

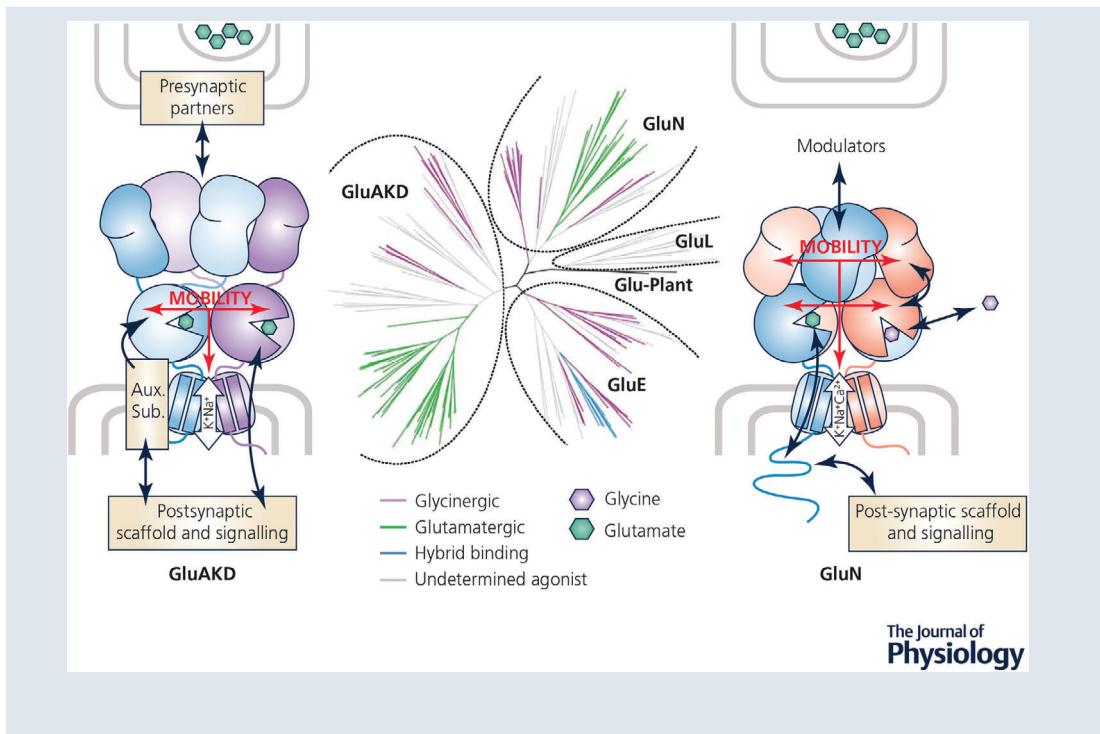
SYMPOSIUM REVIEW

Architecture and function of NMDA receptors: an evolutionary perspective

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Abstract Ionotropic glutamate receptors (iGluRs) are a major class of ligand-gated ion channels that are widespread in the living kingdom. Their critical role in excitatory neurotransmission and brain function of arthropods and vertebrates has made them a compelling subject of interest for neurophysiologists and pharmacologists. This is particularly true for NMDA receptor (NMDARs), a subclass of iGluRs that act as central drivers of synaptic plasticity in the CNS. How and when the unique properties of NMDARs arose during evolution, and how they relate to the evolution of the nervous system, remain open questions. Recent years have witnessed a boom in both genomic and structural data, such that it is now possible to analyse the evolution of iGluR genes on an unprecedented scale and within a solid molecular framework. In this review, combining insights from phylogeny, atomic structure and physiological and mechanistic data, we discuss how evolution of NMDAR motifs and sequences shaped their architecture and functionalities. We trace differences and commonalities between NMDARs and other iGluRs, emphasizing a few distinctive properties of the former regarding ligand binding and gating, permeation, allosteric modulation and intracellular signalling. Finally, we speculate on how specific molecular properties of iGluRs arose to supply new functions to the evolving structure of the nervous system, from early metazoan to present mammals.

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Abstract figure legend Molecular specificities of ionotropic glutamate receptors (iGluRs). The center panel illustrates the phylogenetic tree of metazoan iGluRs with its branches colored according to their agonist specificity. The left and right panels depict the molecular and functional signaling at vertebrate synapses of GluAKD (grouping AMPA, kainate and delta receptors) and GluN (grouping NMDARs) receptors, respectively.

Introduction

With the increasing availability of high-quality genome sequences and the advances in functional genomics, new perspectives have arisen for the exhaustive exploration of protein evolution. This is particularly interesting for the main families of ion channels and receptors whose structure and function have been studied extensively. This is the case for glutamate-gated ion channels, also known as ionotropic glutamate receptors (iGluRs), which play key roles in neurotransmission. In vertebrate, iGluRs are abundant in the central nervous system (CNS) where they mediate the vast majority of excitatory neurotransmission and are essential players of synaptic plasticity which is widely thought to be a cellular substrate for learning and memory (Traynelis *et al.* 2010; Nicoll, 2017). Mammalian genomes encode 18 iGluR subunits that have been extensively characterized, and which, based on their amino acid sequences, pharmacological and functional properties, and molecular structure, have been classified into four groups (Traynelis *et al.* 2010): AMPA, kainate, delta, and NMDA receptors. Within each group, iGluR subunits can assemble as homo- or hetero-tetramers, generating a multiplicity of receptor subtypes. To date, at least 30 distinct iGluR assemblies have been described in native rodent tissue, yet the exact number may be significantly larger (Traynelis *et al.* 2010; Paoletti *et al.*

2013; Zhao *et al.* 2019). This molecular diversity, together with the discrete spatiotemporal expression pattern and signalling properties of each iGluR subunit, contributes to the wide diversity of excitatory responses in the CNS of vertebrates.

Although best known for their critical role in the CNS of vertebrates, the unique sequence and structural signature of iGluRs allowed identification of their presence in many species across the tree of life, including in organisms lacking a nervous system. Thus, hundreds of iGluR homologues have now emerged from sequencing projects, resulting in the discovery of new families of iGluR genes. iGluRs or iGluR-like proteins have been identified in all major families of non-vertebrate animals, in plants, and also in protozoa and even bacteria (Chen *et al.* 1999; Alberstein *et al.* 2015; De Bortoli *et al.* 2016; Li *et al.* 2016; Greer *et al.* 2017; Mayer, 2021). Obviously, these major neurotransmitter-signalling pathways have ancient evolutionary roots that stem from microbial life. In this review, we present the structure and evolutionary origins of iGluRs, focusing on NMDA receptors (NMDARs). This receptor family has always sparked intense interest because of their unique capacity to trigger synaptic plasticity and control information storage within the brain. Interestingly, in humans, NMDAR genes are among the genes most intolerant to variations (Swanger *et al.* 2016; XiangWei *et al.* 2018), and clinical studies have

identified a plethora of disease-causing mutations in patients with moderate-to severe neurodevelopmental phenotypes (Endele *et al.* 2010; Hu *et al.* 2016). Therefore, NMDARs are targets with strong therapeutic potential. From an evolutionary angle, NMDARs are also particularly intriguing, equipped with functionalities that have no equivalent in other iGluRs. This includes high calcium permeability together with voltage-dependent magnesium block of their ion channel pore, slow gating properties, multiple modulatory sites binding endogenous substances, as well as dual agonist dependence, requiring the binding of both glutamate and glycine (or D-serine) for their activation (Paoletti, 2011; Glasgow *et al.* 2015; Hansen *et al.* 2018). These attributes raise fundamental questions about the evolutionary emergence of novel receptor properties as well as agonist specificity of primitive iGluRs. By compiling data from various disciplines – evolution, structural biology, pharmacology and physiology – we discuss the structure and function of NMDARs from an evolutionary perspective, highlighting how these receptors evolved along a common evolutionary path of iGluRs as paramount components of the vertebrate CNS.

Origin of iGluRs: molecular architecture and phylogeny

Modular design. The iGluRs form large tetrameric complexes in which the four subunits assemble around a single membrane-embedded ion channel with a fourfold (or pseudo-fourfold) symmetrical arrangement. In eukaryotes, iGluR subunits display a typical modular architecture composed of four discrete modules or domains (Fig. 1; Mayer, 2006; Traynelis *et al.* 2010; Paoletti, 2011; Sobolevsky, 2015; Zhu & Gouaux, 2017; Hansen *et al.* 2018; Greger & Mayer, 2019): in the extracellular region, a tandem of large globular clamshell-like (or bilobate) domains, the N-terminal domain (NTD) and the ligand binding domain (LBD, also known as ABD for agonist binding domain); a trans-membrane domain (TMD) composed of several transmembrane segments and forming the ion channel; and an intracellular C-terminal domain (CTD) highly variable in length and mostly disordered. Another striking feature of eukaryotic iGluRs, that distinguishes them from other ligand-gated channels, is their layered organization with at the ‘top’ (and the most distal from the pore) the NTDs, at the ‘bottom’ the CTDs and TMD layer, and sandwiched in between the LBDs. All members of the iGluR superfamily also share the same basic gating mechanism whereby agonist binding to the LBDs promotes clamshell closure, which in turn exerts mechanical strain on the short LBD-TMD linkers, eventually pulling open the ion channel pore. Dimerization of the LBDs, and more generally subunit-subunit interfaces, have a key role in

this process, allowing clamshell lobe motions to be transduced into mechanical work on the ion channel (Gouaux, 2004; Mayer, 2005).

The characteristic kit design of (eukaryotic) iGluRs probably arose during evolution through the fusion of separate genes encoding distinct proteins in prokaryotes (Mayer, 2006; Fig. 1). Indeed, early sequence analysis revealed that, apart from the CTD and the last membrane segment (M4), each individual domain (NTD, LBD, M1-M3) shows sequence homology with bacterial proteins of known structures (O’Hara *et al.* 1993; Wo & Oswald, 1995). The M1-M3 pore region resembles an inverted KcsA potassium channel, with the characteristic pore loop (P-loop) involved in ion selectivity. The NTD and LBD are each related to periplasmic binding proteins (PBPs), of the type leucine-isoleucine-valine binding protein (LIVBP or PBP type I) and glutamine-binding protein (QBP or PBP type II), respectively (Tam & Saier, 1993). Those soluble clamshell-like proteins are found in abundance in the periplasmic space of gram-negative bacteria where they contribute to solute uptake (Olah *et al.* 1993). In agreement with this molecular toolbox, individual domains behave in a semi-autonomous fashion, retaining functionality (such as ligand binding) when produced in isolation from the rest of the receptor or when transplanted into chimeric iGluR subunits (Lampinen *et al.* 1998; Furukawa & Gouaux, 2003; Hoffmann *et al.* 2006; Schmid *et al.* 2009; Wilding *et al.* 2014).

GluR0 gating core. In 1999, the identification and functional characterization of the first iGluR found in a prokaryote, GluR0 from the photosynthetic cyanobacterium *Synechocystis* PCC6803, provided spectacular insights into the evolutionary history of iGluRs (Chen *et al.* 1999). GluR0 presents a minimal architecture devoid of the NTD, the M4 segment and accompanying CTD. Yet, it behaves as a functional glutamate-gated (and glutamine-gated) potassium channel. In brief, GluR0 is the result of gene fusion consisting of a type II amino acid PBP member bisected in two discontinuous segments (S1 and S2) by insertion of an inverted P-loop potassium channel (Fig. 1). This unique structural signature, distinct from other neurotransmitter receptors, forms the ‘gating core’ of all eukaryotic iGluRs. At a mechanistic level, it allows coupling ligand binding to gating of the ion channel pore through ‘simple’ opening-closure motions of the LBD (Mayer *et al.* 2001; Mayer, 2006). In prokaryotes, genes encoding GluR0-like proteins are found in proteobacteria and cyanobacteria where they are probably involved in chemotaxis (Lee *et al.* 2008; De Bortoli *et al.* 2016). Because GluR0-like receptors are related to eukaryotic iGluRs in amino acid sequence, architecture and biological function, they are likely to represent the closest descendants of the ancestral precursor that led to the large family of eukaryotic iGluRs.

Origin and distribution of eukaryotic iGluRs. Until now, no GluR0-like short version of iGluRs (nor a PBP on its own) has been reported in eukaryotic genomes. Expanding from the GluR0 scaffold, eukaryotic iGluRs contain a supplementary transmembrane segment right after the agonist-binding PBP fold (i.e. the LBD) that transfers the receptor's C-terminus from the extracellular space to the cytoplasm. This shift in location, which opens opportunities for intracellular interactions with cytoskeletal and signal transduction molecules, has had profound evolutionary and functional implications (discussed below). The other addition is the NTD, corresponding to fusion of another PBP (of type I) upstream of the PBP type II LBD. Hence, eukaryotic iGluRs are two-sided expanded versions of GluR0, with additional domains grafted at both the N and C terminals (Fig. 1).

The iGluRs with the full structural attributes (NTD-LBD-TMD-CTD) are not only found in animals of the unikont supergroup, but also in photosynthetic eukaryotes (bikonts), where they are relatively widespread (Fig. 1). The presence of iGluRs with the same complex

architecture in the two most distant groups of eukaryotes (unikonts and bikonts) suggests that they derive from a common ancestral gene that was present early in the evolution of eukaryotes. Bikont iGluRs are found both in plantae (green algae and plants) and stramenopiles (brown algae, diatoms), showing a large expansion of their gene repertoire in vascular plants, with 22 iGluR genes identified in *Arabidopsis* and no less than 44 in *Pinus taeda* (Chiu *et al.* 1999, 2002; De Bortoli *et al.* 2016). Plant iGluRs have been proposed to participate in many different physiological processes including root development, reproduction, gamete chemotaxis, cellular defense and stomatal closure (De Bortoli *et al.* 2016; Ortiz-Ramírez *et al.* 2017). Moreover, the presence of iGluRs in bikont organelles (chloroplasts and mitochondria) is interesting to put in relationship with the presence of GluR0-related genes in cyanobacteria or proteobacteria (Teardo *et al.* 2011, 2015; De Bortoli *et al.* 2016).

Among unikonts, iGluR genes are present in all groups of metazoans (porifers, placozoans, ctenophores, cnidarians and bilaterians; Fig. 2A and B). In contrast,

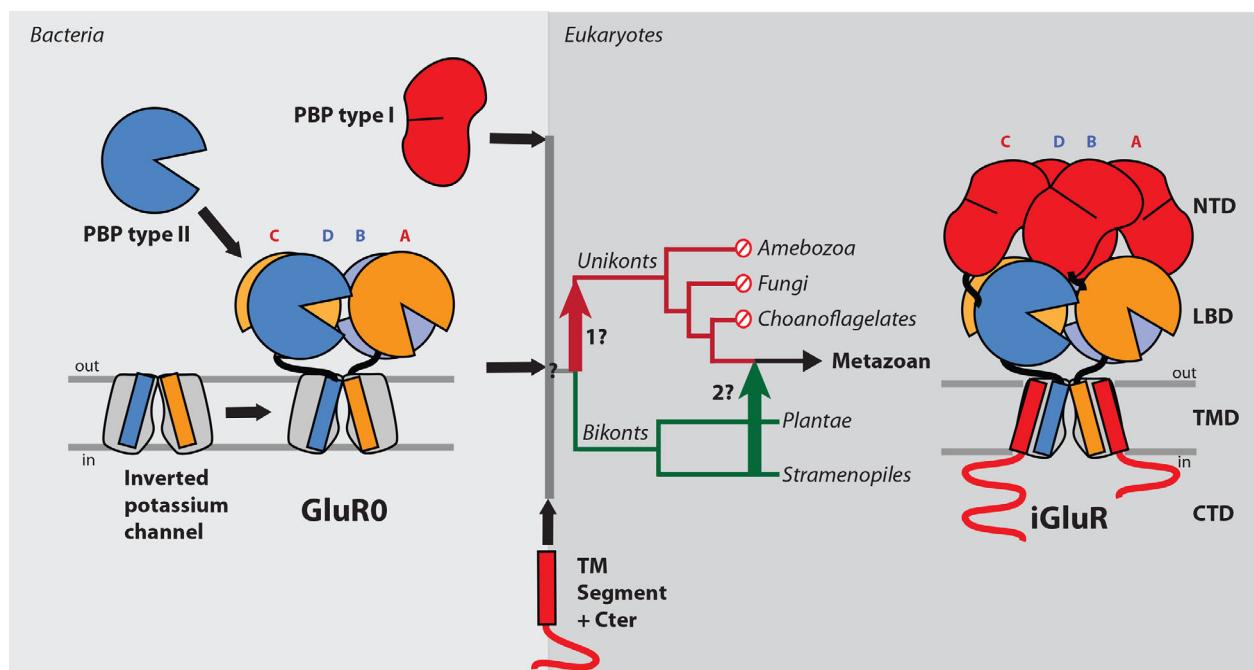


Figure 1. Origin and architecture of iGluRs

Origin and modular architecture of iGluRs from bacteria to metazoans. GluR0: prokaryotic iGluR homologue from the cyanobacterium *Synechocystis PCC6803* consisting in the fusion of a PBP with an inverted potassium channel (Chen *et al.* 1999). PBP, periplasmic binding protein; NTD, N-terminal domain; LBD, ligand-binding domain; TMD, transmembrane domain; CTD, C-terminal domain; iGluR, ionotropic glutamate receptor. The right panel depicts the architecture and evolution of eukaryotic iGluRs, while the left panel depicts iGluR building components found in bacteria. To the best of our knowledge, all these components stem from eubacteria but not archaeabacteria. The grey line between the Bacteria and Eukaryotes panels corresponds to the assembly of several bacterial components to form a full eukaryotic iGluR. The associated question mark reminds us that it is unknown when and in which kingdom this event took place. The red and green arrows with question marks on the right panel represent two hypotheses for the origin of modern metazoan iGluRs (see text). Crossed red circle symbols stand for no gene transmission or gene loss.

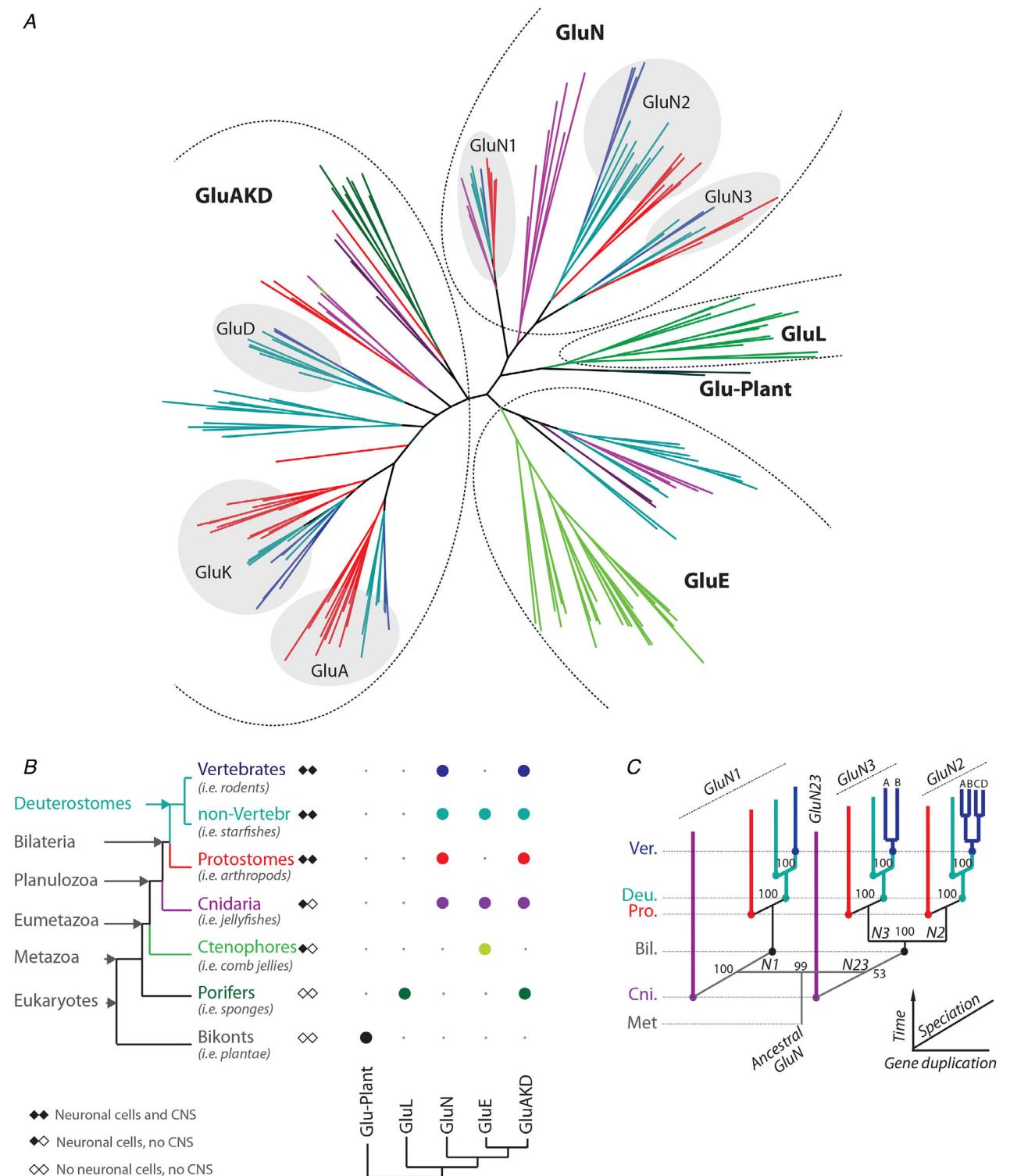


Figure 2. Phylogeny of metazoan iGluRs

A, phylogenetic tree of metazoan iGluR genes with plant iGluRs as outgroup. A radial representation of a Bayesian tree built with 203 iGluR genes is shown. The calculation procedure was performed as described in Ramos-Vicente *et al.* (2018) using the same gene dataset except for partial sequences that were excluded. The branch colour indicates specific metazoan phyla. Blue indicates genes from vertebrates (humans); cyan, other deuterostomes (echinoderms, hemichordates and non-vertebrates chordate); red, representative protostomes (arthropods, annelids, mollusks); magenta, representative cnidarians (corals, anemone and jellyfish); light green, ctenophores; dark purple, placozoans; dark green, porifers (homoscleromorphs and calcareous sponges). The outgroup

none is currently detectable in the genomic database of non-metazoan unikonts such as opitokonts (fungi), choanoflagellates and amebozoa (Fig. 1) (Ramos-Vicente *et al.* 2018). This absence of iGluR genes in species branching at the root of the unikont evolutionary tree singularly differs from the situation observed with pentameric cys-loop receptors (Jaiteh *et al.* 2016), metabotropic receptors (Krishnan *et al.* 2014) and other important components of synapses (Burkhardt *et al.* 2014; Burkhardt, 2015). One possibility is that iGluRs were transmitted from early unikonts to metazoans but lost individually by each branch of non-metazoans (Fig. 1 vertical red arrow) (Liebeskind *et al.* 2015). An alternative, and more parsimonious, hypothesis is that metazoan iGluRs were acquired by horizontal gene transfer from bikonts (Fig. 1, vertical green arrow). Both plantae and stramenopiles possess iGluRs in their genomes, yet these two groups presumably separated around 1–1.5 billion years ago, long before the emergence of metazoans (porifer oldest fossil records dating back ~600 million years; Yin *et al.* 2015). Thus, operational iGluRs were probably already in usage in bikonts before the first metazoans such as commensal sponges emerged.

iGluRs in metazoans. Within metazoans, iGluR genes are ubiquitously present in neural species (eumetazoans: ctenophores, cnidarians and bilaterians) (Fig. 2A and B). They are also found in some aneural metazoans, particularly in calcareous sponges and homoscleromorphs, two of the four main groups of porifers (Ramos-Vicente *et al.* 2018). Sponges lack neurons or synapses yet do harbour homologues of many genes central to synaptic function (Gazave *et al.* 2010; Burkhardt *et al.* 2014; Mah & Leys, 2017; Wong *et al.* 2019). It is worthy of note that the place of porifers at the root of the metazoans is still debated and has been proposed to branch later than the nerve net-containing ctenophores (Francis *et al.* 2017; King & Rokas, 2017). Overall, the presence of iGluRs in unikonts can be traced back to

early metazoans, and iGluR gene diversification at that time coincides closely with the appearance of nervous communication.

At the root of the metazoan iGluR phylogenetic tree, four main branches can be distinguished (Ramos-Vicente *et al.* 2018): GluL, GluE, GluN and GluAKD (Fig. 2A and B – and see legend for naming of groups) The GluL branch groups iGluRs that are found in some porifers only. Their physiological role and functional properties are still completely unknown. The GluE branch corresponds to a new group of iGluRs seemingly absent in vertebrates and protostomia yet present in most other eumetazoans (Ramos-Vicente *et al.* 2018). Currently, it gathers all ctenophore iGluRs (Alberstein *et al.* 2015) and genes present in cnidarians and deuterostomes. Their physiological role remains elusive but some GluE genes are expressed in neurons and encode receptors exhibiting amino-acid gated ion fluxes (Alberstein *et al.* 2015; Ramos-Vicente *et al.* 2018). Whether this recently proposed group is truly monophyletic remains to be established. The GluAKD branch groups the well-known AMPA (GluA), kainate (GluK), delta (GluD) receptors and other unstudied groups (Fig. 2A). Here again, the monophyly of this group remains unproven, given the uncertainties regarding the position of sub-groups at its root. The GluAKD is the only iGluR group in which all metazoan iGluR-containing species are represented from sponges to mammals (except ctenophores). This underlines the potential seniority of the group and may explain the greater divergence between its paralogues and orthologues than in the fourth and last group, the GluN branch that brings together the NMDARs.

NMDAR evolution. The GluN branch that contains all NMDARs stands out from the four branches of metazoan iGluRs as clearly monophyletic (Fig. 2A). GluN genes (the official gene nomenclature is *Grin*) are not detected in ctenophores and sponges but are found in all cnidarians and bilaterians (Fig. 2B). The correspondence of the calculated phylogenetic tree with the consensual tree of

'Glu-Plant' is in black. The four main iGluR groups (GluAKD, GluN, GluL and GluE) are separated by thick dotted lines. Grey shaded circles localize the vertebrate iGluR groups. Note on names of iGluR groups: We use in this review the gene group names GluL, GluE, GluN and GluAKD as first defined by Ramos-Vicente *et al.* (2018) because they are convenient and the existence of these groups is well supported by statistics. Note however that we shortened the name of the 'GluAKDF' group to 'GluAKD'. The F branch or subgroup does exist but many other distinct and still uncharacterized branches are present in the GluAKDF group, which will require further names. For the sake of simplicity, we preferred to keep just the name of the three main branches A, K and D. Please also note that the official gene nomenclature for GluA, GluD, GluN and GluK are *Gria*, *Grid*, *Grin* and *Grik*, respectively. B, distribution of four main iGluR gene groups in the metazoan kingdom. The coloured dots indicate which iGluR group (column) is present in which metazoan phylum. Same colour code as in panel A. Please note that this distribution depends on our present partial knowledge of metazoan genomes. Bottom right: representation of the phylogenetic relationship between the four identified iGluR gene groups rooted with plant iGluR genes. Each node of this schematic tree corresponds to nodes at the base of the Bayesian tree represented in panel B with posterior probabilities of 100 (on a scale 0–100). For each phylum, the presence of neuronal cells and of a centralized nervous system (CNS) is indicated. C, origin and diversity of NMDA receptors (NMDAR) genes (GluN). Schematic representation of the GluN genes evolution from a single putative eumetazoan ancestral gene to all currently known GluN genes. Same colour code as in panels A and B. Numbers indicate posterior probabilities (scale 0–100) of the corresponding nodes.

life is especially striking for the GluN1 subunit (Fig. 2C). Indeed, GluN1 metazoan orthologues, from mammals to cnidarians, share a minimum of 46% amino acid sequence identity (*versus* 26% at best between the same distant orthologues for any subunit of the GluAKD branch). It thus appears that GluN genes, once assembled in the early history of eumetazoans, have been submitted early on to a much stronger conservative selection pressure than any other iGluR subtype. Moreover, GluN genes have been conserved, duplicated and transmitted only in organisms containing a nervous system (cnidarians and bilaterians). In that respect, GluN is the only monophyletic iGluR group that is specific to neural organisms (ctenophore apart).

The pool of mammalian GluN genes comprises one GluN1 gene and two GluN3 genes (GluN3A-B), encoding glycine-binding NMDAR subunits, and four GluN2 genes (GluN2A-D) encoding glutamate-binding NMDAR subunits. Sequence similarities indicate that GluN2 and GluN3 subunits are closer paralogues than with GluN1. The duplication of GluN3 genes and the tetrlication of GluN2 genes result from the dramatic whole genome duplication (WGD) event that occurred in early vertebrates around 500 million years ago (Mya) (Fig. 2C; see also Fig. 6A and Sacerdot *et al.* 2018). A step back in time, protostomes and deuterostomes inherited one GluN1, one GluN2 and one GluN3 gene from the same bilaterian ancestor. The situation differs in cnidarians, however. There, a GluN1 subunit is readily found but all other GluN subunits form a cnidarian-specific subgroup. This subgroup, named here GluN23, appears to cluster before the separation between the GluN2 and GluN3 subunits (Fig. 2A and C). We conjecture that the splitting of distinct GluN2 and GluN3 gene groups may be an interesting marker of CNS evolution. Finally, one of the most distinctive features of bilaterian NMDARs is their obligate heteromeric assembly. While AMPA and kainate receptors can form functional homotetrameric complexes, NMDARs require strict association of two GluN1 subunits with two non-GluN1 subunits (either GluN2 or GluN3). Structures of full-length vertebrate NMDARs convincingly showed that GluN1 subunits and GluN2 subunits occupy non-equivalent positions in the assembled tetramer (Karakas & Furukawa, 2014; Lee *et al.* 2014; Lu *et al.* 2017). The distinct evolutionary pathway of GluN1 and GluN2-3 genes, as well as the high evolutionary conservation of the GluN1 gene, suggest that heteromeric assembly might be an ancient property of NMDARs.

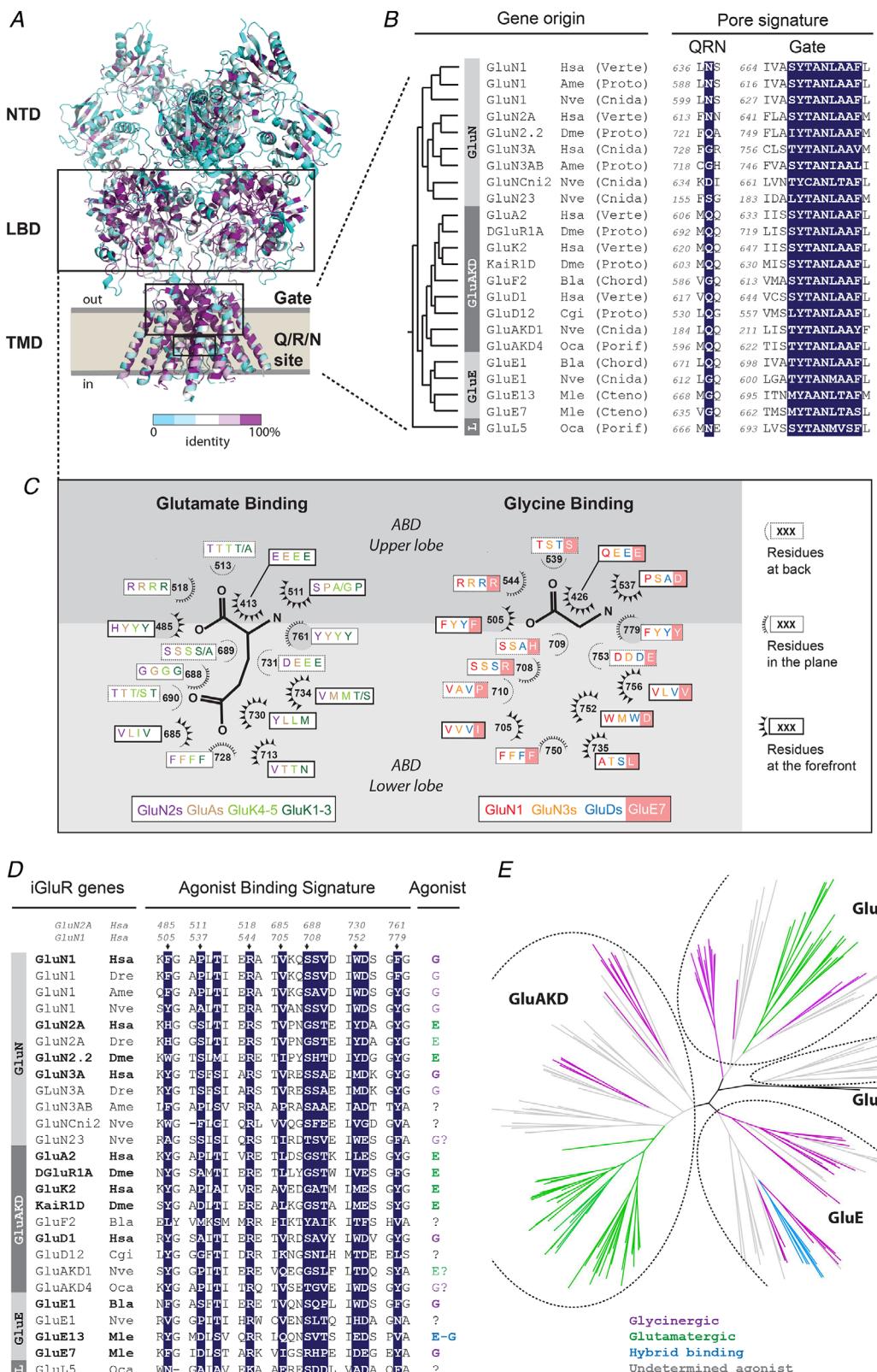
Pore signatures. As all other ion channels, iGluRs are defined by their permeation and gating properties that reflect the types and amounts of ions translocating through the ion channel pore and their access to this permeation pathway. Mapping the amino acid sequence homology between all human iGluR subunit paralogues

identifies the LBD and ion channel pore as the most conserved regions (Fig. 3A), while the NTD and CTD are the least conserved (the latter greatly diverging in length, see below and Fig. 5E). Among the 18 subunits, the NTD share only 22% mean of pairwise sequence identity, as opposed to 42% for the LBD and 47% for the M1-M3 region. Thus, the region that embeds and controls the gating and permeation machinery – the GluR0-like core (LBD + M1-M3) – is the most conserved. In contrast, the receptor's 'periphery', including the NTD, several surface loops, the fourth transmembrane segment (M4), and the CTD, is poorly conserved. Interestingly, these least conserved regions correspond to additions specific to eukaryotes in evolution (Fig. 1). Functionally, they also correspond to regions that are not directly involved in channel gating and permeation, but provide opportunities for subunit-specific regulations.

Lining the ion channel pore and central to its gating is the canonical SYTANLAAF motif in the TMD third helix (M3) that is highly conserved motif in all iGluR subunits (Fig. 3B). Located at a point of contact between the four subunits, the M3 bundle crossing forms a constriction point that seals the channel closed at rest (i.e. in the absence of agonist) and expands via an iris-like motion during gating to allow the ions through (Twomey *et al.* 2017; Poulsen *et al.* 2019), a mechanism likely to be inherited from potassium channels (Jiang *et al.* 2002). The SYTANLAAF motif is particularly intolerant to natural mutations and is a hot spot for disease-causing mutations (Zuo *et al.* 1997; Swanger *et al.* 2016; Amin *et al.* 2021). Mutations in this motif disrupt receptor gating, the most extreme leading to either constitutively open (such as the Lurcher mouse mutant; Zuo *et al.* 1997; Hansen *et al.* 2009) or closed (Davies *et al.* 2017) channels, alterations expected to dramatically impair receptor signalling. Sitting below the upper SYTANLAAF constriction is a second narrow constriction formed by the extended regions of the M2 re-entrant loops, another motif inherited from potassium channels (Wo & Oswald, 1995; Kuner *et al.* 2003). This region, and most particularly the Q/R/N site located at the tip of the re-entrant loop, is a critical determinant of iGluR permeation, serving as selectivity filter. While in mammalian AMPA and kainate receptors, the presence of a glutamine (Q) or an arginine (R) dictates the channel rectification properties, in most mammalian NMDARs, an asparagine (N) plays essential role in controlling calcium permeability and magnesium block (Fig. 3B; Traynelis *et al.* 2010; Wollmuth, 2018).

Beyond glutamate binding: glycine and NMDAR co-agonism

In 1987, Johnson and Ascher made the unexpected observation that NMDARs are capable of binding glycine, in addition to glutamate, and that glycine causes

**Figure 3. Sequence conservation and glycine agonism**

A, amino acid conservation profile between the 18 human iGluR orthologues. The template structure corresponds to that of GluN1/GluN2B NMDAR (Karakas & Furukawa, 2014; Esmenjaud *et al.* 2019). Positions with >90% identity are coloured in magenta, ~75% in white, and <60% in cyan. NTD,

strong potentiation of the receptor activity (Johnson & Ascher, 1987). Subsequent studies rapidly established that glycine binding, together with glutamate, is an absolute requirement for NMDAR activation (Kleckner & Dingledine, 1988; Benveniste *et al.* 1990; Clements & Westbrook, 1991). This discovery has had multiple and profound implications in neurobiology. First, it revealed that glycine is not exclusively an inhibitory neurotransmitter (through its action on pentameric glycine receptors). Second, it offered a new target for drug development against neurological and psychiatric disorders linked to NMDAR dysfunction. Third, it promoted the pharmacological concept of co-agonism whereby the simultaneous presence of two ligands of different chemical nature is mandatory for receptor activation. To our knowledge, co-agonism by two different neurotransmitters as observed with NMDARs is unique among all CNS receptors involved in intercellular signalling. Co-agonism at NMDARs endows these receptors with unique capabilities of sensing and adjusting to their extracellular microenvironment. This may take shape as unique crosstalk between excitatory (glutamatergic) and inhibitory (glycinergic) synapses (Ahmadi *et al.* 2003), or between neurons and glycine transporter enriched glial cells (Harvey & Yee, 2013). Strong evidence also indicates that D-serine, in addition to glycine, is another NMDAR co-agonist endogenously found in the vertebrate CNS, being particularly abundant in the forebrain (Oliet & Mothet, 2009; Wolosker, 2018).

Distribution of glycinergic iGluR subunits. A significant subset of vertebrate iGluR subunits binds glycine (or D-serine) and not glutamate (or with extremely low affinity). This is the case of the NMDAR subunits GluN1, GluN3A and GluN3B (Chatterton *et al.* 2002), and of the

GluD1 and GluD2 subunits (Naur *et al.* 2007; Fig. 3C–E). While conventional NMDARs made of GluN1 and GluN2 subunits display ‘mixed’ pharmacology, binding glutamate (on GluN2) and glycine (on GluN1), other vertebrate glycine binding iGluRs appear to operate as ‘pure’ glycine receptors. This is the case with GluD receptors (although for these receptors direct binding of glycine is not sufficient to drive ion channel opening) (Hansen *et al.* 2009), and with GluN1/GluN3 receptors. These latter, known as non-conventional NMDARs and originally described in heterologous expression systems (Chatterton *et al.* 2002), were recently identified in the mouse forebrain as forming neuronal glycine-gated excitatory conductances (Grand *et al.* 2018; Otsu *et al.* 2019).

Global analysis of the iGluR phylogenetic tree reveals that, in each of the GluE, GluAKD and GluN branches, both glycinergic and glutamatergic subunits co-exist (Fig. 3E). Interestingly, glycinergic subunits appear systematically closer to the root of the tree than glutamatergic ones. This is the case for GluN1 in the NMDAR GluN branch and for GluDs in the GluAKD branch, but also for the GluE branch that encompasses glycine-gated iGluRs from ctenophores (Fig. 3E; see also Alberstein *et al.* 2015). This raises the intriguing possibility that iGluRs from the earliest metazoan lineage were not glutamatergic but rather glycinergic (Alberstein *et al.* 2015). However, many iGluRs from early metazoans have not been functionally characterized. Therefore, the information about their ligand selectivity is lacking and cannot also be easily inferred from their amino acid sequences, which are too divergent (see below). This is, for instance, the case for sponge iGluRs of the entire GluL branch, and of the cnidarian GluN23 subgroup in the GluN branch. The question of the ligand specificity of the first metazoan iGluRs thus remains open. One

N-terminal domain; LBD, ligand-binding domain; TMD, transmembrane domain; CTD, C-terminal domain. Note that conservation is strongest in the gating core region (LBD+ inner part of the TMD). *B*, the SYNTANLAAF signature sequence and the Q/R/N site of the iGluR ion channel pore. Alignment of sequences from 23 genes representative of metazoan iGluR diversity. Positions highlighted in blue are highly conserved amino acids and/or amino acids that play critical functional role (Q/R/N site). Species (and group) acronyms are the following: Hsa, *Homo sapiens* (Chordata, vertebrates); Dre, *Danio rerio* (Chordata, vertebrates); Bla, *Branchiostoma lanceolatum* (Chordata); Lgi, *Lottia gigantean* (Protostomes); Ame, *Apis mellifera* (Protostomes); Dme, *Drosophila melanogaster* (Protostomes); Cgi, *Crassostrea gigas* (Protostomes); Nve, *Nematostella vectensis* (Cnidaria); Mle, *Mnemiopsis leidyi* (Ctenophora); Oca, *Oscarella carmela* (Poriferia). *C*, iGluR agonist binding pocket: schematic mapping of the ligand binding pocket of selected glutamatergic iGluR subunits (left) and glycinergic iGluR subunits (right) within the LBD clamshell. The position of the residues compared to the plane of the agonist is mentioned according to the legend on the right. The associated residue numbers are those of human GluN2A (left) and human GluN1 (right). The four coloured amino acids (in one letter code) at each position correspond to the residues found in the four iGluRs mentioned in the colour bar below the two binding sites. All selected sequences are from vertebrate genes except GluE7 highlighted in rose that is from ctenophores. *D*, evolution of iGluR agonist binding signature. Same representation as in *B* and same species (and group) acronyms. Positions highlighted in blue are those involved in the agonist-binding pocket. The top rows in italic indicate the amino acid numbering for the human GluN2A and GluN1 subunits (as in panel *C*). In the last column are indicated the experimentally validated (bold) and predicted (non-bold) agonist-binding specificity: ‘G’ for glycine (magenta), ‘E’ for glutamate (green). A question mark is present when the specificity is difficult to predict or unclear. *E*, distribution of agonist (glutamate and glycine) specificity of metazoan iGluRs. Same phylogenetic tree as in Fig. 2A. The term ‘Hybrid binding’ corresponds to receptor subunits that bind both glutamate and glycine.

cannot dismiss the possibility that ancestral metazoan iGluRs did not clearly discriminate between glutamate and glycine (or other amino acids), as found with GluE13 from the ctenophore *Mnemiopsis leidyi* (Alberstein *et al.* 2015; Fig. 3*D*) or with AvGluR1 from the rotifer *Adineta vaga* (Janovjak *et al.* 2011; Lomash *et al.* 2013).

Molecular determinants of agonist specificity. Contrasting with many other ligand-gated ion channels where agonists bind at subunit interfaces (Lemoine *et al.* 2012), at iGluRs, each subunit harbours its own agonist binding site formed by the clamshell-shaped LBD. A wealth of high-resolution structures, as well as binding and functional studies, have demonstrated that agonists activate the receptor by binding the LBD central interlobe cleft and stabilizing the domain in a closed cleft conformation (Armstrong & Gouaux, 2000; Armstrong *et al.* 2003; Pøhlsgaard *et al.* 2011; Yao *et al.* 2013; Møllerud *et al.* 2017). Interestingly, a limited set of amino acids from both the upper and lower lobes make direct interactions with the agonist molecule, therefore playing key roles in agonist selectivity and affinity. The recognition of the amino-acid backbone over other molecules is mainly determined by two highly conserved upper lobe residues, an arginine (R518 in hGluN2A, R544 in hGluN1) binding the amino acid α -carboxylate, and an acidic residue (D731 in hGluN2A, D753 in hGluN1) binding the amino acid α -amine (Fig. 3*C* and *D*). These two conserved positions act as an anchoring site for amino acids and are found in almost all the metazoan iGluR genes but also in iGluRs from photosynthetic eukaryotes. Their presence is generally considered as a defining criterion for an iGluR to operate as an amino acid sensitive receptor.

The selectivity between glutamate and glycine relies heavily on residues from the lower lobe. The pocket where the glutamate lateral chain binds is occluded in glycine-selective iGluR subunits, sometimes filled by bulky hydrophobic residues such as W752 in hGluN1 (Fig. 3*C* and *D*; Furukawa & Gouaux, 2003; Naur *et al.* 2007; Yao *et al.* 2008). Accordingly, the size of the glycine-binding cavity is significantly smaller than that of glutamate, pointing to a selectivity mechanism based, at least in part, on steric hindrance (Mayer, 2006). This may be an over-simplification, however. Indeed, the residue at the position homologous to hGluN1 W752 is a methionine in the glycinergic hGluN3A and hGluN3B subunits, but a methionine is also present in the glutamatergic hGluK1-3 subunits. Thus the residue occupying the position homologous to hGluN1 W752 does not appear to be a simple faithful predictor of glutamate *vs.* glycine binding selectivity. We note that another site in the LBD lower lobe might be a better predictor (although again not absolute). At positions homologous to G688 of hGluN2A, a glycine residue is present in most glutamate-sensitive iGluR subunits, while, a serine is usually found instead

in glycinergic iGluRs (Fig. 3*C* and *D*). As the G688 of hGluN2A is in close contact with the lateral chain of the bound glutamate (Furukawa *et al.* 2005), we propose that a LBD with the small glycine residue can accommodate the long side chain of glutamate while a LBD with the longer serine residue at the same position cannot. Any L amino acid larger than glycine would be likely to generate a steric clash. In contrast, amino acids with the D stereochemistry would fit because of their reoriented side chain, probably accounting for the robust D-serine (or D-alanine) binding at many glycine sites of iGluR members. Extensive sequence comparison reveals, however, that there are exceptions to the above-mentioned ‘rule’ (for instance with certain iGluRs from ctenophores; Fig. 3*C* and *D*; see also Alberstein *et al.* 2015). This highlights the complexity of the quest for a universal signature sequence for glycine *vs.* glutamate selectivity in iGluRs (if at all).

Focusing more specifically on NMDARs reveals that GluN1 encoding genes from distant organisms are likely to share a glycinergic profile. Indeed, the residues directly participating in the ligand binding pocket show strong conservation from glycine-binding mammalian GluN1 to cnidarian GluN1 (Fig. 3*D*). Similarly, the specificity for glutamate in GluN2 encoding genes appears well conserved between deuterostomes and protostomes (e.g. *Drosophila*). In agreement with this observation, electrophysiological recordings of GluN1/GluN2 receptors assembled from *Drosophila* genes clearly indicate a dual requirement for glutamate and glycine for activation (Xia *et al.* 2005). Therefore, distinct GluN agonist sensitivity and co-agonism seem to be ancient properties of NMDARs, dating at least 500 Mya, before the separation between protostomes and deuterostomes. However, it is unclear whether NMDAR co-agonism can be traced back to cnidarians. In these organisms, a glycinergic GluN1 encoding gene is clearly identified, but the agonist specificity of the group of GluN2 genes remains undetermined (although potential glycinergic signatures are detectable; Fig. 3*D*). In the absence of well-defined glutamate binding signatures, one cannot exclude the possibility that cnidarian GluN receptors are glycinergic only, like the newly identified vertebrate GluN1/GluN3 receptors (Grand *et al.* 2018; Otsu *et al.* 2019).

Distinctive structural features of NMDARs

Over the last 20 years, our knowledge about the structural biology of iGluRs has increased enormously. It started with the first X-ray structures of an isolated ligand-binding domain (Armstrong *et al.* 1998), reaching a milestone with the first X-ray structure of an entire AMPA receptor (Sobolevsky *et al.* 2009), and culminating recently with multiple full-length structures solved by single-particle cryo-EM. Atomic or quasi atomic structures of iGluRs in

various activity states are now available for AMPA (Dürr *et al.* 2014; Twomey *et al.* 2017, 2018; Herguedas *et al.* 2019; Zhao *et al.* 2019), kainate (Meyerson *et al.* 2014; Kumari *et al.* 2019), delta (Elegheert *et al.* 2016; Burada *et al.* 2020) and NMDA (Tajima *et al.* 2016; Zhu *et al.* 2016; Lu *et al.* 2017; Jalali-Yazdi *et al.* 2018; Zhang *et al.* 2018) receptors, all from vertebrates (*Xenopus*, rodents or human). These structures invariably show the typical layered architecture and the dimer-of-dimers organization of the receptor's extracellular region. Moreover, AMPA, kainate and NMDA receptors also display the typical domain 'swapping' (also known as 'subunit crossover') such that subunits change dimerization partner between the NTD and LBD levels.

Compaction. A striking difference when comparing full-length structures of NMDARs and non-NMDA iGluRs (GluAKD receptors) is the receptors' compaction. While in NMDARs the NTD layer tightly packs on the LBD layer, in GluAKD receptors the two layers appear almost disconnected with the NTDs 'floating' above and minimally interacting with the LBDs (Fig. 4A). Analysing the compaction of 67 full-length structures from the two families reveals a significantly more extended extracellular region in GluAKD receptors than in NMDARs (Fig. 4A; right panel). Therefore, at synaptic sites, GluAKD receptors, and to a lesser extent NMDARs, form massive complexes protruding almost halfway through the synaptic cleft (assuming a synaptic cleft width of ~30 nm). Not unsurprisingly, several GluAKD receptors, including kainate (Matsuda *et al.* 2016) and delta (Matsuda *et al.* 2010; Elegheert *et al.* 2016; Fossati *et al.* 2019) receptors can engage in trans-synaptic interactions, with important functional consequences for synapse maturation and plasticity (Yuzaki & Aricescu, 2017). Similar trans-synaptic contacts have not been described for the more compact NMDARs.

Organization and functional impact of the NTDs. Comparison of the NTD tetramer organization from AMPA, kainate, delta and NMDA receptors reveals that the overall arrangement of the four NTDs is remarkably similar between the GluAKD family members but diverges markedly from that in NMDARs (Fig. 4B; top views). The difference between the two groups of receptors (GluN vs. GluAKD) first stems from the NTD conformation itself. In NMDARs, individual NTDs adopt a peculiar twisted conformation, with no equivalent in any other LIVBP-like clamshell domain, where the two constitutive lobes are rotated one relative to the other by ~45° (Karakas *et al.* 2009, 2011; Stroebel *et al.* 2011; Karakas & Furukawa, 2014; Lee *et al.* 2014). This unique geometry directly affects NTD dimerization. In GluAKD receptors, the two NTDs are virtually glued together making extensive interactions

between their upper and lower lobes (Fig. 4B; side views). They behave essentially as a rigid body. In contrast, in NMDARs the NTD dimer arrangement is much less tightly packed. The upper lobes form the main dimer interface while the lower lobes are separated, permitting structural reconfigurations (Fig. 4B; Karakas *et al.* 2011; Zhu *et al.* 2013; Karakas & Furukawa, 2014; Lee *et al.* 2014).

The NTDs of AMPA and kainate receptors have little influence on the receptor gating properties (Möykkynen *et al.* 2014; Yelshanskaya *et al.* 2016), compatible with the very loose connection between the NTD and LBD layers in these receptors. In other words, motions of the LBDs occurring during receptor activation and desensitization (LBD closure and LBD dimer interface disruption, respectively; Armstrong & Gouaux, 2000; Sun *et al.* 2002) are expected to be minimally impeded by the distal NTDs. This structural and functional decoupling between the NTD and LBD layers probably contributes to a distinctive feature of AMPARs, their exceptionally high speed of operation which is uniquely matched for fast signal propagation within neuronal networks (Baranovic & Plested, 2016). The situation differs for NMDARs. There, the extensive interlayer contacts between the NTDs and LBDs provide the physical basis for the strong functional coupling known to occur between the two layers. NMDAR NTDs form allosteric hubs whereby local conformational changes – either spontaneous or driven by binding of allosteric modulators – are transmitted to the downstream gating machinery to affect receptor channel activity (Gielen *et al.* 2009; Yuan *et al.* 2009; Mony *et al.* 2011; Zhu *et al.* 2013; Tajima *et al.* 2016; Esmenjaud *et al.* 2019). Reciprocally, LBD motions affect NTD motions and binding of modulatory ligands (Zheng *et al.* 2001; Zhu *et al.* 2013). Therefore, the extracellular region of NMDAR acts as an integrated allosteric unit, whereby the NTD and LBD layers reciprocally influence one another. Obviously, this coupling has been rendered possible by the receptor's strong compaction, unique among vertebrate iGluRs.

Figure 4C provides an illustration of the differential conformational landscape of NMDARs vs. AMPA and kainate receptors. Compiled from 73 full-length iGluR structures, it reveals that NTD conformational dynamics is higher in NMDARs (as assessed by lower lobe-lower lobe distances within NTD dimers), while LBD quaternary rearrangements appear of larger amplitude in AMPA and kainate receptors (as assessed by inter-LBD dimer distances; Fig. 4C). With their NTDs and LBDs knitted together, NMDARs are endowed with strong allosteric capacity translating into high sensitivity to their extracellular environment, a feature that is important for synapse and brain function (see below). This capacity, however, probably came at the expense of speed, the structural constraints imposed by the NTDs on the LBDs limiting NMDAR gating kinetics.

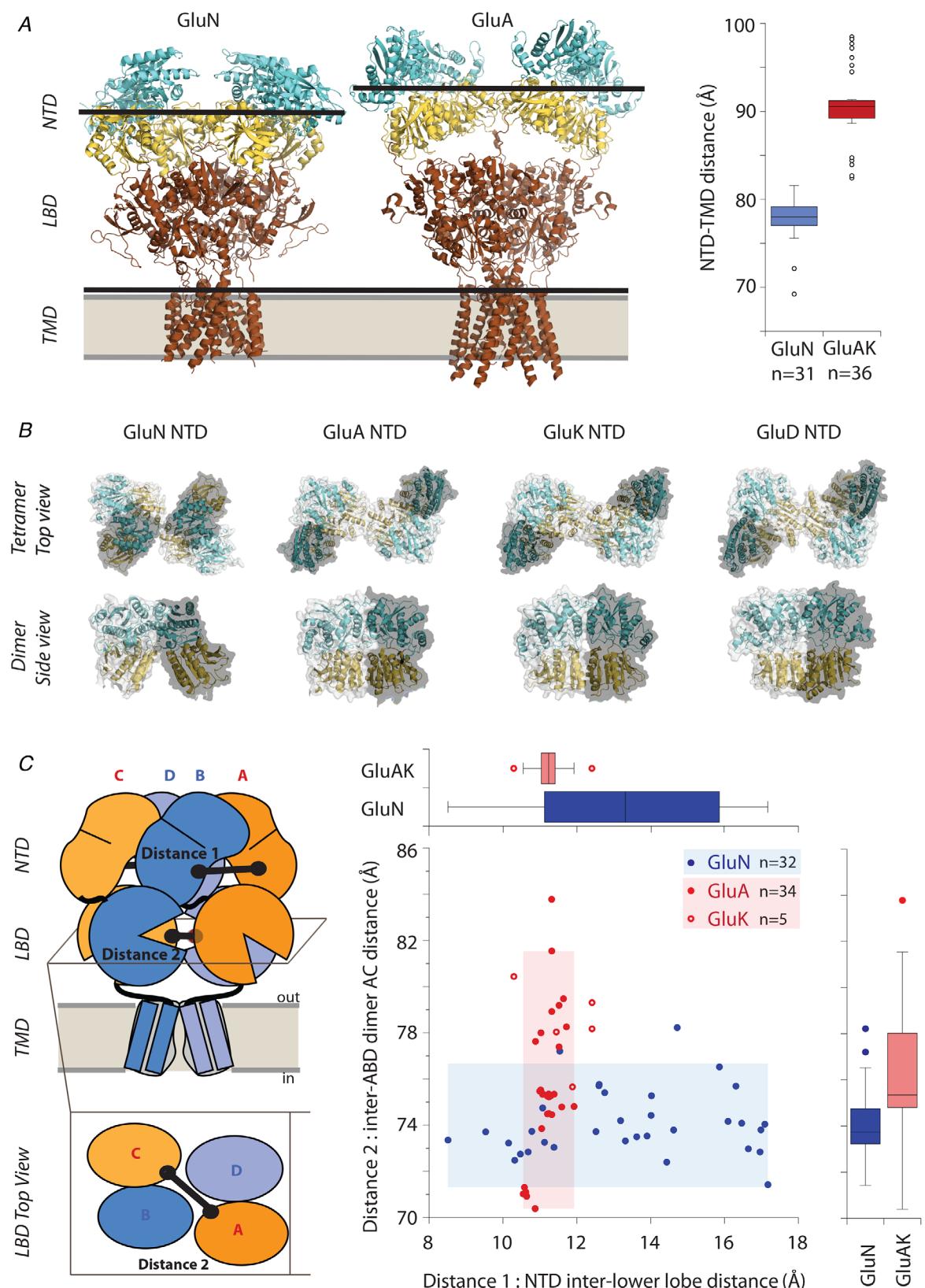


Figure 4. Distinctive structural features of NMDARs

A, the receptor extracellular domain is more compact in NMDARs than in AMPA and kainate receptors. Left: comparison between the structures of the GluN1/GluN2B NMDAR (GluN, PDB 4PE5; Karakas & Furukawa, 2014)

Domain evolution shaped NMDAR properties

As presented above, NMDARs developed a specific molecular architecture and an elaborate inter-domain communication that has no equivalent in other iGluRs, at least in vertebrates. Because the receptor gating core (LBDs + TMD) is highly conserved (Fig. 3A) and poorly tolerant to variations (Swanger *et al.* 2016), structural elements underlying the functional differences between receptor families usually occur as modifications or extensions at the receptor's surface or in less conserved regions. Domain extensions such as surface-exposed loops are classical markers of functional divergence in large multimeric complexes. We present below several illustrative examples of such loops and other domain extensions that distinguish NMDARs from other iGluRs. Important functional consequences for receptor function and neuronal signalling are discussed.

NTD loops and zinc sensitivity. The NTD is the least conserved folded domain of iGluRs (Fig. 3A). While in AMPA and kainate receptors the NTDs seem functionally 'inert', in NMDARs they form major regulatory domains undergoing strong conformational dynamics (Zhu & Paoletti, 2015). Notably, the NTD region of some NMDAR subunits provides binding sites for small ligands acting as subunit-specific allosteric modulators of the receptor channel activity. The NTD region of GluN2B-containing NMDARs (GluN2B-NMDARs) bind two agents found endogenously in the CNS: zinc and polyamines, the former acting as an allosteric inhibitor (Rachline *et al.* 2005), and the latter as an allosteric potentiator (Mony *et al.*

2011). It also binds ifenprodil and derivatives, a large family of synthetic GluN2B-selective antagonists with therapeutic potential (Williams *et al.* 1990; Perin-Dureau *et al.* 2002; Karakas *et al.* 2011; Stroebel *et al.* 2016). The NTD region of GluN2A-NMDARs also binds zinc, but with much greater affinity than GluN2B-NMDARs (nM vs. μ M sensitivity; Paoletti *et al.* 1997; Traynelis *et al.* 1998; Fayyazuddin *et al.* 2000). Zinc is enriched at many excitatory synapses in the forebrain and co-released with glutamate in an activity-dependent manner (Paoletti *et al.* 2009). Animal and human studies have uncovered specific evidence for the GluN2A NTD-zinc interaction in pain processing, and in synapse function and brain development (Nozaki *et al.* 2011; Lemke *et al.* 2013; Vergnano *et al.* 2014; Anderson *et al.* 2015; Serraz *et al.* 2016).

High-resolution crystal structures of GluN2A and GluN2B NTD-zinc complexes reveal that in both subunits zinc binds at the periphery of the NTD clamshell cleft. The Zn^{2+} ion is coordinated by residues from the upper and lower NTD lobes, thus stabilizing a closed-cleft conformation (Fig. 5A–C; Karakas *et al.* 2009; Romero-Hernandez *et al.* 2016). One histidine residue (H44 in rat GluN2A) from a GluN2A-specific upper lobe loop (the 'zinc loop'; Romero-Hernandez *et al.* 2016) appears particularly critical in conferring high zinc sensitivity. Interestingly, sequence alignments reveal that this histidine is replaced by a positively charged residue (arginine or lysine) in most non-mammalian vertebrate GluN2A subunits (Fig. 5B), indicative of a lack of high-affinity zinc binding in these subunits. Knowing that neither GluN2C nor GluN2D NTD from mammalian

and the GluA2 AMPA receptor (GluA, PDB 3KG2; Sobolevsky *et al.* 2009). LBD and TMD in brown, NTD upper lobes in cyan and NTD lower lobes in yellow. Black lines indicate equivalent NTD interlobe positions in the two receptors. NTD; N-terminal domain; LBD, ligand binding domain; TMD, transmembrane domain. Right: measure of the extracellular domain compaction in available full-length iGluR structures. Only those structures that contain an intact interface between the two constitutive NTD dimers and for which resolution is sufficient to define accurately the reference points for measurements of distances were considered. Note that structures of receptors with modified NTD-LBD linkers were included (without affecting the overall distribution of distances). Distances were measured between the TMD ion channel gate and the NTD interlobe as represented by the two thick black lines in the structures. The TMD reference point corresponds to the coordinates of the midpoint between the four alanine residues of the SYTANLA_AF pore motif. The coordinates of the NTD reference corresponds to the midpoint between the four NTD hinge regions. *B*, quaternary arrangement of the NTD region differs between NMDARs (GluN) and AMPA/kainate/delta receptors (GluA/KD). Upper row: NTD tetramer top view. Lower row: NTD dimer side view. NTD structures (PDB codes), from left to right: GluN1/GluN2B receptor (4PE5 Karakas & Furukawa, 2014), GluA2 (3KG2; Sobolevsky *et al.* 2009), GluK2 (5KUF; Meyerson *et al.* 2014) and GluD2 (5KCA; Elegheert *et al.* 2016). Note that, within a NTD dimer, the two individual NTDs are more loosely associated in NMDARs than in AMPA/kainate/delta receptors. *C*, conformational profiles of extracellular domains differ between NMDARs (GluN) and AMPA/kainate receptors (GluA/K). Two collective variables corresponding to two sets of inter-domain inter-subunit distances were computed from full-length structures of 32 NMDARs, 34 AMPA receptors and 5 kainate receptors. This corresponds to all currently available full-length iGluR structures excluding those with a broken inter-NTD dimer interface, or with an extreme disruption of the central pseudo 2-fold axis of symmetry, or with insufficient resolution for measurements of distances. Distance 1 is a marker of the NTD layer flexibility and reports the separation between the centres of the NTD lower lobes within the NTD dimers. Distance 2 is a marker of the LBD layer flexibility and reports the separation between the LBD of subunits A and C within the tetramer. Box plots in the right panel derive from the scatter point values displayed in the Distance 1–Distance 2 graph. The two pairs of subunits in the tetramer are marked A & C and B & D. Note that in the NTD layer, NMDARs display greater flexibility than AMPA and kainate receptors, while it is the reverse in the LBD layer.

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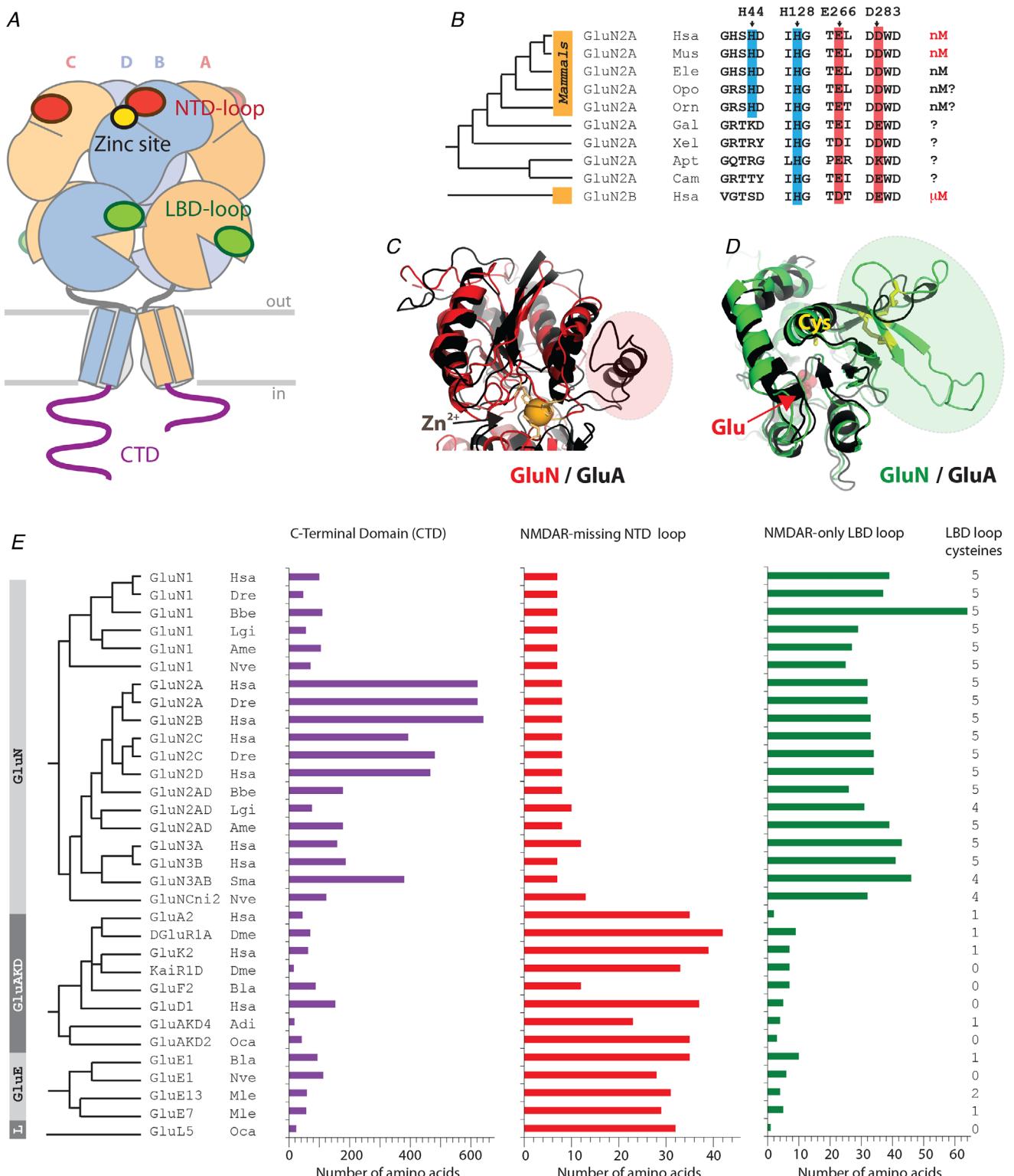


Figure 5. Domain evolution shapes NMDAR properties

Figure 5. Domain evolution shapes NMDAR properties
A, localization of NMDAR specific structural motifs. The green and red ovals correspond to loops present or absent in NMDARs when compared to other iGluRs. The yellow sphere localizes the modulatory zinc binding site of GluN2A and GluN2B NMDAR subunits. A & C, pair of GluN1 subunits; B & D, pair of GluN2 subunits. B, the NTD inhibitory zinc binding site of GluN2A and GluN2B subunits is a recent acquisition in the evolution of NMDARs. Alignment of zinc binding site signature sequences from various GluN2A subunits representative of the diversity of vertebrate GluN2A subunits. The human GluN2B subunit is also included for comparison

NMDARs harbours zinc binding site, we suggest that the GluN2A-specific high affinity zinc modulation of NMDARs probably emerged long after the second WGD event, with the emergence of placental mammals about 100 Mya (see Fig. 6A). NMDAR NTD dynamics, and therefore NMDAR zinc inhibition, relies on the high flexibility of the NTD interlobe hinge (Gielen *et al.* 2009; Zhu *et al.* 2013, 2016; Tajima *et al.* 2016; Jalali-Yazdi *et al.* 2018). In AMPA, kainate and delta receptors, a large loop ‘decorates’ the NTD hinge at a locus close to the zinc-binding site of NMDARs (Fig. 5C). This loop is systematically absent in NMDARs, even in NMDARs orthologues that diverged 600 Mya (Figs 5C, E and 6A). It is tempting to speculate that the absence of this NTD loop contributed to the emergence of a zinc-binding site in NMDARs with its unique peripheral location close to the clamshell hinge.

LBD loops and interaction between glutamate and glycine binding. The LBDs together with the connecting linkers to the TMD form the core machinery transducing agonist binding into channel gating. In AMPA and kainate receptors, the two constitutive LBD dimers operate largely independently and binding of glutamate to one LBD site has little influence on the binding of glutamate to a neighbouring LBD site (Robert *et al.* 2001; Jin *et al.* 2003). Moreover two glutamate binding events (presumably one on each dimer), out of four possible, are sufficient to gate the channel (although not at its maximal level;

Rosenmund *et al.* 1998). In conventional GluN1/GluN2 NMDARs, four binding events are mandatory for channel opening (Gibb *et al.* 2018), and binding of glutamate influences the binding of the co-agonist and vice versa through a negative allosteric interaction (Mayer *et al.* 1989). This coupling is mediated in a large part by a GluN2 LBD surface loop (Fig. 5D; Regalado *et al.* 2001; Durham *et al.* 2020), which protrudes at the interface between the two constitutive GluN1-GluN2 LBD heterodimers (Karakas & Furukawa, 2014; Lee *et al.* 2014). Comprising 30–40 residues, this loop contains several β -sheets tightly maintained by a pool of highly conserved cysteine residues engaged into disulfide bonds. Comparison of iGluR sequences and structures reveals that the presence of the loop constitutes a hallmark of NMDARs, with no equivalent in AMPA, kainate and delta receptors (Fig. 5D–E). This NMDAR-only loop strategically positioned at subunit-subunit interfaces participates to inter LBD-dimer contacts, and to the greater compactness of the LBD ring in these receptors (Lee *et al.* 2014). Unsurprisingly, it is also important for receptor assembly. Mutation of one of the conserved cysteines, as found in patients suffering from neuro-developmental disorders, is deleterious for receptor expression (Serraz *et al.* 2016; Swanger *et al.* 2016). Owing to its multiple contacts with the NTD lower lobes, the NMDAR-only loop is also appropriately positioned to participate in interlayer coupling and long range allosteric communication (Esmenjaud *et al.* 2019).

purposes. Residue numbers correspond to those of the human GluN2A subunit. Highlighted positions correspond to residues known to coordinate the Zn²⁺ ion (red acidic residues, blue histidine residues). Species acronyms: Hsa, *homo sapiens*; Mus, *Mus musculus*; Ele, *Loxodonta africana* (elephant); Opo, *Monodelphis domestica* (opossum); Orn, *Ornithorhynchus anatinus* (platypus); Gal, *Gallus gallus* (chicken); Xel, *Xenopus laevis*; Apt, *Apterodon leptorhynchus*; Cam, *Callorhinichus milli* (Australian ghostshark). The experimentally measured (red) or predicted (black) zinc affinity is mentioned in the right column for each GluN1/GluN2 receptor subtype. A question mark is present when no reliable prediction can be done. C, superimposition of the NMDAR GluN2A NTD (GluN red, PDB 6MMV subunit B; Jalali-Yazdi *et al.* 2018; a zinc ion was added and positioned in the clamshell as in PDB 5TPW; Romero-Hernandez *et al.* 2016) with the NTD of the AMPA receptor GluA2 (GluA black, PDB 3HSY subunit A; Rossmann *et al.* 2011). The views are centred on the NTD upper lobes. The orange sphere localizes the Zn²⁺ ion and orange sticks the zinc binding residues. The pink shaded area corresponds to a NTD loop absent in NMDARs. D, superimposition of NMDAR GluN1 LBD (GluN green, PDB 4TLL subunit A; Lee *et al.* 2014) and the AMPA receptor GluA2 (GluA black, PDB 4U2Q subunit C; Dürr *et al.* 2014) (black). The LBD is represented with the upper lobe at the forefront and the lower lobe at the back. The pink spheres localize the agonist molecule (glutamate) which lies in the interlobe cavity. Yellow sticks are from the cysteine residues within the GluN loop (see panel E column at the far right for number of cysteine residues). The green shaded area corresponds to the LBD loop found in NMDARs only. E, comparison of the size in amino acid number of three distinct regions among various iGluRs. From left to right: the intracellular CTD, the extracellular NTD loop missing in NMDARs (see panel C) and the extracellular NMDAR-only LBD loop (see panel D). The sequence length of each signatures (localized in panel A) from 32 genes representative of iGluR diversity are plotted as histograms. For each sequence, two conserved residues in the alignment of the 32 complete transcripts were selected as starting and ending limits. The numbers in the far right column correspond to the number of conserved cysteine residues in the NMDAR-only LBD loop. Species (and group) acronyms are the following: Hsa, *Homo sapiens* (Chordata, vertebrata); Dre, *Danio rerio* (Chordata, vertebrata); Bbe, *Branchiostoma belcheri* (Chordata); Bla, *Branchiostoma lanceolatum* (Chordata); Lgi, *Lottia gigantea* (Protostomes, mollusc); Sma, *Strigamia maritime* (Protostomes, arthropod); Ame, *Apis mellifera* (Protostomes, arthropod); Dme, *Drosophila melanogaster* (Protostomes, arthropod); Nve, *Nematostella vectensis* (Cnidaria); Adi, *Acropora digitifera* (Cnidaria); Mle, *Mnemiopsis leidyi* (Ctenophora); Oca, *Oscarella carmela* (Porifera). Note that the longest intracellular C-terminal regions are from vertebrate NMDAR subunits.

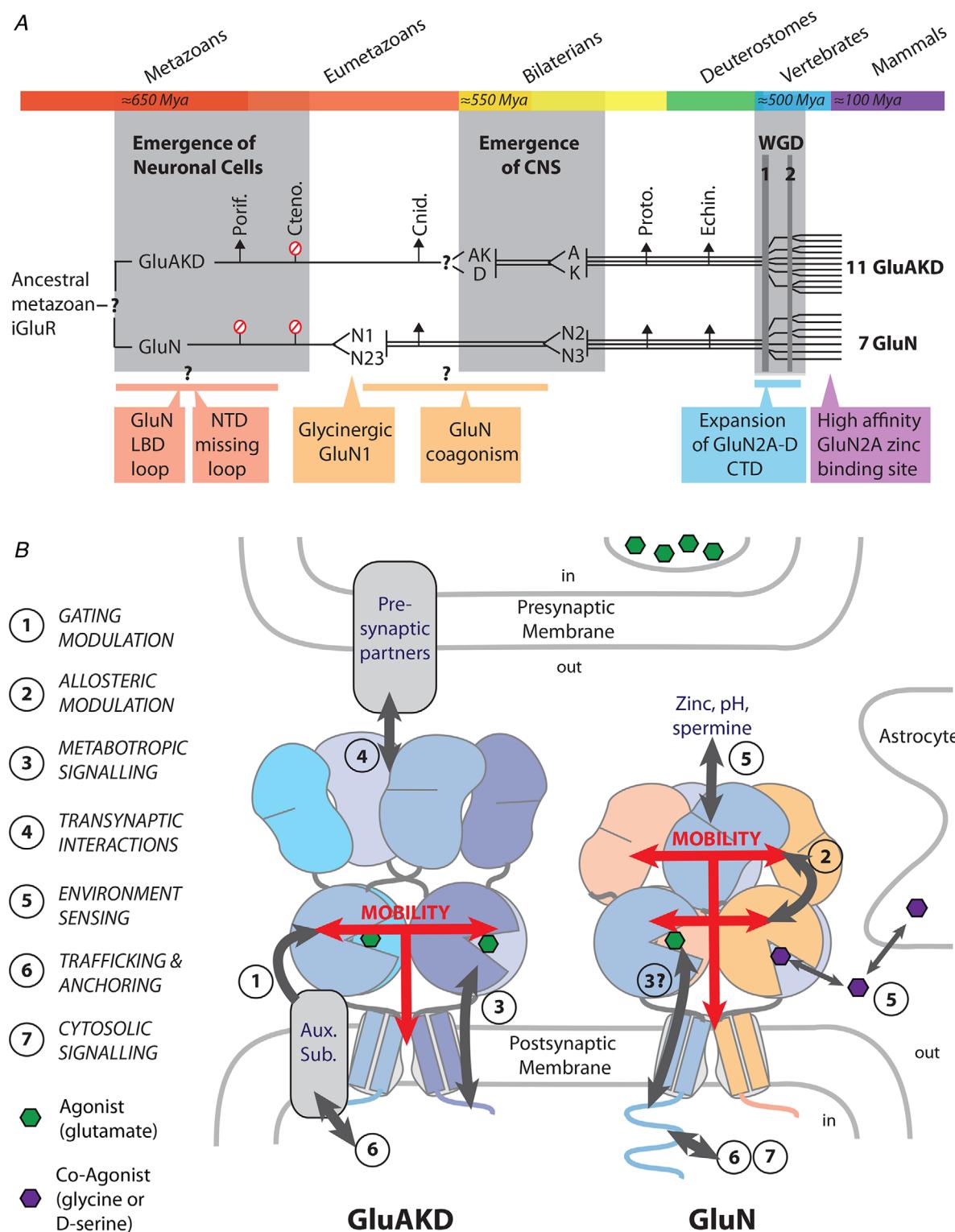


Figure 6. Evolution of iGluRs and synaptic transmission

A, evolutionary timeline and diversification of NMDARs (GluN, 7 genes in mammals) and AMPA, kainate and delta receptors (GluAKD, 11 genes in mammals) from early metazoan to modern mammals. The dates associated with the simplistic coloured timeline correspond to estimates, with approximately +/- 50 million years uncertainty associated with ongoing paleontological and phylogenomic debates. Vertical arrows indicate orthologous transmission of genes to the above-mentioned groups. Crossed red circle symbols stand for no gene transmission or gene loss. The boxes indicate important events in the architectural and functional evolution of NMDARs. Question

CTD extension and intracellular signalling. Contrasting with the other domains of the receptor, the CTD that lies after the last transmembrane segment (M4), is predicted to extend in the cytoplasm as an intrinsically disordered domain (Ryan *et al.* 2008; Choi *et al.* 2013). Multiple sequence alignment comparison reveals that the C-terminal tails of vertebrate GluN2 subunits are remarkably long and divergent (Ryan *et al.* 2008). While in most iGluR subunits, including invertebrate chordates NMDAR subunits, the CTD is made of just a few tens of amino acids, in vertebrate GluN2A-D subunits, it accounts for almost one-third of the protein coding sequence, totalizing ~420 amino acids in mammalian GluN2C and GluN2D subunits, up to ~630 amino acids in mammalian GluN2A and GluN2B subunits (Fig. 5E). This, together with the differential CTD length between GluN2A/GluN2B and GluN2C/GluN2D subunits, suggests that the C-terminal expansion occurred during, or not long before, the first vertebrate WGD event (Fig. 6A). Sequence wise, the CTD of vertebrate NMDARs is by far the most divergent region of the whole receptor, with only nine residues conserved between the four paralogues (GluN2A-D), primarily at the two extremities of the domain (exit of M4 and very last few amino acids). This great diversification of NMDAR intracellular domains during evolution suggests specific functional adaptations in intracellular signalling properties for modern NMDARs (Ryan *et al.* 2008, 2013; Coba *et al.* 2009).

Years of research combining genetic, biochemical and physiological approaches have established that (vertebrate) NMDAR C-terminal tails are essential for receptor function. In mice, deleting the GluN2B CTD is as harmful as knocking out the whole subunit, resulting in lethality (Mori *et al.* 1998; Sprengel *et al.* 1998), while the suppression of the GluN2A CTD impairs synaptic transmission (Steigerwald *et al.* 2000). NMDAR CTDs associate with numerous enzymes and scaffolding proteins forming large protein complexes that are critically involved in neuronal and behavioural plasticity (Salter & Kalia, 2004; Lau & Zukin, 2007; Sheng & Kim, 2011; Sanz-Clemente *et al.* 2013; Frank & Grant, 2017). NMDAR CTDs contain signals involved in receptor targeting and anchoring at synaptic sites, thus controlling receptor sub-unit composition and abundance at excitatory synapses.

Vertebrate GluN2 CTDs also contain a plethora of phosphorylation sites that can alter receptor activity and cellular trafficking. Obviously, the multiplicity of interacting partners (>50 different proteins identified in NMDAR super-complexes) (Frank & Grant, 2017) and possible posttranslational modifications are a major source of functional diversity among NMDAR subtypes. Experimental evidence indicates that the CTDs of GluN2A and GluN2B, the two most abundant GluN2 subunits in the adult vertebrate CNS, confer differential binding to enzymes such as CaMKII (GluN2B preferring) or to PSD MAGUK proteins such as PSD-95 (GluN2A preferring) and SAP-102 (GluN2B preferring) (Lau & Zukin, 2007; Sheng & Kim, 2011; Sanz-Clemente *et al.* 2013; Frank & Grant, 2017). These and other GluN2 CTD-specific interactions translate into different forms of long-term synaptic plasticity, differential effects on neuronal survival and ultimately differential learning capacities (Ryan *et al.* 2008; Martel *et al.* 2012; Paoletti *et al.* 2013). Interestingly, the extension of the C-terminal tails in NMDARs appears contemporary with the WGD that led to the diversification of the synaptic proteome repertoire in vertebrates 500 Mya (Fig. 6A; Emes & Grant, 2012; Grant, 2016; Sacerdot *et al.* 2018).

Perspectives: iGluR emergence, synapses and the nervous system

Large-scale genome analysis tells us that the evolutionary history of iGluRs, essential molecular actors for information processing and storage in our brain, originated several billion years at the start of the tree of life. Indeed, the evolutionary precursors to mammalian iGluRs are to be found in prokaryotes that encode in their genome ancestral iGluR-like gene products with amino acid sequence and function (i.e. gating of an ion channel pore) related to eukaryotic iGluRs. What exact roles iGluRs play in prokaryotes and ancestral eukaryotes is not clear, but one can easily imagine it involves environment sensing. About 650 Mya, eukaryotic iGluRs gene were somehow transmitted (Fig. 1) to ancestral metazoan organisms. Those organisms were probably lacking neurons, since the iGluR gene can be found in some aneural metazoans that underwent differentiation during this period (e.g. sponges; Fig. 6A). At this stage the role

marks indicate uncertainties about dating. WGD, whole genome duplication. *B*, NMDARs (GluN) and AMPA, kainate and delta receptors (GluAKD) co-cluster at excitatory synapses but have evolved distinct functional and signalling properties. The red arrows indicate inter-domain mobility that couples to the gating process and regulates the receptor channel activity. See text for further information. Note that in contrast to AMPA and kainate receptors, no *bona fide* auxiliary subunit of NMDARs has been identified to date. This provided an illustrative example on how closely related protein families adopted different strategies during evolution to carry out specific biological functions (receptor trafficking and anchoring). Note also that, in addition to their classical roles as ligand-gated ion channels, several iGluRs (including kainate and delta receptors as well as NMDARs) have been proposed to signal through non-ionotropic mechanisms involving agonist-induced activation of intracellular signalling pathways independently of ion fluxes through the receptor ion channel ('metabotropic signalling').

of those iGluRs remains enigmatic. It is, however, clear that iGluRs became ubiquitous components of neural metazoan genomes as soon as nervous systems emerged in the forms we know nowadays (diffuse nerve net or CNS; Fig. 2B). Concomitantly, an essential step in iGluR gene diversification occurred with the separation between the GluN and GluAKD branches. Actually, GluN and GluAKD genes appear to be good molecular markers of the global evolution of the nervous system, being present during all phases of neural evolution and diversifying gradually as the nervous system increased complexity (Fig. 6A).

Among iGluR genes, NMDAR GluN genes stand out as a monophyletic branch specific to neural organisms. As previously mentioned, evidence indicates that GluN genes have been under particularly strong purifying selection during evolution, indicative of critical roles in early neural organisms, even before the emergence of complex centralized nervous systems. At the protein level, this means that structural motifs found in modern NMDARs and specific to this receptor family (see Fig. 5) are not recent ‘inventions’ but rather direct inheritance from the most ancestral GluN encoded subunits. Hence, it seems that planulozoan (cnidarian plus bilaterian) GluN ancestral receptors with their unique LBD loop were already equipped for distinct subunit-subunit interactions absent in other iGluRs (Fig. 5D and E). Moreover, the particularly high conservation of the GluN1 subunit and its glycine binding motif in all planulozoans suggests that glycinergic agonism (or co-agonism) may have already been present in the GluN receptor family before the separation between cnidarians and bilaterians (Figs 3D and E and 6A). Since GluN2 and GluN3 genes are present as clear individual entities both in protostomes and deuterostomes but not in cnidarians (Fig. 2C), the duplication of the ancestral GluN23 gene probably occurred in bilaterian ancestors after the separation with cnidarians. The individualization of the GluN2 and GluN3 genes thus overlaps with the emergence of a CNS (Fig. 6A). Glutamate and glycine co-agonism in NMDARs, intimately linked to intercellular signalling and neurotransmission (Schell, 2004; Oliet & Mothet, 2009; Henneberger *et al.* 2013; Fig. 6B) may have appeared during this period, although this remains speculative. On a related matter, the recent realization that several evolutionarily distant iGluRs are unresponsive to glutamate but act as glycine receptors, in such organisms as ctenophores (Alberstein *et al.* 2015) and mammals (GluN1/GluN3 and GluD receptors; Grand *et al.* 2018; Otsu *et al.* 2019; Gantz *et al.* 2020), has strong implications for our understanding of neurotransmission. Firstly, it uncovered an unsuspected excitatory action of glycine on neuronal activity; secondly, it suggests that glycine was selected to act as a neurotransmitter early in evolution. Thirdly, as pointed out by Alberstein *et al.* (2015), it raises the intriguing possibility that binding of glycine was a

common feature of primitive iGluRs predating the binding of glutamate (see Fig. 3E). Since many genomics studies and databases still consider the presence of iGluR genes as a marker of glutamatergic signalling, the importance of glycinergic signalling through iGluRs should be stressed within the scientific community, in particular to those colleagues working on non-bilaterian species.

Among the four original metazoan branches of iGluRs, the GluN and GluAKD receptor families are the two branches that were transmitted to vertebrates where they became essential components of the CNS. NMDARs encoded by the GluN genes and AMPA receptors encoded by the GluA genes are the lynchpin of fast excitatory neurotransmission and synaptic plasticity. Both receptor types co-cluster at glutamatergic synapses and have evolved unique structural and functional properties tailored to match their distinct, yet complementary, roles in neurotransmission (Fig. 6B). AMPA receptors with their uniquely fast gating kinetics act as electrical switches mediating rapid point-to-point excitatory transmission (Baranovic & Plested, 2016). NMDARs, in contrast, operate at a slower timescale, capable of synaptic integration (Paoletti *et al.* 2013; Iacobucci & Popescu, 2017). NMDARs trigger long-term changes in synaptic strength reflected in a large part by changes in the number of postsynaptic AMPA receptors (Paoletti *et al.* 2013; Nicoll, 2017). Insuring fast gating kinetics and tightly controlled receptor trafficking is thus of paramount importance for AMPA receptors. For NMDARs, intracellular signalling and sensing of the local environment are vital. From an evolutionary perspective, it is enlightening to realize how iGluR structure and mechanisms diversified and adapted to fulfill these ‘requirements’ (Fig. 6B). In AMPA receptors, the gating core region (LBD + TMD) is quasi disconnected from the large NTD region, allowing the gating machinery to proceed at high speed, unconstrained (Fig. 4A and C). On the other hand, regulation of AMPA receptor trafficking and anchoring at synaptic sites has been ‘externalized’ to a slew of partner proteins including auxiliary subunits, associating with the receptor’s TMD, and trans-synaptic adaptors, contacting the receptor’s distal NTD region (Fig. 6B; Sia *et al.* 2007; Greger *et al.* 2017; Yuzaki & Aricescu, 2017; Chen & Gouaux, 2019; Greger & Mayer, 2019). In NMDARs, specific evolution in the receptors’ sequence and architecture resulted in distinct functionalities. This is the case for the packed NTD + LBD extracellular domain conferring exquisite sensitivity to the extracellular micro-environment through binding of various endogenous small molecule compounds acting as co-agonists (glycine, D-serine) or allosteric modulators (zinc, protons, polyamines) (Fig. 6B). The compaction of the extracellular region may also contribute to the slow gating kinetics of NMDARs (compared to AMPA receptors). This is also the case for the unique atomic composition of the

ion channel selectivity filter, where a set of asparagine residues (Q/R/N site) endow NMDARs with exquisite sensitivity to local changes in membrane potential through extracellular Mg²⁺ pore blockade, a property of critical importance for the induction of synaptic plasticity and associative learning. Finally, the major extension of the CTD in NMDAR GluN2 subunits provides expanded opportunities for controlling the receptor trafficking but above all for linking NMDAR activation to a variety of intracellular signalling pathways (Fig. 6B). By sensing and integrating local extracellular, membrane and intracellular signals, modern NMDARs are certainly one of the most complex molecular machines in neurotransmission. Hundreds of millions of years of evolution and selection were required to shape such machines that govern the fate of brain circuits.

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Additional information

Competing interests

None.

Author contributions

Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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