with aberrant NMDAR synaptic localisation or activation of specific downstream signalling pathways (Gardoni and Di Luca, 2006; Ballarin and Tymianski, 2018; Franchini et al., 2020). The use of interfering peptides has been instrumental in preclinical studies, revealing the physiological significance and specificity of a variety of protein-protein interactions involving the NMDAR complexes. The same approach can be applied to disease states to induce the establishment of physiological compositions of NMDARs and has been shown to represent a valid pharmacological tool in preclinical models. Additional clinical studies remain necessary to validate this pharmacological approach in patients.

Recent studies have also suggested that NMDAR interactions with different synaptonuclear protein messengers might play a role in the pathogenesis of brain disorders. Although the molecular mechanisms regulating these events require additional careful examination, the modulation of synaptonuclear messenger activity might represent an innovative approach for both neurodevelopmental and neurodegenerative brain diseases.

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Declaration of competing interest

The authors declare no conflicts of interest.

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