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Invited review

# Evolution of glutamatergic signaling and synapses

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ABSTRACT

Glutamate (Glu) is the primary excitatory transmitter in the mammalian brain. But, we know little about the evolutionary history of this adaptation, including the selection of L-glutamate as a signaling molecule in the first place. Here, we used comparative metabolomics and genomic data to reconstruct the genealogy of glutamatergic signaling. The origin of Glu-mediated communications might be traced to primordial nitrogen and carbon metabolic pathways. The versatile chemistry of L-Glu placed this molecule at the crossroad of cellular biochemistry as one of the most abundant metabolites. From there, innovations multiplied. Many stress factors or injuries could increase extracellular glutamate concentration, which led to the development of modular molecular systems for its rapid sensing in bacteria and archaea. More than 20 evolutionarily distinct families of ionotropic glutamate receptors (iGluRs) have been identified in eukaryotes. The domain compositions of iGluRs correlate with the origins of multicellularity in eukaryotes. Although L-Glu was recruited as a neuro-muscular transmitter in the early-branching metazoans, it was predominantly a non-neuronal messenger, with a possibility that glutamatergic synapses evolved more than once. Furthermore, the molecular secretory complexity of glutamatergic synapses in invertebrates (e.g., *Aplysia*) can exceed their vertebrate counterparts. Comparative genomics also revealed 15+ subfamilies of iGluRs across Metazoa. However, most of this ancestral diversity had been lost in the vertebrate lineage, preserving AMPA, Kainate, Delta, and NMDA receptors. The widespread expansion of glutamate synapses in the cortical areas might be associated with the enhanced metabolic demands of the complex brain and compartmentalization of Glu signaling within modular neuronal ensembles.

-"*I want to know how God created this world. I am not interested in this or that phenomenon, in the spectrum of this or that element. I want to know His thoughts; the rest are details.*" -Albert Einstein

-"… ***when God created the world, it was done so with glutamic acid in mind!***" – Veron R. Young & Alfred M. Ajami

## 1. Introduction

It is estimated that over 99 % of all synapses in the mammalian brain use chemical transmission vs. electrical coupling by gap junctions (Greengard, 2001). There are more than 20 low molecular weight neurotransmitters. But, it is surprising that most mammalian brain synapses use L-glutamate (Glu) as an excitatory neurotransmitter (Micheva et al., 2010). Why did Glu 'conquer' such a special place in our brain? Understandably, about half of the neuroscientists today work with different aspects of Glu signaling.

Forty years ago, the 'glutamate' revolution in neuroscience was

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**List of abbreviations**

| | |
|---|---|
| AAA | L-2-amonoadipic acid |
| ATD | amino terminal domain (=NTD) |
| Asp | Aspartate |
| Basal Metazoans | five major animal superclades such as Ctenophora, Porifera, Placozoa, Cnidaria and Bilateria |
| CNS | Central Nervous System |
| GABA | Gamma-aminobutyrate |
| Glu | Glutamate |
| Glt | Glutamine |
| iBMK | immortalized baby mouse kidney |
| iGluRs | ionotropic glutamate receptor superfamily |
| LUCA | Last Universal Common Ancestor |
| LBD | a ligand-binding domain of iGluRs |
| mGlu | metabotropic glutamate receptors |
| Mya | Million years ago |
| NTD | *N*-terminal domain of iGluRs |
| NO | Nitric oxide |
| scRNA-seq | single-cell transcriptome sequencing |
| SBP | Substrate-binding Proteins |
| TCA | Tricarboxylic acid cycle (= the Krebs cycle) |
| TMD | a transmembrane domain of iGluRs |

underway with the landmark review summarizing synaptic functions of amino acids (Watkins and Evans, 1981). By May 2021, PubMed contained >170,000 papers describing different aspects of glutamate chemistry and biology. ~95,000 reports related to neural functions of glutamate, and ~55,000 are related to glutamatergic (Glu-mediated) neurotransmission. Nonetheless, most of these efforts were focused on mammals, with a smaller fraction of studies using other vertebrates and two basal chordate lineages (tunicates and cephalochordates).

In contrast, there are '*only*' ~650 papers directly or indirectly associated with the transmitter functions of Glu in invertebrates. Most of these researchers are using *Drosophila* (~250 articles), *C. elegans* (~150), *Aplysia,* and its kin (~150) as models; plus ~50 papers are related to other worms. These model species represent just four (out of 35) extant animal phyla: Arthropoda, Nematoda, Mollusca, Annelida.

Even these limited comparative examples revealed non-canonical solutions for the use of Glu, which are remarkably different from what is described in classical neuroscience textbooks. In invertebrates, Glu often acts as an *inhibitory* neurotransmitter (Cleland, 1996; Kehoe et al., 2009; Kehoe, 2000) using pentameric cys-loop receptor channels (Cymes and Grosman, 2021; Jaiteh et al., 2016; Kehoe et al., 2009; Lynagh et al., 2015). *L-glutamate operates as the neuromuscular transmitter in most animals on the planet* (e.g., in arthropods (Jan and Jan, 1976) and nematodes (Dent et al., 1997); fewer neuro-muscular glutamatergic synapses were identified in molluscs (Fox and Lloyd, 1999). But, how and why did we (mammals, vertebrates) arrive at using acetylcholine as a neurotransmitter for our somatic muscles? At least some glutamatergic synapses in molluscs are molecularly more complex than in our brain (see below). Finally, D-glutamate might also act as a signaling molecule, in addition to D-aspartate (Moroz et al., 2020b).

We know virtually nothing about Glu-signaling in the remaining 30 animal phyla. But these phyla represent the largest and the most incredibly diverse forms of neuronal organization and functions (Bracken-Grissom et al., 2014; Brusca and Brusca, 2003; Bullock and Horridge, 1965; Mackie, 1990; Moroz, 2015a, 2018; Nielsen, 2012).

The growing comparative genomic and other 'omics' data only recently started to uncover multiple paths of recruiting this ancestral molecule in signaling functions, which possibly has over 3.5 billion years of history. This manuscript emphasizes comparative aspects of Glu-signaling. We will start with a brief history and later focus on molecular components of Glu-mediated transmission in animals and other eukaryotes. The universal core of cellular metabolism and nitrogen utilization in living systems (Smith and Morowitz, 2004) paved the way for selecting Glu as a ubiquitous extracellular messenger in all domains of life. The recruitment of Glu into different forms of neural organization (from chemosensation to cognitive functions) might also be based upon constraints impeded by the energetic demands of metabolism and the chemical properties of this versatile signal molecule. It would be impossible to cover the multifaced evolution of the versatile Glu signalings across time and species in a single manuscript. Thus, we will emphasize the major transitions reflecting the long recruitment history of Glu into the transmitter and integrative functions.

## 2. A brief history of glutamate signaling

The deciphering of glutamate signaling has a hundred years of history (Fig. 1). The story started in 1909 with Prof. Kikunae Ikeda's discovery (Ikeda, 1909, 2002) of monosodium glutamate as a chemical responsible for the umami taste (Halpern, 2002). The finding implied the critical role of glutamate (Glu) in chemosensation and created trillion-dollar segments of the food industry. However, the identification of the umami-specific taste receptors (metabotropic Glu receptors mGlu4 (Chaudhari et al., 2000) and mGlu1 (San Gabriel et al., 2005), plus G-protein receptor complexes T1R1+3 (Lopez Cascales et al., 2010; Nelson et al., 2002; Yasumatsu et al., 2015; Zhang et al., 2008; Zhao et al., 2003)) occurred a century later (Kurihara, 2015). Today the deciphering functions of glutamate in chemoreceptive systems are still one of the frontiers in neuroscience.

In the 1950s, chemical transmission was established as the primary communication mechanism between neurons (Valenstein, 2002, 2005). The question was raised about what neurotransmitters would act in the mammalian brain. Takashi Hayashi originally proposed the synaptic role of Glu (Hayashi, 1954). This hypothesis was based on two sets of facts: (i) Glu could induce convulsions after intracerebroventricular and intracarotid injections in dogs and monkeys (Hayashi, 1954); and (ii) Glu contributes to cognitive functions (Weil-Malherbe, 1950). Curtis, Phillis, and Watkins showed that Glu depolarized central neurons and, therefore, could be the excitatory transmitter in the CNS (Curtis et al., 1959, 1960a, 1960b, 1961, 1961; Curtis and Watkins, 1960, 1961, 1963). However, it took more than 20 years before the transmitter role of glutamate had been established and widely recognized (Watkins and Evans, 1981).

The universal place for Glu in cell biochemistry formed the early doubts and the long-time acceptance of its localized signaling functions in the CNS. Indeed, Glu is abundant in every cell and plays a critical role in cellular metabolism. Jeff Watkins himself elaborated primary doubts held by most neuroscientists against the hypothesis that Glu was the central neurotransmitter. Among these negative preconceptions was a viewpoint that neurotransmitter (signaling) substances must be at *low* concentrations, act *locally* in specific synaptic pathways, have a *single action*, and have specific degrading/inactivation enzymes, etc. (Watkins, 2000; Watkins and Jane, 2006). These particular criteria were validated for the classical transmitter functions of acetylcholine and noradrenaline (Valenstein, 2002, 2005). L-Glu did not fit the dominant portrait of a neurotransmitter. It took time to develop selective pharmacology for Glu receptors as powerful tools to confirm this type of neuronal communications (Watkins and Evans, 1981).

High endogenous Glu concentrations and multiplicity of its effects contrasted with the accepted view in the 1950–1970s how other classical low molecular weight transmitters (such as acetylcholine, serotonin, dopamine, etc.) operated. But the very same ('negative') arguments, which were used against the neurotransmitter role of L-Glu, are the foundation that explains the recruitment of Glu as one of the earliest signaling molecules across the entire Tree of Life. In this capacity, from the very dawn of the evolution of the first cells, ancient transmitter roles of Glu, ATP, and Nitric Oxide (NO) were developed in parallel (Moroz,

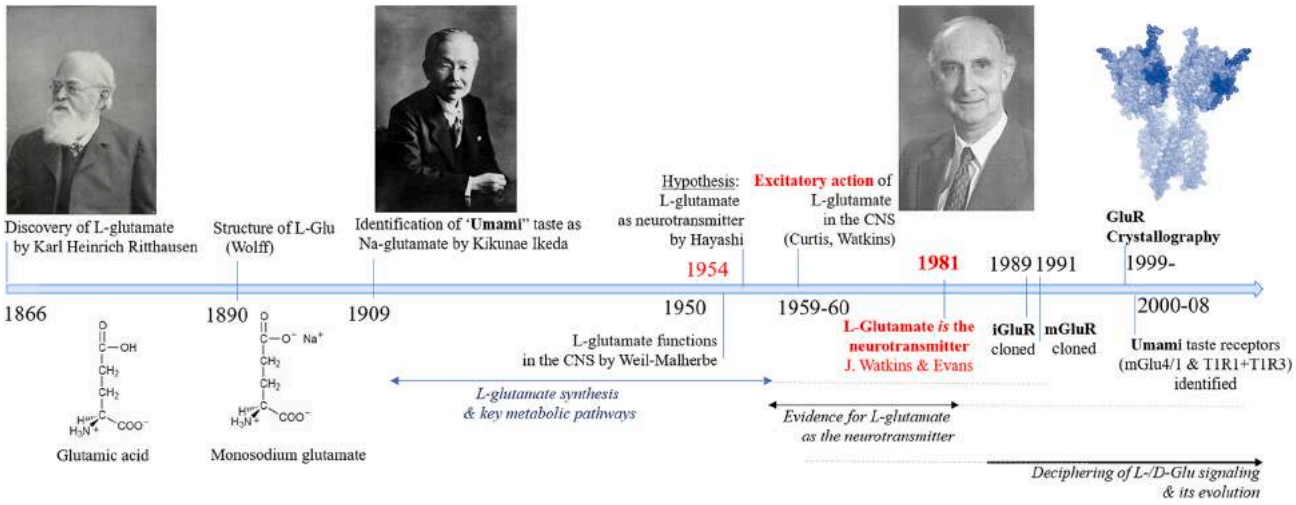


**Fig. 1.** A brief history of glutamatergic signaling in mammals. The discovery of glutamate (Glu) in 1866 was followed by identifying monosodium glutamate as a chemosensory agent in the human taste (Ikeda, 1909). However, the identification of these taste receptors was about 100 years later, when metabotropic mGlu4, mGlu1, and 7TM receptors (T1R1+T1R3) were cloned (Kurihara, 2015). As sensors for Glu, together with other co-activator/s (5′ nucleotides), they provided the molecular bases for the distinct umami sensation in humans. Synaptic functions of Glu had been suggested by Hayashi (Hayashi, 1954). This hypothesis was based on his observation that Glu induced convulsions after the central injections and earlier studies about the involvement of Glu in cognitive functions (Weil-Malherbe, 1950). The concept of chemical transmission in the brain had been accepted by the end of the 50s. What is the central transmitter in the CNS? Watkins and colleagues in the Eccles laboratory showed that L-Glu had excitatory action (Curtis et al., 1959; Curtis and Watkins, 1961). Nevertheless, the acceptance of this innovative idea took over 20 years. The significant objections were related to the fact that Glu is a crucial amino acid for dozens of metabolic pathways across all domains of life (see Fig. 2 and text for details). How is the universality of Glu functions in virtually every cell transformed into precise synaptic communications in the brain? After the development of specific agonists and antagonists, both 'fast' ionotropic (iGluRs - (Hollmann et al., 1989)) and 'slower' metabotropic receptors (mGluR - (Houamed et al., 1991; Masu et al., 1991)) were cloned (Hollmann and Heinemann, 1994). And their structural organizations had been revealed using crystallography (Armstrong and Gouaux, 2000; Armstrong et al., 1998; Chen et al., 1999; Kunishima et al., 2000; Mayer, 2006; Mayer et al., 2001; Sun et al., 2002). In invertebrates, glutamate-mediated signaling is more diverse and perhaps is more complex than in vertebrates. Deep comparative and evolutionary roots of glutamate-mediated transmission are also poorly explored. But the very origin and parallel evolution of glutamatergic signaling and synapses are inherently linked to a crucial role of glutamate in metabolism and bioenergetics.

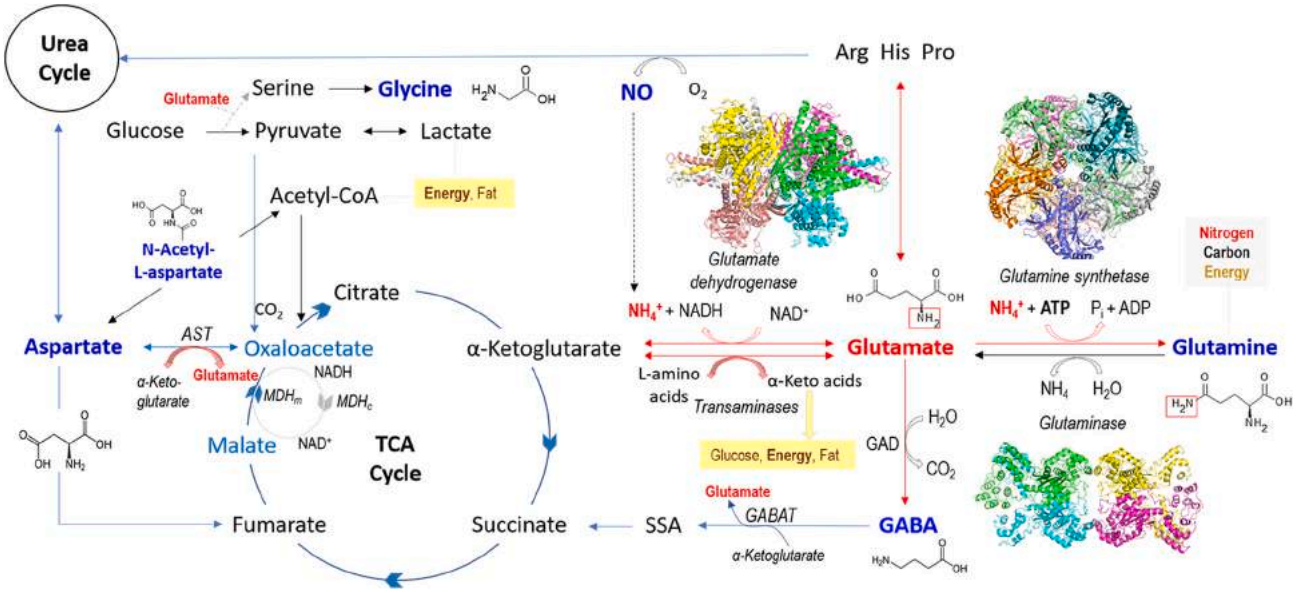


**Fig. 2.** The Krebs cycle and glutamate metabolism as evolutionary sources of transmitter signaling. The schematic diagram of universal biochemical pathways emphasizes L-glutamate (Glu) as a critical metabolite in bioenergetics, nitrogen assimilation, and amino acid synthesis. Both Glu and glutamine are nitrogen donors for amino acids (including ubiquitous transamination mechanisms). All enzymes of these pathways are highly conserved across species. 3-D organizations of three key enzymes are our reconstructions from *Trichoplax adhaerens*. This nerveless placozoan has the simplest-known animal organization but complex behaviors (see text). The co-factor/metabolite controls of glutamate dehydrogenase, glutaminase, and glutamine synthetase are more complicated than the regulation of most neuronal components. GABA, Aspartate, *N*-acetyl-L-aspartate, Glycine, and Nitric Oxide (NO) are also known as universal extracellular low molecular weight transmitters (bold blue) across all domains of life. Their syntheses are inherently linked to glutamate metabolisms and cellular bioenergetics. Thus, these transmitters might evolve as signal molecules from the intermediate metabolism. And these are essential components of nearly every prokaryotic and eukaryotic cell. Intracellular metabolite concentrations are unpredictable from these biochemical pathways. However, these concentrations can be determined experimentally using metabolomic tools, which gave surprising results, including the highest intracellular glutamate concentration (see Fig. 3 and text).

2009; Moroz et al., 2021). And in all cases, these transmitter functions were derived and deeply embedded in the architecture of cellular bioenergetics and nitrogen metabolism (Fig. 2). The enormous diversity of systemic and signaling mechanisms of these three simple molecules are astonishing and can be traced to injury responses of primordial cells (Moroz et al., 2021). The discovery of Glu receptors' extraordinary diversity further emphasized both the ancestry and numerous alternative ways of Glu-mediated signaling.

In summary, Glu-related molecular and system innovations deserve special attention for two reasons. First, innovations within the molecular architecture of Glu receptors, synapses, and signaling pathways open novel perspectives, when countless experiments of Nature can be used for the needs of synthetic biology and synthetic neuroscience of the future. Second, the deep evolutionary history of Glu-mediated signaling, in many ways, determines the constraints of the overall molecular architecture of the living cell establishing the foundations for the very origin of neurons, synapses, and even basal cognition.

Here, we will address three fundamental questions: (i) why and how did glutamate achieve nearly universal usage in all living organisms? (ii) why and how was glutamate recruited to be an intercellular signal molecule/neurotransmitter? And (iii) why and how glutamate became the primary neurotransmitter in our brain? The last question is not so trivial. In invertebrates and the most basal chordate, amphioxus or *Branchiostoma* (Candiani et al., 2012), only a relatively small fraction of neurons (~5–20 %) use glutamate as the neurotransmitter vs. >50–80 % of the mammalian brain (Micheva et al., 2010).

## 3. Glutamate at the crossroads of the cellular metabolism

It would not be hyperbole to say that Glu is one of the ancestral 'protomolecules' critical for establishing the core of early cellular metabolism more than 3.8 billion years ago. Fig. 2 illustrates the position of L-Glu at the intersection of bioenergetic and synthetic pathways for carbon and nitrogen utilization. Glutamate is made from the tricarboxylic acid cycle (TCA or Krebs cycle) intermediate α-ketoglutarate (=2-oxoglutarate) by reversible reductive amination with either ammonium or glutamine as the nitrogen sources.

Glutamine synthetase is one of the most ancient functioning enzymes (in the history of gene evolution (Kumada et al., 1993)), with multiple regulatory sites and having its descendants even in the lens of our eyes (Wyatt et al., 2006). In this intermediate metabolism architecture, Glu can be an important energy source by itself. Indeed, many cells can use Glu as their sole carbon source, possibly even preferring it over glucose. Glutamine metabolism (in part via Glu) also provides carbon and energy sources, and TCA cycle intermediates (DeBerardinis et al., 2007). In this capacity, Glu also serves as the most dynamic and vital among anaplerotic substances (i.e., molecules responsible for re-filing of the catalytic intermediates of the TCA cycle (Brunengraber and Roe, 2006)). For example, a decline in intramuscular Glu at the start of exercise in vertebrates is an example of its anaplerotic functions.

The most acknowledged glutamate-glutamine function is nitrogen assimilation (Young and Ajami, 2000, 2001). Glu donates about half of the cellular nitrogens (Walker and van der Donk, 2016), including activities of glutamate transaminases. Glutamine (Glt) similarly serves as the nitrogen donor for about half of the nitrogens in purines and pyrimidines (Walker and van der Donk, 2016). All of this makes this tightly coupled Glu-Glt pathway the primary route for nitrogen assimilation in all domains of Life and globally for the planetary nitrogen cycle.

In addition to its role in ribosomal and nonribosomal protein synthesis, L-Glu participates in more than two hundred enzymatic and non-enzymatic reactions, with 107 regulator molecules and metabolic intermediates of these pathways (Yelamanchi et al., 2016). This complex homeostatic system is precisely controlled with at least two dozen different kinases, phosphatases, deacetylases, and posttranslational modifications (Yelamanchi et al., 2016). As a result, the Glu-associated pathways are highly adaptable systems in numerous responses to specific and general/stress environmental factors.

Glu is used as a building block in the biosynthesis of the broad spectrum of complex molecules, starting with glutathione – one of the major antioxidants. Thus, Glu-dependent anabolic and catabolic fluxes are the true "hub" of signaling adaptations and innovations such as antibiotics and other immune components (Walker and van der Donk, 2016; Young and Ajami, 2000).

Glu is an organic anion and cannot cross the membrane. This function is attributed to glutamine (Glt), which acts as the coupling partner for Glu for the most fundamental processes of cellular metabolism: bioenergetics, sources for carbon, and nitrogen (Fig. 2). L-Glu is the most abundant metabolite in bacteria and mammals (Figs. 3 and 4), with its intracellular concentrations reaching 96 mM in *E. coli* (Bennett et al., 2009), 44 mM in yeast, and 64 mM in the immortalized baby mouse kidney (iBMK) epithelial cells (Park et al., 2016). It is more than 20–40 % of all other metabolites combined.

The ionization state of this triprotonated amino acid, Glu, depends on pH. The predominant form of Glu at physiological pH is a singly-negative anion $-OOC-CH(NH_3^+)-(CH_2)_2-COO^-$ potentially forming a monohydrate salt as in potassium glutamate. Thus, L-Glu acts as one of the principal osmolytes, providing a unique intracellular microenvironment. Such high Glu concentration and the overall architecture of cellular metabolisms emphasize other features of this extraordinary molecule. We will list some relevant for the evolution of Glu signaling and neural functions.

For example, Glu is the most abundant amino acid in the alpha-helix secondary structure of proteins (Young and Ajami, 2000). Thus Glu constitutes 11–22 % of all amino acids in animal proteins and up to 40 % in plants as wet-weight (Giacometti, 1979). These values are essential for nutrition and predation in animals. The combination of Glu with sea salts and nucleotides is the basis for umami taste in humans and chemosensation in early and extant animals.

High concentrations of Glu are also linked to its role as a protecting group or scaffold (Young and Ajami, 2000) in many homeostatic mechanisms, immunity, and signaling. Glu can be added to a target (=glutamylation). Some molecular transformations can be performed on the glutamylated molecule, and then the Glu is removed unchanged. From a chemical standpoint, glutamylation may block *N*-oxidation (Young and Ajami, 2000). Second, Glu can provide a scaffold for substrate recognition, and that the enzymes, acting on the glutamylated substrates, recruited from other pathways involving Glu. Third, reversible polyglutamylation supports highly dynamic cell signaling, second messengers, and immunity processes.

Several structural proteins and cofactors contain polyglutamate chains (Mahalingan et al., 2020; Martinez-Limon et al., 2016). Glutamylation of tubulin (Bigman and Levy, 2020; Edde et al., 1990) is one of the most recognized mechanisms controlling cytoskeleton dynamics in cell division (van Dijk et al., 2007), motile (Kubo and Oda, 2017; Wang et al., 2021), non-motile (sensory) cilia (Kimura et al., 2018; Yang et al., 2021), molecular motors for axonal transport (Kubo et al., 2010), synapse functions (Akella and Barr, 2021; Ikegami et al., 2007), and nucleosome assembly (proteins NAP1 and NAP2 - (Regnard et al., 2000)).

The observed high concentrations of L-Glu might also be considered the critical intracellular milieu of all mentioned processes, forming the unique chemical micro-environment for cellular homeostatic and regulatory functions. Why and how had L-Glu occupied such a vital place in the cell metabolisms and signaling? Why is there such preference for L-Glu (5-carbon amino acid) versus its homologs, L-2-amonoadipic acid (AAA, 6-carbon) and L-aspartate (L-Asp, 4-carbon) or D-Glu?

## 4. Glutamate in early life evolution: natural selection of glutamate as metabolite and signal molecule

Both D- and L-Glu are synthesized abiotically in Nature (Miller, 1957) and found in meteorites (Glavin et al., 2011, 2021). There is a

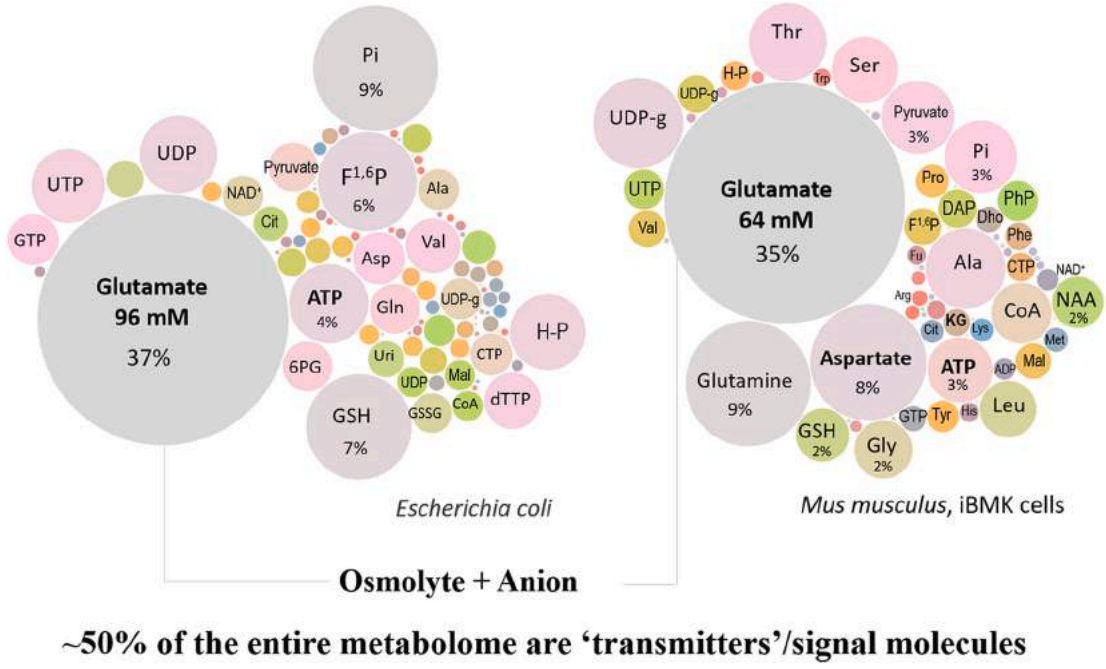


**Fig. 3.** Glutamate as the most abundant metabolite across the tree of life: Intracellular metabolomics from bacteria to mammals. Comparing the metabolomes between a prokaryotic cell (*E. coli*) and a mammalian cell iBMK from mouse (Park et al., 2016). In both cases, glutamate is the major intracellular metabolite with the highest concentration compared to other molecules. Absolute intracellular concentrations are shown together with % of a particular molecule in the entire metabolome for both cell types. 50 % of the whole metabolome are evolutionarily conserved pan-signal molecules.

slight excess of L-Glu (and other L-amino acids) in meteorites, and astronomical sources of circular polarization might account for the observed enantiomeric excess in interstellar chiral molecules (Bailey et al., 1998). The chiral amplification might sustain the preservation of the dominant enantiomers due to a partial transfer of enantioselective chiralities to all molecules derived from it (Breslow and Levine, 2006). These findings led to the hypotheses of extraterrestrial origins of L-enantiomers before the birth of Life on our planet.

However, the alternative "Earth-based" hypotheses could be more favorable, starting with the primordial RNA world. L-amino acids could dominate in metabolism due to the selection of D-ribose (and not L-ribose) in the early RNA molecules (Ricardo et al., 2004), starting more than 4.3 billion years ago (Benner et al., 2020). Thus, RNA is selective for L-enantiomers (Bailey, 1998). Glutamylated-tRNA is also involved in numerous enzymatic reactions (Walker and van der Donk, 2016; Young and Ajami, 2000).

Multiple lines of evidence suggested that Glu was produced on the early Earth (>3.5–4 billion years ago), in its reduced atmosphere by various energy sources (Harada, 1974): electrical discharges and photochemically (UV light), ionizing radiation, and even meteorite impact-shock waves (Chyba and Sagan, 1992). Under those conditions, the formation of copolymers of Glu and Asp could occur on the surface of minerals (Harada, 1974).

The polymerization on rocks is a highly 'innovative' process for the origin of life (Orgel, 1998). On the surface of anion exchange minerals such as hydroxyapatite or illite (Hill et al., 1998), the negatively charged residues of Glu can form a template lattice, similarly with modern technologies of solid-phase syntheses (Young and Ajami, 2000). It was shown that the efficiency of Glu is greater than Asp for these processes (Hill et al., 1998), and Glu is a significantly more temperature stable molecule than Asp (Abelson, 1959).

Proteinoid structures, produced via copolymerization of Glu and Asp with 16 of the other natural amino acids, show weak catalytic activity, including hydrolyses, decarboxylation, and amination. This abiotic chemistry can be ancestral for the origin of transamination activity, as we know it today (Young and Ajami, 2000).

By further reconstructing the early Earth conditions, it was demonstrated that sunlight-driven photolysis of Glu produced succinate via α-ketoglutarate as an intermediate. Similarly, sunlight-driven Asp photolysis made malonate via its intermediate oxaloacetate (Waddell and Miller, 1991). All these compounds are essential components of the TCA cycle (Fig. 2). Thus, the intermediates of the TCA cycle, possible in its reversed orientation (Morowitz et al., 2000; Nunoura et al., 2018; Smith and Morowitz, 2004), could emerge, as a consequence of preexisting early chemical evolution, by reassembling of available organic compounds including those derived from photochemical, oxidative decarboxylation of Glu and Asp. Two of the smallest α-keto-acids (such as glyoxylate and pyruvate with 2- and 3-carbons respectively) may have been prebiotically available for the abiotic synthesis of oxaloacetate (4-carbon) and α-ketoglutarate (5-carbon) and a series of α-ketoacid analogs of the reductive TCA cycle without the need for metals or enzyme catalysts (Stubbs et al., 2020).

Of note, the simplest amino acid, glycine, together with α-ketoglutarate, can also efficiently and non-enzymatically produce Glu (without metal-catalyzed abiotic reductive amination) with glyoxylate as a co-product (Fahrenbach and Tran, 2020; Stubbs et al., 2020). This mimics the modern transamination pathways for the synthesis of amino acids. The primordial TCA intermediates can be one of the first autocatalytic systems, with glyoxylate acting as both a reducing agent and the carbon source; whereas glycine and 4- and 5-carbon α-ketoacids produced Asp and Glu, respectively as well as recovered glyoxylate in this early cycle (Stubbs et al., 2020).

The TCA cycle (Martinez-Reyes and Chandel, 2020), specifically its reductive (CO2-assimilating) version, is one of the oldest (pre)metabolic pathways (Martin, 2020; Smith and Morowitz, 2004). The reverse TCA cycle is found in methanogenic Archaea, Clostridia, Chlorobi, Aquifex, and other early-branching prokaryotes. Comparative genomics points to its presence in the last universal common ancestor (LUCA) and even before (Braakman and Smith, 2012), possibly predating even genome-type organization. Mineral-catalyzed reverse TCA was even argued to precede any RNA and peptides (Fahrenbach and Tran, 2020; Morowitz et al., 2000; Muchowska et al., 2019; Smith and Morowitz,

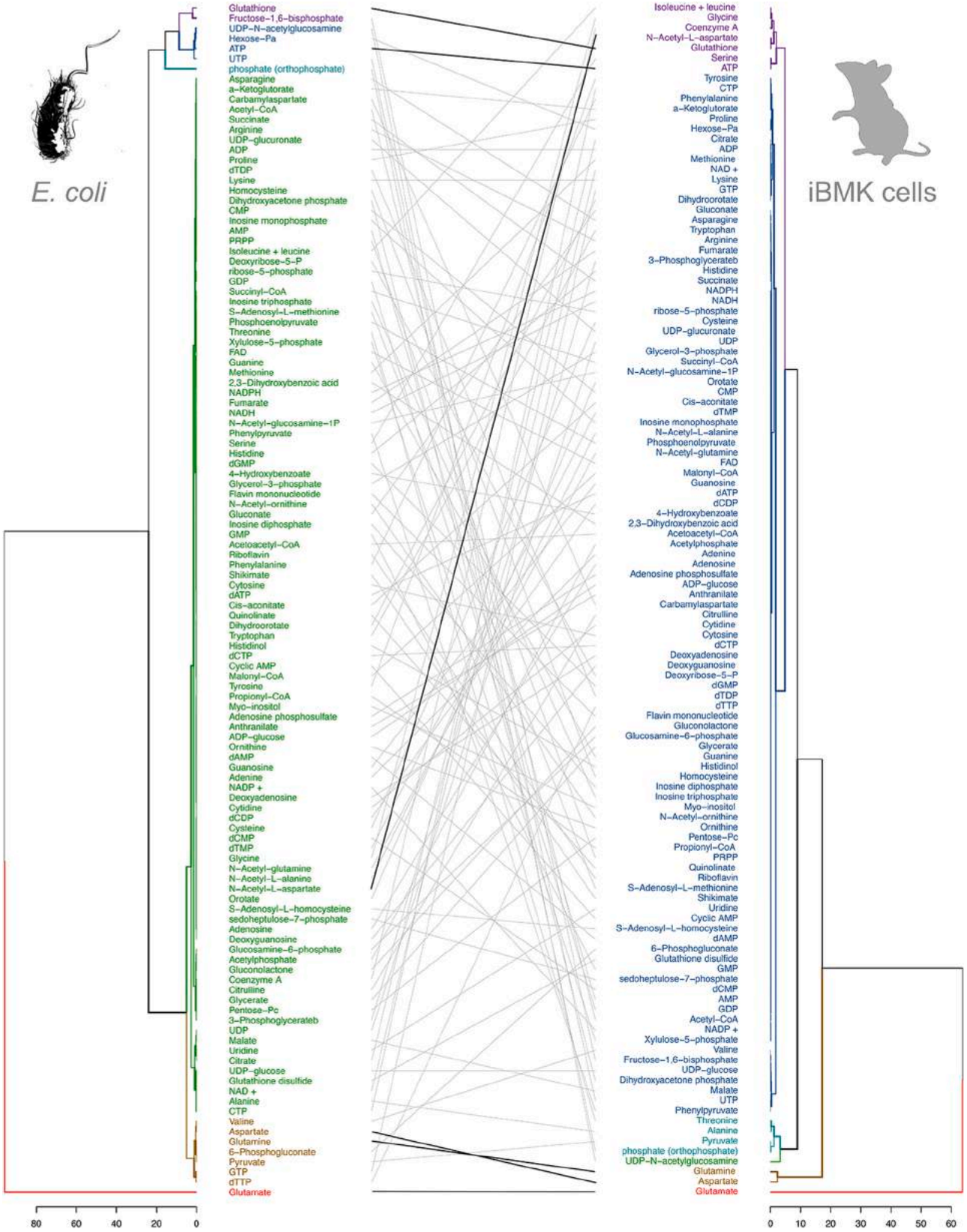


**Fig. 4.** A comparing the hierarchy of intracellular metabolites based on their absolute concentrations for bacteria (*E. coli*) and iBMK cells (*M. musculus*); . The tanglegram shows six clusters with concentrations for $10^{-2}$ to $10^{-7}$ M: red ($10^{-2}$ M), orange ($10^{-3}$ M), green ($10^{-4}$ M), cyan4 ($10^{-5}$ M), dodger blue ($10^{-6}$ M), darkorchid ($10^{-7}$ M). Lines for the same metabolite connect similar sub-trees in each species. Glutamate is a noticeable outgroup for both *E. coli* and mouse metabolomes with an absolute concentration of about 96 mM. Scale bar: *E. coli* - 96 mM, iBMK cells - 63.8 mM. Although concentrations of metabolites between prokaryotes and eukaryotes vary, Glu is the most abundant metabolite followed by aspartate, ATP, pyruvate, glutamine. The tanglegram was constructed using 'dendextend' packages (Galili, 2015; Park et al., 2016)).

2004; Stubbs et al., 2020).

By modeling prebiotic environments >3.8 billion years ago, it was shown that TCA cycle intermediates (inherently coupled with Glu/Asp) were readily produced via several abiotic pathways in the early atmosphere or using mineral/surface chemistry. For example, HCN oligomer hydrolysis (Eschenmoser, 2007), photocatalytic oxidation of formamide on $TiO_2$ crystals (Saladino et al., 2011), and photocatalytic $CO_2$ reduction on ZnS crystals (Zhang and Martin, 2006) closely mimick the enzymatic reverse TCA cycle. Similar to non-enzymatic pathways without catalysts described before, ketoacid intermediates of the reverse TCA cycle can be abiotically aminated to Glu, Asp, and Ala via photocatalytic reactions on ZnS crystals (Wang et al., 2012) or in alkaline ferrous oxyhydroxide-catalyzed fashion. Glu and Asp also produced alongside 11 other proteinogenic amino acids in cyanosulfidic protometabolism scenario (Patel et al., 2015).

By modeling early prebiotic conditions in the presence of $Fe^{2+}$, Muchowska et al. (2019) showed that just two compounds (aqueous glyoxylate and pyruvate) at 70 °C, under an inert atmosphere, could produce 9 of 11 metabolites of TCA within 3 h. There are five universal metabolic precursors for all life pathways: acetate, oxaloacetate, succinate, and α-ketoglutarate, and pyruvate itself. Moreover, by adding metallic iron ($Fe^0$) with hydroxylamine ($NH_2OH$ - an intermediate of global nitrogen fixation), this abiotic system, within 1 h, produced four amino acids: glycine, aspartate, glutamate, and alanine (Muchowska et al., 2019). Intriguingly, three of them were widely recruited as signal molecules/neurotransmitters later in the evolution.

In conclusion, early in chemical evolution, Glu was not exclusively derived from the intermediates of a reverse TCA cycle (Morowitz et al., 1995; Stubbs et al., 2020). There were several effective parallel abiotic synthetic pathways to produce Glu. Even in these early protometabolic cycles, Glu might co-play the unifying and buffer roles as anaplerotic sources of carbon, energy, and nitrogen in the formation of evolving families of self-assembled autocatalytic circles (Orgel, 2000; Schuster, 2000; Wachtershauser, 1990). The extant oxidative TCA cycle, which is located in mitochondria, might by itself be a result or a co-product of early eukaryotic evolution and symbiogenesis (Ryan et al., 2020).,

In any case, by testing various chemical solutions for bioenergetics, it was demonstrated that the oxidative TCA cycle had been a typical case of 'opportunism' in molecular evolution and the best possible chemical design for cellular energy production (Melendez-Hevia et al., 1996). "The modern design of the Krebs (=TCA) cycle is in fact a *unique solution,* arrived at not by process of optimization, but by assembling "pieces'' of chemical steps previously functioning for amino acid biosynthesis. Therefore, this conclusion is strongly dependent on the universality of biosynthesis of amino acids - glutamate and aspartate particularly." (Melendez-Hevia et al., 1996).

In other words, ***L-Glu was at the frontmost position of shaping the metabolic pathways in protocells outperforming Asp and AAA in their chemical efficiency***. The structural, physical, and chemical parameters of Glu provide additional possibilities for its opportunistic recruitments into both biochemical and signaling pathways. Seven such features can be summarized as following (Young and Ajami, 2000):

(1) The odd, 5-carbon chain length of Glu orients the 2-amino and 1-carboxylic oxygen atoms on different planes of symmetry. In Glu, the oxygen atoms form a "pocket" (Fig. 5, arrow) that is not present in its even carbon chain homologs (Asp and AAA). Consequently, the interatomic distance between the oxygens in the glutamate pocket is the smallest compared to Asp and AAA, thus contributing to tighter hydration and cation retention as a coordinating Glu structure.

(2) The five-carbon skeleton of Glu permits the keto-enol tautomers and transient, shared-electron activation states at the 2,3- and 4,5-positions to be in conjugation during active site binding under enzyme catalysis. This architecture is not so for Glu homologs (4-carbon Asp and 6-carbon AAA).

(3) Glu induces helicity to a greater extent than its homologs. In this capacity, Glu is a principal contributor to the hydrogen bonding, hydration, and coordination-chelation phenomena leading to the characteristic tighter packing of chain folds that permits α-helices to function as molecular springs (Kohn et al., 1995a, 1995b; Young and Ajami, 2000).

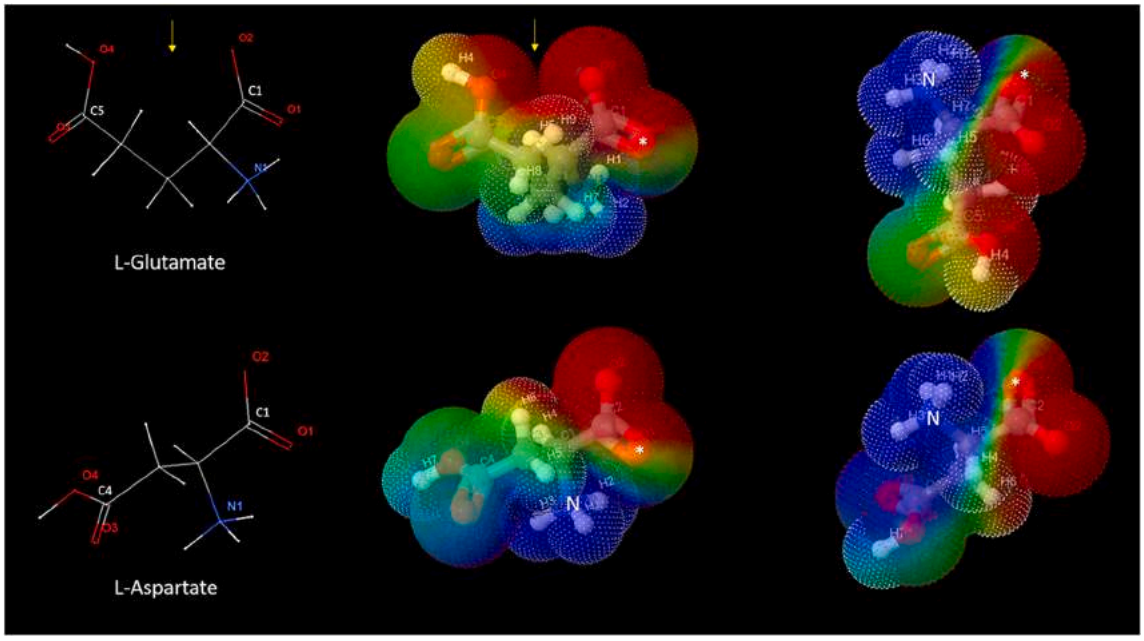


**Fig. 5.** The structures of glutamate (Glu) **and aspartate** (Asp). Amino acids Asp and Glu have 4-carbon and 5-carbon skeletons, respectively. However, their 3D organization and the 3D distribution of polar groups differ substantially; it is reflected in their distinct recruitments in metabolic pathways and signaling, including pharmacological properties. Two right images are carbon skeletons of Glu and Asp. The central and left images show van der Waals surfaces and molecular electrostatic potential (range −0.1 -0.1). The atomic structural data were obtained from the Cambridge Crystallographic Data Center. https://www.ccdc.cam.ac.uk/structures/?ccdc-check=881ded3bf3ea70f6813dada69c8a8eee.

(4) In the protonated form, Glu dynamically stabilizes the coiled-coils by modulating their hydration state in response to pH changes. This Glu-associated pH-dependence facilitates dynamic protein folding and unfolding with countless functional outcomes.
(5) The extra methylene group of Glu contributes to a greater dipolar moment and, therefore, leads to a dramatically greater hydrophobic effect (1.73 kcal/mol) than Asp (1.13 kcal/mol) or Gly (1.18 kcal/mol). Deprotonated Glu is highly hydrophilic by a fivefold greater factor than Asp. As a result, such a broad amphiphilic nature makes Glu unique among the polar amino acids (Bordo and Argos, 1991; Karplus, 1997).
(6) As a direct consequence of its structure, the biochemical reactivity of Glu is also broader than Asp (Young and Ajami, 2000). For example, Glu can efficiently release its nitrogen to transaminating amino acids, whereas aspartate exchanges its nitrogen only with Glu.
(7) By analyzing all possible D- and L-Glu conformations under identical solvation levels, Gonzalez et al. demonstrated that the D-Glu is less flexible (i.e., more stable structurally) than the L-Glu enantiomer (González et al., 2009). As a result, a more adaptable and conformationally flexible L-Glu would be a "preference" for living systems, overperforming its D-Glu enantiomer in diverse metabolic and signaling functions (see also (Moroz et al., 2020b) and below).

These and many other examples further emphasize the primordial and opportunistic recruitment of L-Glu in more than 200 metabolic pathways (Yelamanchi et al., 2016), making it the most abundant and versatile metabolite from bacteria to humans (Figs. 3–5).

## 5. Early evolution of Glu signaling

### 5.1. Glu as a significant osmolyte and folded protein stabilizer

With 20–100 mM intracellular concentration in prokaryotes and eukaryotes (Bennett et al., 2009; Park et al., 2016), Glu acts as the major osmolyte in the form of potassium glutamate (KGlu) - the cytoplasmic salt. These are not neutral osmolites. Hight $K^+$ is critical for protein and ribosomal functions and neutralization of negative charges in nucleic acids. The presence of free Glu anions in the cytoplasm is equally essential for maintaining a broad spectrum of basal cellular functions. KGlu stabilizes folded proteins, protein-protein, and protein−nucleic acid complexes (Sengupta et al., 2016). *In vitro*, KGlu ranks with Hofmeister salts KF and $K_2SO_4$ (the most efficient) in driving protein folding and assembly (Cheng et al., 2016). Replacement of KCl by KGlu dramatically enhances protein-DNA interactions *in vitro* (Deredge et al., 2010; Leirmo et al., 1987). Even a simple buffer consisting primarily of KGlu, with concentrations comparable to their endogenous intracellular levels, is suitable for the activity of almost all restriction endonucleases, DNA methylases, and many other DNA-modifying enzymes (Hanish and McClelland, 1988).

Analysis of the stabilizing effects of KGlu provides quantitative predictions of its natural selection as an intracellular salt. First, KGlu is an effective cytoplasmic osmolyte because of the dominant influence of unfavorable interactions of KGlu with anionic and amide oxygens and hydrocarbon groups on the water-accessible surface of cytoplasmic biopolymers. Second, KGlu is a potent stabilizer of folded proteins because of the dominant effect of unfavorable interactions of KGlu with hydrocarbon groups and amide oxygens exposed in unfolding (Cheng et al., 2016). Consequently, Glu may generally *promote interactions* between intrinsically disordered regions of proteins, as shown for single-stranded DNA binding protein (SSB) that binds to single-stranded DNA intermediates formed during genome maintenance (Kozlov et al., 2017). This property opens unprecedented evolutionary opportunities for molecular and system innovations, including novel signaling mechanisms.

There is a tight interdependence of $K^+$ and $Glu^-$ accumulation during osmotic adaptations in prokaryotes. A rapid increase in the osmolarity in the extracellular medium stimulates Glu synthesis in *E. coli* with a lag of only about a minute, which is also dependent on $K^+$ uptake. Other early responses also include an increase in ATP concentrations (Ohwada and Sagisaka, 1987). Moreover, the synthesis of Glu appears to be required to attain normal values for the electrical membrane potential (McLaggan et al., 1994). Thus, glutamate synthesis increases virtually simultaneously after stress, which provides a counter ion in the cytoplasm. Other organic osmolytes could be accumulated more slowly either by uptake from exogenous sources or by endogenous synthesis, and then the intracellular concentration of KGlu falls.

At low $K^+$ concentrations or other limitations with K-transporters, after the fast initial rise of [Glu], arginine can also be produced from Glu. In this case, the positively charged arginine might act as a substituent of $K^+$ (Gundlach et al., 2018), potentially contributing to the original $K^+$ role to buffer the negative charge of nucleic acids. In bacteria and archaea, a significant fraction of osmotic homeostasis and adaptive stress responses are mediated by cyclic-di-AMP signaling as the second messenger (Stulke and Kruger, 2020). Interestingly, comparable functions related to stress adaptations in eukaryotes can be associated with cyclic AMP (Erkenbrack et al., 2018).

In summary, the osmotic stress responses in prokaryotes with the recruitment of Glu could evolve as a powerful mechanism to mitigate the destabilizing effect of ionic strength on nucleic-acid-protein interactions and systemic interactions among cellular molecules and their complexes (Burg and Ferraris, 2008; Record et al., 1998). It was recently described that KGlu induced self-assemblies of proteins into high-order oligomers (Purohit et al., 2021). As models, Purohit and colleagues used sliding clamps – the evolutionary conserved oligomeric ring-shaped proteins that increase the efficiency of DNA replication. This finding opens a new dimension in the study of protein-protein interactions and making novel protein assemblies. These adaptations, mentioned above, paved the way to further evolution of intra- and intercellular signaling and Glu signaling in particular.

### 5.2. Hypothesis of early extracellular signaling with Glu

Under the osmotic stress conditions, for example, the intracellular Glu concentrations might increase to 250–300 mM (McLaggan et al., 1994; Richey et al., 1987). The corollary of such high intracellular Glu concentrations is 'needs' for its homeostasis, compartmentalization, and detection. Indeed, any injury, damage, or death of early prokaryotic cells could lead to the substantial elevation of extracellular concentration of Glu. Stress-inducing 'leakage' of Glu during different metabolic, feeding, reproductive stages, together with the polar (anionic) nature of Glu, is an ideal 'pre-adaptation' or exaptation (Gould and Vrba, 1982) toward its future selection as a perfect molecule for intercellular communication. Thus, Glu signaling can be as old as Life itself and can be derived from injury sensing (Moroz, 2009). Under this scenario, as the injury/-stress signal, Glu might act parallel with ATP and NO (Moroz et al., 2021), securing the inherent coupling between bioenergetics and nitrogen metabolisms. In general, the origins of most (neuro)transmitters as extracellular signals could be traced to the very architecture of the intercellular metabolism, often targeting both injury-induced regenerative responses and the utilization of all available resources, including signal molecules as nutrients (Moroz, 2009, 2021; Moroz et al., 2021).

The second corollary is a metabolic co-dependence between concentrations of Glu (especially in prokaryotes) and the membrane potential or bioelectricity functions. It was shown that the bacterial biofilms, as they start to grow, show remarkable *emerging properties* such as social cooperation (Flemming et al., 2016). The rise of complexity often induces nearly universal conflicts (cooperation vs. competition) within the community of cells. For example, the bacterial biofilm periphery protects the center from external, potentially dangerous factors

and starves them simultaneously. And this conflict is resolved through the emergence of long-range metabolic and electrical coupling between the central and peripheral cell populations, where Glu plays an essential role (Liu et al., 2015). Here, the Glu recruitment is derived from its already mentioned roles as the vital metabolite, osmolyte, nitrogen, carbon, energy sources, and the interdependence among Glu, $K^+$, and membrane potentials. Tightly coupled metabolic (Martinez-Corral et al., 2018) and electrical oscillations result in periodic halts of the population growth, increasing nutrient availability for the sheltered inferior cells (Liu et al., 2015). In other words, metabolic states in the biofilm center use interdependent electrical (Prindle et al., 2015) and chemical signals to transmit information about *stress* to the periphery. Glu-dependent brain-like bioelectricity communications in bacterial populations can be modeled with two important conclusions. (i) Oscillations emerge from the interplay between Glu and electrical signaling, and (ii) Glu metabolism and associated stress release determine oscillation onset sizes and periods (Martinez-Corral et al., 2019).

The emerging cooperative architecture with unexpected behavior features can increase overall fitness and be expanded to other systems. First, a relatively simple ancestral electrochemical $K^+$ -Glu signaling could contribute to the independent origins of multicellularity both in prokaryotes and eukaryotes. Second, the modeled self-organizing emerging behaviors are similar to the complex collective dynamics that characterize neural ensembles (Martinez-Corral et al., 2019). It is tempting to propose that these universal processes (and molecular ensembles) also contributed to the independent origins and parallel evolution of neural systems in Metazoa. Indeed, recent studies on ctenophores indicated that Glu could be one of the first low molecular weight neurotransmitters (Moroz, 2015b; Moroz et al., 2014; Moroz and Kohn, 2016), together with neuropeptides and ATP (Moroz, 2009, 2014). These scenarios will be discussed below. But how Glu could be sensed as a signaling molecule in prokaryotes.

## 6. Origins of Glu receptors in prokaryotes: modular organization of Glu receptors is inherently linked to nutrition-related architecture

Previous chapters illustrated that injury, stress, nutrition, chemoreception - all these processes are interdependent and Glu-dependent. The overall architecture of the metabolic and nutrition pathways leads to very high intracellular Glu concentrations. These high Glu concentrations can be non-specifically 'released' extracellularly following injury or stress. By itself, Glu is a powerful nutrient, carbon, nitrogen, and energy source. As a result, sensing of Glu is a nearly universal requirement for most prokaryotic cells or organisms to be competitive in most environmental situations. Furthermore, Glu and $K^+$ abundance and their inherent links to bioelectricity were derived from the ancestral metabolic architecture. Thus, it is logical to propose that the vital exaptation for the origin of Glu receptors were K-channels themselves.

It is universally accepted that prokaryotic K-channels are ancestral to most ion channel families due to the exceptional preservation of their transmembrane domain (Miller, 2000; Moran et al., 2015; Yu et al., 2005). The discussed importance of potassium ions and Glu in cellular homeostasis and stress responses also substantiates the original hypothesis that the first ionotropic glutamate receptors (iGluRs) resulted from a fusion of two proteins: (i) the ancestral potassium channel and (ii) a nutrition ligand-binding domain (LBD) (Arinaminpathy et al., 2003; Felder et al., 1999; Ger et al., 2010; Tikhonov and Magazanik, 2009), see also (Ramos-Vicente et al., 2021).

The transmembrane domain (TMD) of eukaryotic iGluRs can be derived from the bacterial potassium GluR0 type channels (Chen et al., 1999), and eventually, be traced to prokaryotic potassium channels of the KscA class (Arinaminpathy et al., 2003; Chen et al., 1999; Tikhonov and Magazanik, 2009). Fig. 6 shows some extant transmembrane proteins, which can also be derived from prototypes of the first channels (corresponding to TM1, TM2 (=P-loop), and TM3 segments of KscA) and transporters. There is a domain similarity between $K^+$ transporters and predicted prokaryotic iGluRs.

Equally important to supporting potential lateral gene transfer in the evolution of iGluRs was finding homologs of K-channels in viruses. The chimeric fusion of a small viral type of K-channel to the Glu-binding LBD of the mammalian AMPA subunit formed a functional glutamate-gated channel (Schonrock et al., 2019). Gouaux and colleagues proposed that the prokaryotic iGluRs might arise by inserting an inverted K-channel pore between SBP domains (Chen et al., 1999). However, it might also be possible that some potassium channels in Archaea have 'non-inverted' orientations of K-channels; such predicted structure is similar to the extant transmembrane region of iGluRs (Fig. 6B).

Similar functional iGluR architectures were found both in bacteria and cyanobacteria. We also found it in Archaea (Fig. 6A). The finding of archaeon iGluRs suggests that the fusion events widely occurred in prokaryotes and might also be subject to lateral gene transfer between different prokaryotic lineages. In prokaryotes, iGluRs are likely tetrameric membrane proteins. Crystallography is not performed for most prokaryotic Glu receptors, and their ligand-binding specificity, ion selectivity, and kinetics are largely unknown (except for GluR01 in the cyanobacterium *Synechocystis* – (Chen et al., 1999)). The diversity of their structures in prokaryotes predicts affinities to different ligands as it was described for plants (Forde and Roberts, 2014).

Ligand-binding domain (LBD, also known as the amino-terminal domain ATD) with two lobes (binding Glu or other amino acids), is derived from the periplasmic prokaryotic substrate-binding proteins (SBP - (Scheepers et al., 2016); specifically from its subfamily of lysine-arginine-ornithine-binding periplasmic proteins (Forde and Lea, 2007). These LBDs could be responsible for binding/gating as many as 13 amino acids (in addition to L-Glu) in the *Arabidopsis* iGluRs (Forde and Roberts, 2014). The SBP family (with LBD homologs) includes one of the broadest diversity of amino acid- and nutrient-binding proteins involved in sensing metabolites, solute uptake, and signal transductions (Scheepers et al., 2016). The 'selection' of lysine-arginine-ornithine-binding motifs further supports the primordial interdependence between Glu-/amino acid signaling and energy-/nitrogen assimilation in early cells.

Importantly, the original functional characterization of GluR0 showed that it is not exclusively a Glu-gated channel (Chen et al., 1999). In addition to L-Glu (but not D-Glu), GluR01 is similarly gated by L-glutamine, L-adipic acid, L-homocysteine, serine, threonine, alanine, and with less affinity by glycine, D,L-a-aminopimelic acid, and is practically insensitive to L-aspartate. These findings emphasize the SBP ancestry of LBD as nutrient sensors, often involved in chemoreception. Accordingly, early iGluRs were likely evolved as more general amino acid sensors. But potentially higher biologically derived Glu concentrations made this class of proteins as functional glutamate receptors.

Regardless of precise ligand specificity, SBPs represent a blueprint of all extant iGluR LBDs by sharing a common ligand-binding mechanism and likely parallel origins via fusion of respective transmembrane domains.

The functional compatibility of bacterial binding proteins to the gate of the channel pore of an iGluR was directly confirmed by reconstructing chimeric receptors *in vitro*. Bernard and Bodo validated two key features of the modular Glu receptor architecture: (a) the evolutionary conservation of ligand binding mechanism between SBPs and iGluR LBDs, (b) the formation of an LBD dimerization interface as a critical step in iGluR evolution to couple ligand binding to channel gating (Bernhard and Bodo, 2021). They further hypothesize that iGluRs evolved by the fusion of class F amino acid-binding proteins (SBP family) with potassium channels.

Structurally another class, Cys-loop pentameric Glu receptors or GluClR (Kunishima et al., 2000), might also have their ancestry in prokaryotes (Corringer et al., 2012; Jaiteh et al., 2016; Tasneem et al., 2005), with LBDs, derived from bacterial periplasmic proteins, as in iGluRs. This class of receptors provides complementary pathways of Glu

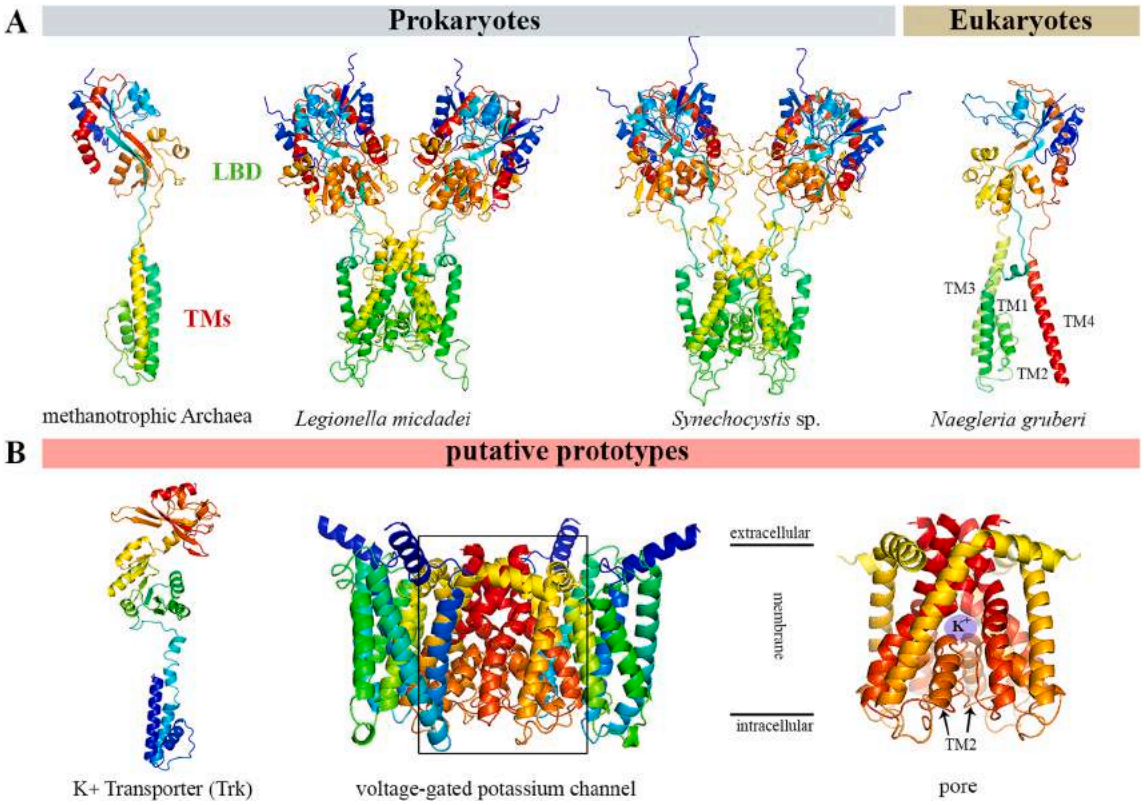


**Fig. 6.** Modular organization of iGluRs in prokaryotes and eukaryotes as sensors of the environment. A. The same domain organization is predicted from sequence information both in prokaryotes and unicellular eukaryotes. Two domains of iGluR are recognized in prokaryotes and some unicellular eukaryotes: LBD - ligand-binding domain, TM – transmembrane domain. A nearly identical organization exists between bacteria and cyanobacteria. For the first time, we identify a putative iGluR in Archaea (see text for details). TM4 domain is the eukaryotic innovation, as shown in *Aureococcus* (red sequence in C-terminus). The organizations of **iGluRs** are shown for uncultured methanotrophic archaeon (WP_010870874.1), bacterial *Legionella micdadei* (CEG60559), basic GluR0 from the cyanobacteria *Synechocystis* sp. (BAA17851 sequences See supplements S1, S2 for sequences). Color codes: *N*-terminus (blue), C-terminus (red). In Archaea, Bacteria and Cyanobacteria *N*-terminal starts in LBD, and C-terminal is also located in LBD. In eukaryotes, C-terminal is in TM domain (TM4). **B.** 'Putative prototypes' section shows predicted ancestral protein structures critical for the evolution of iGluRs. Potassium transporter (Trk) and voltage-gated potassium channel (center, (AFV23945)) from Archaea share similar protein topology and orientation with transmembrane domains of iGluRs. The right image shows the pore structure of the predicted archaeon potassium channel (AFV23945) with the position of the potassium ion. Of note, the archaeon potassium channel pore orientation is the same as in iGluRs of both prokaryotes and eukaryotes. The predicted potassium channel sequence (CBH37362.1) was obtained and reconstructed from an uncultured archaeon (methanotrophic ANME-1 group, environment samples (Meyerdierks et al., 2012)). The potassium transporter (AFV23945.1) is from another archaeon *Methanolobus psychrophilus* R15 (Chen et al., 2016)).

sensing early in evolution, and some of the members are gated by alkaline pH (Hu et al., 2018). The precise functions of each class of Glu receptors are still unknown.

The growing evidence indicates that Glu might control bacterial behaviors, including chemotaxis (Brown and Berg, 1974; Craven and Montie, 1985; Mesibov and Adler, 1972; Ordal and Gibson, 1977; Yang et al., 2015), to its theoretical limits (Brumley et al., 2019) as well as *social-type* communications within biofilms (Liu et al., 2015; Martinez-Corral et al., 2018). The stress responses in bacteria also recruit γ-aminobutyrate (GABA) as a part of systemic adaptations with a prominent Glu/GABA coupled transporter. The glutamate decarboxylase (GAD) system facilitates intracellular pH homeostasis by consuming protons in a decarboxylation reaction that produces GABA from Glu, crucial for acid tolerance in bacteria genera (Feehily and Karatzas, 2013).

## 7. Diversification of Glu receptors in eukaryotes

The eukaryotic cell is likely a result of a symbiosis between an archaeal host and an α-proteobacteria (mitochondrial precursor) plus sulfate-reducing bacteria. The rise of planetary oxygen level (about 2 billion years ago) might had triggered this innovation, and eukaryotes were evolved within Archaea (Liu et al., 2021; Lopez-Garcia and Moreira, 2019, 2020, 2020; Nasir et al., 2021; Zaremba-Niedzwiedzka et al., 2017) as a cooperation-type adaptation to oxygen toxicity and stress. Under this widely accepted reconstruction, the oxidative TCA cycle (coupled with Glu-Glt metabolism and consuming/scavenging oxygen in mitochondria) became the major source of energy production. The emerged cellular architecture, with internal membranes, released eukaryotes from many bioenergetic constraints of the prokaryotic world. As such, mitochondria triggered both the growth of cell sizes and remarkable genome expansion, eventually producing more than 3000 novel eukaryotic protein families (>200,000 genes) and 5–10 times more novel protein folds compared to prokaryotes (Koonin et al., 2004; Lane and Martin, 2010). Also, this apparently autocatalytic process of eukaryogenesis, re-utilized intermediate TCA metabolites for extensive intercellular and intracellular signaling functions, including epigenetic control of the genome operation (Ryan et al., 2019, 2020). Again, Glu signaling is not an isolated event in the history of life; its evolution have always been deeply integrated with metabolism and energetics both in prokaryotes and eukaryotes.

Considering the inherent coupling between Glu/nutrition/energy and bioelectricity described for prokaryotes (Liu et al., 2015, 2017; Martinez-Corral et al., 2018, 2019), it would be reasonable to propose that such interactions also contributed to the eukaryogenesis, and the origins of multicellularity in eukaryotes. The latter result can be

naturally derived from the evolution of social type organization within unicellular organisms. Similar to prokaryotic biofilms, any aggregation or symbiosis would eventually lead to competition-cooperation cycles for resources and energy with Glu-Glt and amino acids as natural signaling molecules under such stress conditions. The recent modeling of cooperation vs. competition strategies shows that the aerobic organisms with greater ATP yield and a low rate of production (vs. fermentation – low yield and high rate) would have selective advantages to cooperate for shared resources (Pfeiffer et al., 2001). In other words, the *oxidative* mitochondrial TCA cycle (coupled to Glu-Glt and other amino acids) facilitated the evolutionary transition from unicellularity to multicellularity. These events occurred more than ten times independently in the evolution of eukaryotes (Knoll, 2011), and iGluRs as versatile amino acid/nutrient sensors could naturally be involved in these processes. Indeed, by comparing iGluRs across major eukaryotic clades, we noted a correlation between the complexity of these receptors and multicellularity (Fig. 8). Below, we will briefly emphasize some aspects of these observations.

### 7.1. Parallel evolution of eukaryotic iGluRs

The iGluRs in eukaryotes have two notable differences with their prokaryote orthologs. The first eukaryotic innovation is a novel TM4 segment (Fig. 6), which might support the stability of iGluR and be required for tetramerization as shown for AMPA receptors (Salussolia et al., 2013).

The second innovation is an additional *N*-terminal domain (NTD) (Figs. 6 and 7, see also (Mayer, 2021b)) and NTDs are derived from prokaryotic SBP (also referred as periplasmic binding proteins) of the leucine-isoleucine-valine-binding periplasmatic subfamily (Felder et al., 1999; Forde and Lea, 2007). As evolutionary derivatives of bacterial periplasmic binding proteins, NTDs are also distantly related to LBD of iGluRs (Felder et al., 1999; Kumar et al., 2009; Tikhonov and Magazanik, 2009) and respective ligand-binding domains of metabotropic Glu receptors (Corringer et al., 2012; Jaiteh et al., 2016; Kunishima et al., 2000; Lynagh et al., 2015; O'Hara et al., 1993; Tasneem et al., 2005). However, the NTD has a Venus-flytrap fold, while LBDs have clamp-shell like structures emphasizing their significant evolutionary and functional divergence (Mayer, 2006, 2011, 2021a, b).

Nevertheless, not all eukaryotes possess both LBD and NTD within the same iGluR (i.e., TM + LBD + NTD). In the unicellular Pelagophyceae algae, *Aureococcus*, iGluRs are similar to their homologs in prokaryotes with only LBD (Fig. 7), lacking the NTD domain (i.e., two-domain structure: TM + LBD).

Next, we screened for the presence of iGluRs across the eukaryotic Tree of life (Fig. 8 and Supplement 1). This comparative analysis suggests that iGluRs were lost in three major eukaryotic lineages, which led to euglenas and parasitic trypanosomes, (forming the clade Euglenozoa), amoebas (including the colonial forms such as myxozoa), as well as fungi (Fig. 8, red cross). The reason(s) for such loss of iGluRs are unclear, but it might be related to the type of feeding and heterotrophic metabolism. Photosynthetic/autotrophic species usually preserved iGluRs from their prokaryotic ancestors.

The eukaryotic phylogeny (Fig. 8) also suggests that NTD domains might be incorporated into iGluRs at least four times independently. Intriguingly, these four events often occurred in the lineages leading to multicellular organisms: some brown algae, red algae, green algae, and animals (Metazoa). It is also well-known that multicellularity evolved many times (Brunet and King, 2017; Colizzi et al., 2020; Knoll, 2011; Parfrey and Lahr, 2013). And the correlation with the rise of the organismal complexity and the complexity of iGluRs (by adding NTDs) might be interdependent events associated with shared nutrient/energy cooperativity as suggested by models discussed above (Liu et al., 2015, 2017; Martinez-Corral et al., 2018, 2019).

NTDs are not needed for ligand bindings, but these domains perform a broad spectrum of modulatory functions: binding to ions, small and large molecules, interactions between subunits, tetramerization, etc. (Mayer, 2006, 2011, 2011; Stroebel and Paoletti, 2020). There are extensive diversifications among different iGluRs (Price et al., 2012; Wudick et al., 2018), some with accessory subunits (Ramos-Vicente et al., 2021; Zhao et al., 2019). The functional significance of these diversification events across eukaryotes needs to be further explored.

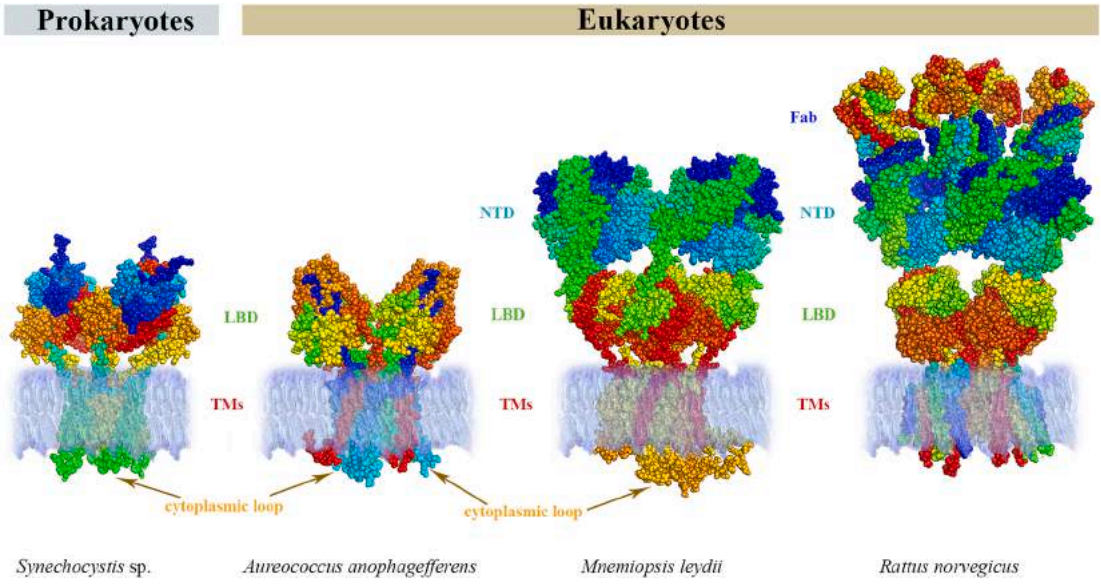


**Fig. 7.** Diversity of ligand-gated ionotropic glutamate receptors (iGluRs) in prokaryotes and eukaryotes. Mammals have two extracellular domains. Representative organization of ionotropic glutamate receptors in prokaryotes and eukaryotes: the cyanobacterium, *Synechocystis* sp., the pelagophyte unicellular algae, *Aureococcus anophagefferens*, the comb jelly, *Mnemiopsis leydyi,* and the rodent, *Rattus norvegicus* (imaging by PyMol software with *N*- terminus domain – blue, C-terminus domain - red). – Transmembrane domains (TMs) anchor receptors in the cell membrane. LBD – extracellular domains with the ligand-binding site. NTD – amino-terminal domains. iGluRs also contain an intracellular cytoplasmic loop. Some receptors have auxiliary subunits (Ramos-Vicente et al., 2021). iGluRs have a tetrameric structure as illustrated for *Synechocystis* sp. (PDB:5weo), *Aureococcus* (PDB:6ruq), *Mnemiopsis* (PDB:5kuf), and *Rattus* (PDB:6njm) with the 'Fab' domain as an additional part of the native AMPA receptor complex (Zhao et al., 2019). Abbreviations: TMs – transmembrane domains, LBD – ligand-binding domain, NTD – *N*-terminus domain. Color: blue – *N*-terminus, red – C-terminus.

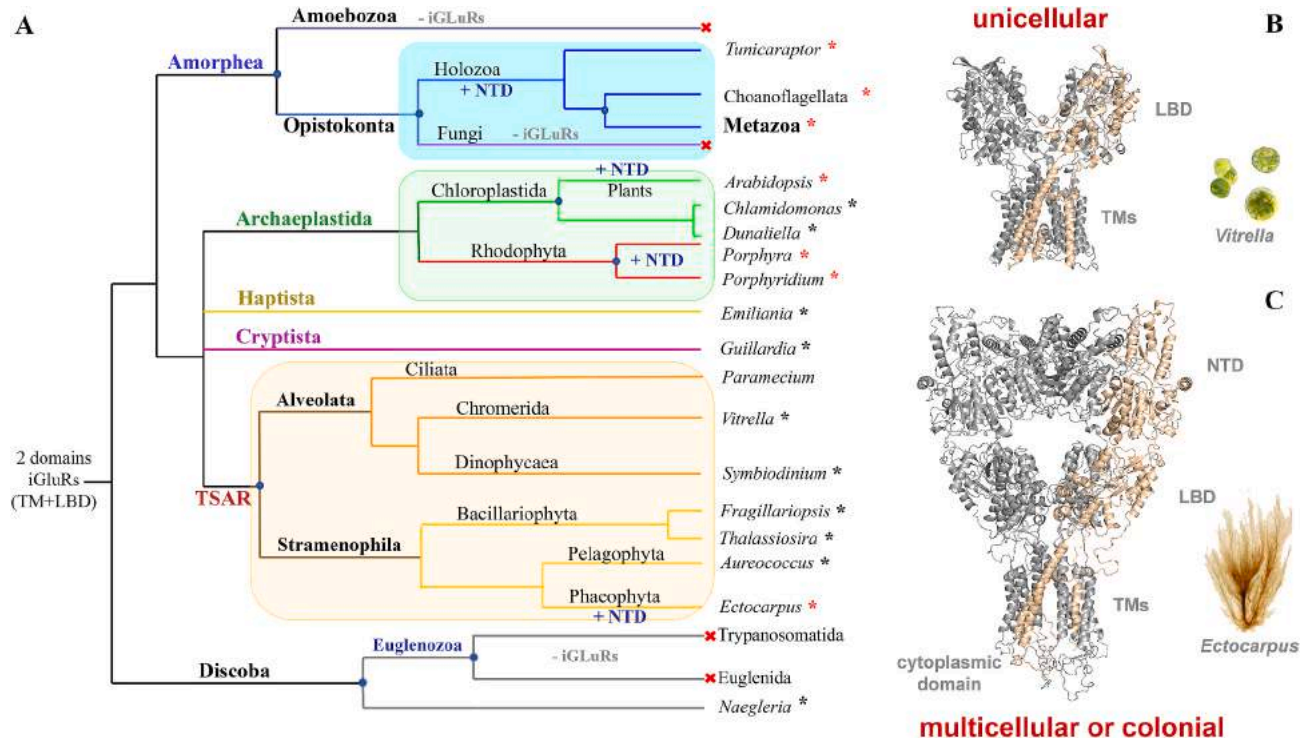


**Fig. 8.** Early diversification of iGluRs: 3-domain organization of iGluRs correlates with independent origins of multicellularity in eukaryotes (Knoll, 2011). The diagram shows phylogenetic relationships among the major eukaryotic lineages (kingdoms) and the presence of iGluRs (asterisks). Black asterisks – unicellular eukaryotes, red asterisks – iGluRs in multicellular eukaryotes. Inserts illustrate the organization of the iGluRs in the unicellular alga (*Vitrella brassicaformis*, Alveolata) and the multicellular filamentous brown alga (*Ectocarpus siliculosus,* Phaeophyta). Four acquisitions of the NTD domains occurred in different eukaryotic lineages (+NTD). Two domain iGluRs (TM + LBD) were present in the last common eukaryotic ancestor, and they could be lost in Euglenozoa, Fungi, and Amoebozoa (-iGluRs, red cross marks). Abbreviations: NTD – *N*-terminal domain, LBD – ligand-binding domain, TMs – transmembrane domains; red stars – the presence of iGluRs, the golden chain on the model – chain A. All sequences are in the Supplementary Tables S1 and S2.

### *7.2. Classification of iGluRs*

The broad, cross-kingdom phylogenomic classification of iGluRs is not yet established. Two recent comparative genome-scale analyses significantly expanded the canonical, vertebrate-centered classification (Ramos-Vicente et al., 2018). These authors recognized four subfamilies of metazoan iGluRs with ten classes combined (Ramos-Vicente et al., 2021). Here, we readdress this question by exploring the eukaryotic diversity of iGluRs.

The Eukaryote tree (127 sequences, see Supplement 1 and Supplementary Tables 1–2) predictably shows that the eukaryotic receptors are not grouped according to their taxonomic affiliation (Fig. 9). The majority of eukaryotic lineages revealed extensive parallel evolution of the iGluRs within each major clade stressing the early adaptive diversification, which probably occurred in the first eukaryotes with multiple gains and losses over more than 2 billion years. This tree recovered 22 clades of iGluRs (Fig. 9), including all 10 previously recognized (Ramos-Vicente et al., 2018, 2021).

This expanded phylogeny suggests that the Lambda iGluRs might be one of the oldest among eukaryotes or metazoans. Remarkably, the Lambda iGluRs were only identified in sponges (Porifera), but they might be lost in the rest of the animals. Alternatively, the Lambda group could be a sponge-specific innovation that diverged enough to be placed basally in the phylogenetic tree. The convergent evolution of Lambda-type receptors in sponges with higher plants is possible (as well as lateral gene transfer), but their functions are unknown.

The NMDA-type receptors were not found outside metazoans; they were also not detected in the sequenced ctenophores and sponges. Thus, likely they are metazoa-specific.

Archaeplastida (green plants and red algae – Fig. 9) possess at least 4–5 clades of iGluRs (see also (Chen et al., 2016; De Bortoli et al., 2016) for green plants), and many of them can be gated by more than a dozen amino acid ligands, in addition to Glu (Alfieri et al., 2020; Forde and Roberts, 2014). Their ion selectivity and kinetic properties are equally diverse (Alfieri et al., 2020). In plants, the multiplicity of systemic Glu functions are associated with stress responses, osmotic regulations, wound healing, chemoreception, chemotaxis, growth, photosynthesis, endosymbiosis, root development, reproduction, gamete recognition, immunity, and many others, including relatively fast adaptive reactions (Chen et al., 2016; De Bortoli et al., 2016; Forde, 2014; Forde and Lea, 2007; Forde and Roberts, 2014; Goto et al., 2020; Li et al., 2019, 2020; Ortiz-Ramirez et al., 2017; Robertson and Tartar, 2006; Singh et al., 2016; Wang et al., 2019a; Yoshida et al., 2016). All of these functions might be derived from the deep ancestry of Glu signaling described above and the systemic role of Glu in extant prokaryotes as a stress/injury messenger.

Of note, intracellular concentrations of Glu in plants are also high and might reach 30–100 mM in the phloem – thus, virtually any damage would locally increase Glu, which can be a critical intercellular messenger in the long-distant propagation of wound signaling events (Toyota et al., 2018).

In contrast to better-studied land plants, little is known about Glu signaling in other eukaryotic lineages. iGluRs are incredibly diverse across eukaryotes. Both LBD and NTD were found in multicellular/colonial brown algae (these organisms evolved as results of secondarily symbiotic events among eukaryotes) and red algae, with two or three possible events of independent acquisition of NTDs. Despite the superior diversity of unicellular eukaryotes, our screening point to the presence of TM + LBD receptors (as in prokaryotes) in the majority of these lineages, suggesting that this might be the primary architecture of iGluRs in the common ancestor of eukaryotes.

As a less likely alternative hypothesis, NBD might be present in

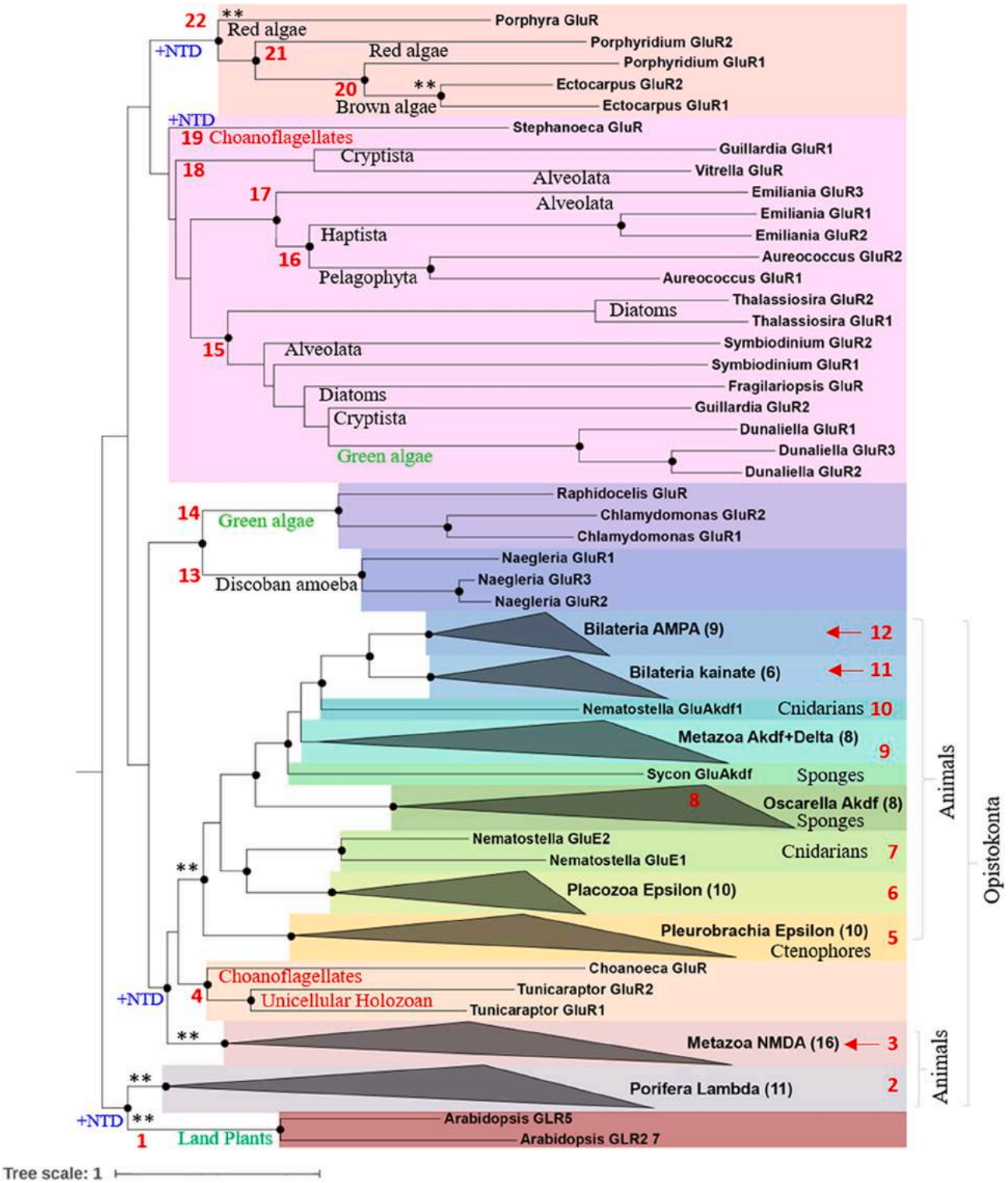


**Fig. 9. Twenty-two distinct phyletic lineages of iGluRs were identified in eukaryotes.** Phylogenetic tree of iGluRs across representatives of the main eukaryotic groups (Maximum Likelihood, see Supplementary tables for 121 sequences). Black dots in branches denote ultrafast bootstrap approximation (UFBoot) support values above 70 %. Lineage-specific duplications are shown as triangles. Full trees with uncollapsed nodes and numerical bootstrap support values are in Supplemental Fig. 1 and Supplementary Tables S1 and S2. NTD domains are present in four separate clades, and their acquisition occurred in the lineages leading to multicellular and/or colonial organisms: such as animals, plants, colonial choanoflagellates, multicellular red and brown algae. The classes of iGluRs such as Lambda, NMDA, Epsilon, Akdf and Delta correspond to the classification proposed elsewhere (Ramos-Vicente et al., 2018).

iGluRs of the common eukaryotic ancestor and then be lost in unicellular eukaryotic lineages (e.g., in the lineages leading to Discoba or some unicellular algae). A variation of this alternative explanation could also be a hypothetical situation when the ancestral eukaryote had both TM + LBD and TM + LBD + NTD iGluRs and that some lineages retained one or the other receptor architecture. Taxonomically broader sampling would be required to clarify the outlined hypotheses of gains or losses of NBD domains. For example, if TM + LBP + NTD iGluRs would be discovered in prokaryotes and/or early branching *unicellular* eukaryotic lineages (e.g. in Discoba, diatoms or Alveolata, Haptista or Cryprista), it would give more support for the hypothesis NTD losses in reported cases.

The majority of iGluRs in unicellular eukaryotes form separate branches (Figs. 8 and 9): they exemplify either ancestral or highly diverged lineages of Glu receptors with only LBD (i.e., without NBD) as in prokaryotes. Even representatives of unicellular green plants such as *Chlamydomonas* and *Dunaliella* are as far from each other as from receptors in higher plants. *Dunaliella* iGluRs are clustered close to receptors in phylogenetically different types of algae (diatoms, ochrophytes, haptophytes). *Chlamydomonas* receptors have been grouped with their counterparts, the free-living amoeboflagellate *Naegleria gruberi* (a sister to the "brain-eating amoeba" *N. fowleri*). *Naegleria* are descendants of one of the earliest eukaryotic lineages, preserving many ancestral features (Fritz-Laylin et al., 2011). Thus, two-domain iGluRs (TM + LBD) in representatives of Discoba might reflect the prototypic organization of Glu receptors for all eukaryotes (Fig. 10).

Interestingly, the choanoflagellates, a sister lineage to Metazoa, possess at least two ancient subfamilies of iGluRs, not recognized in animals (Figs. 8 and 9). One subfamily was found in colonial

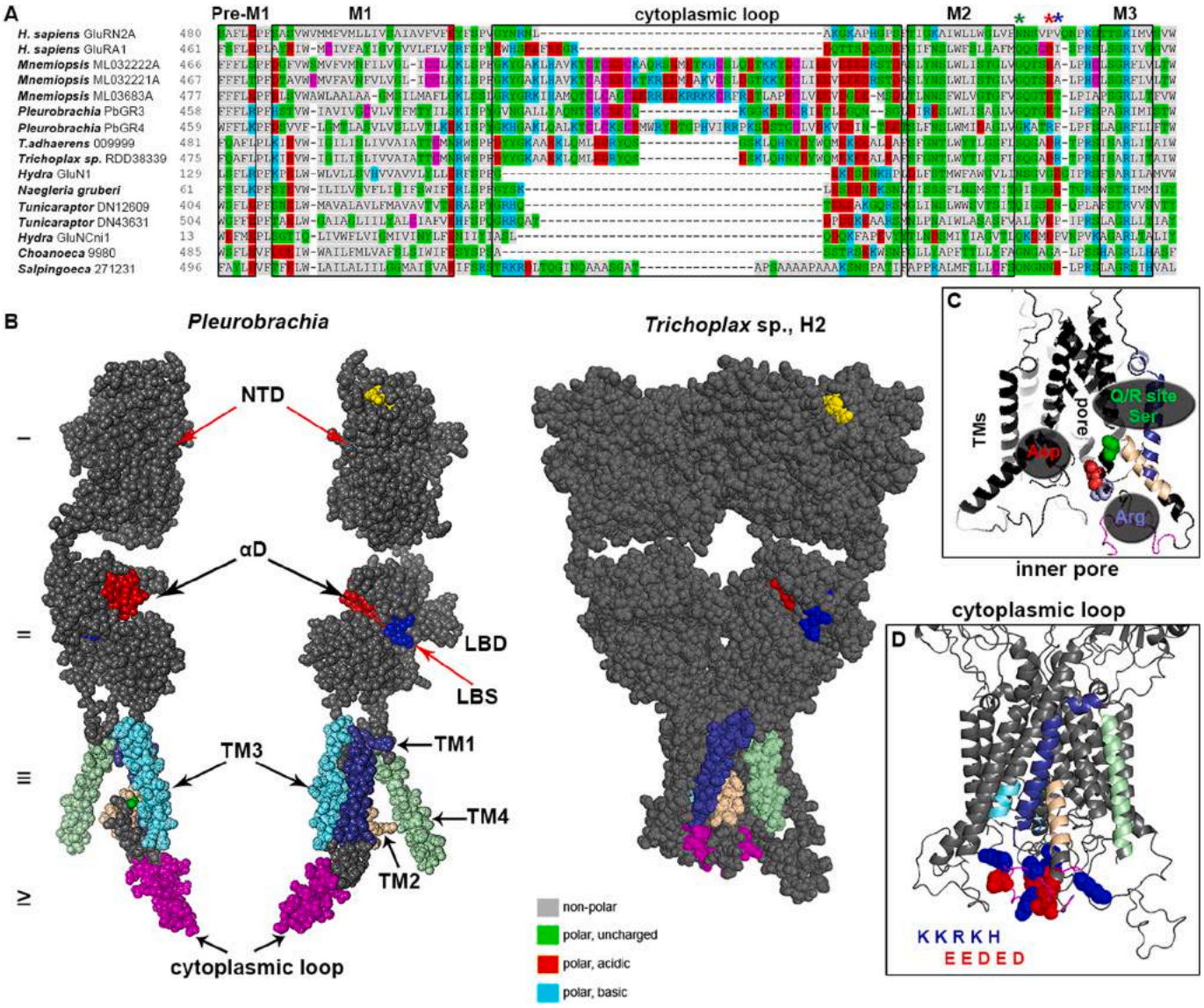


**Fig. 10.** Domain organization of iGluRs. A. Alignments of the transmembrane domains and cytoplasmic loop (cysteine [cys]-rich loop) part of iGluRs. Cys-rich loop presents between M1 and M2 in ctenophore iGluRs with 4–5 cysteines (Alberstein et al., 2015). Humans, Porifera, Placozoa, choanoflagellates, and Amoebozoa have no cysteines in similar cytoplasmic loop regions but contain many polar charged amino acids (Arg, Lys, His, Asp, Glu). **B**. 3D organization of iGluR from the ctenophore *Pleurobrachia bachei* (PbGR3 in the alignment A; one monomer is shown at different rotation angles) and the placozoan *Trichoplax* sp. (H2 haplotype, RDD38339 in the alignment; tetrameric structure). NTD - *N*-terminal domain (NTD, I), LBD – ligand-binding domain with the ligand-binding site (LBS) and α-COOH group (αD) (II), TM – transmembrane domain (III), and cytoplasmic loop of iGluRs (IV). **C**. *Trichoplax* sp. (RDD38339). A region inside the pore with the Q/R site for potential RNA editing (it is serine Ser633; green asterisk in A, green dots in B and C). Of note, two polar charged amino acids are located nearby (Arg638 - blue asterisk in A, and aspartate Asp637, red asterisk). **D**. Cytoplasmic loop in *Trichoplax* sp. iGluR (RDD38339) has 5 basic (blue) and 5 acidic (red) amino acids (with length variations in different species).

choanoflagellate *Choanoeca,* and the recently discovered *predatory* unicellular *Tunicaraptor* (Tikhonenkov et al., 2020); this subfamily can be a holozoan innovation. Phylogenetically, these receptors are placed between the NMDA and the rest of iGluRs in animals (except sponges, Fig. 9 and 10). Such reconstruction suggests that this group of iGluRs might be lost in the metazoans. The second subfamily of iGluRs, found in the loricate *Stephanoeca*, might be even more ancient due to its clustering with unicellular algae (Fig. 9). Both iGluR subfamilies contain LBD and NTD, perhaps with different evolutionary histories. The identification of 3 domain iGluRs (TMD + LBD + NTD) in Holozoa is essential: it is a case of the increased complexity of iGluRs in the lineages leading to coloniality (e.g., *Choanoeca perplexa* and *Stephanoeca urceolata*) and multicellular animals. Species of the genus *Choanoeca* are colonial organisms with cooperative alternating swimming and feeding behaviors as well as photosensitivity (Brunet et al., 2019). *Stephanoeca urceolata* is a loricate choanoflagellate with a complex life cycle. This species possesses both LBD and NTD, but *Diaphanoeca grandis* (Eriksen et al., 2019) have only the LBD extracellular domain. Interestingly two model species, *Monosiga brevicollis* and *Salpingoeca rosetta*, lost iGluRs. Unfortunately, no functional studies associated with Glu signaling have been performed using representatives of this group, so critical to understand animal origins.

In summary, the emergence of multicellularity, coloniality, or complex life cycle correlates with the greater complexity of iGluRs. Moreover, there are several events of independent diversification of iGluRs in different lineages of multicellular eukaryotes. This trend is especially dramatic in the land plants, where the number of iGluR genes can be around 20 in *Arabidopsis* and 44 in pines (possibly a reflection of the large genome size 22 Gb). Thus, the observed parallel diversification of iGluRs can be a result of their recruitments in numerous functions (neofunctionalization), as mentioned earlier for *Arabidopsis* with two large receptor classes. Even more profound diversification of iGluRs is observed in animals (or Metazoa) as the best-studied case of multicellular organization. Although there are some secondary simplifications and gene loss events, the number of genes encoding iGluRs in such lineages as arthropods can exceed two hundred (Polinski et al., 2021). It is impossible to cover the entire extent of Glu signaling in animals, and we will highlight some critical issues and models.

## 8. iGluR and Glu signaling in metazoa: a brief overview of lineage-specific innovations

### 8.1. Introduction

A set of global geological events during the Cryogenian (720-635 Mya) and Ediacaran (635-541 Mya) periods such as the Sturtian and Marinoan glaciations, oxygenation of oceans, a breakout of continental plates, etc. provided substantial ecological stresses on Neoproterozoic biota in most habitats (Knoll and Sperling, 2014). These conditions facilitated the independent formation of the multicellular organization in many eukaryotic lineages and triggered the origin of animals by the end of the Ediacaran period. However, affinities of Ediacaran animal-like fossils to modern phyla are still elusive. The emergence of extant phyla in fossil records occurred about 540–510 million years ago, marking the famous Cambrian explosion.

The lack of clear links among phyla in fossil records generated hundreds of hypotheses of early animal evolution. The consensus exists that all animals can be classified within only five basal metazoan lineages (Figs. 12 and 17): Ctenophora (comb jellies), Porifera (sponges), Placozoa, Cnidaria (polyps and jellyfishes), and Bilateria (which consist of about 30 phyla including Chordata to which we belong). Cnidaria and Bilateria are sister lineages with the prominent neuro-muscular organization. In contrast, little is known about the physiology, signaling, and behaviors of the remaining three basal metazoan lineages. Even phylogenetic relationships among Ctenophora, Sponges, and Placozoan are topics of numerous, still unresolved, debates. These lineages were claimed to be a descendent of the first animals or a sister group to all other metazoans (Halanych et al., 2016; Jekely and Budd, 2021; Kapli and Telford, 2020; Laumer et al., 2019; Li et al., 2021; Moroz et al., 2014; Redmond and McLysaght, 2021; Telford et al., 2016; Whelan et al., 2015, 2017). No convincing Precambrian fossils were found for sponges (with skeletons) and fragile ctenophores or placozoans. The separation of basal metazoan lineages likely occurred within a relatively short geological interval, perhaps several million years or less, due to global environmental catastrophic events in Precambrian, with the parallel evolution of multiple animal traits over 550 million years. Here, we will primarily focus on Glu signaling in basal metazoans.

### 8.2. How to make a glutamatergic cell?

We hypothesize that Glu (+glycine/amino acids) signaling was the inherent part of intercellular communications in early nerveless animals equipped with a diverse array of iGluRs and transporters. Adaptations to stress responses and injury-related Glu signaling predated the animal organization. And it is also preserved in extant metazoans in the form of glutamatergic nociceptive pathways (Carr and Zachariou, 2014; Maricq et al., 1995; Schafer, 2015; Walters, 2018; Walters et al., 2004; Walters and Moroz, 2009; Wittenburg and Baumeister, 1999). Glu-mediated communication pathways (perhaps with Gly sensing (Stroebel and Paoletti, 2020)) were sequentially recruited in a broad spectrum of sensory and effector functions within each basal metazoan lineage. In this reconstruction, important insights provide specific mechanisms underlying non-specific stress responses in other eukaryotes.

The most remarkable example of the evolutionary conserved eukaryotic Glu communications is the neural-like electro-chemical defense signaling in plants (Koselski et al., 2020; Lapeikaite et al., 2020; Muday and Brown-Harding, 2018; Toyota et al., 2018). It was elegantly shown in *Arabidopsis* using fluorescent $Ca^{2+}$ and Glu reporters that when herbivores attack and damage cells and tissues (e.g., caterpillar feeding), Glu is released from the injury site. Plants sense these local signals: within 2s, a dramatic increase of intracellular $Ca^{2+}$ was detected at the herbivory site, and this information is rapidly transmitted within 1–2 min to distant places to activate defense responses in undamaged leaves (Toyota et al., 2018). The concentration of Glu at the injury site was estimated as ~50 mM. Remarkably, applications of the same Glu (50 mM) concentration, but not other amino acids or sorbitol (osmotic control), mimicked the elevation of $Ca^{2+}$ signals induced by the tissue damage. Rapid propagation of injury-induced electrical signals depends upon at least two types of Glu gated ion channels GLR3.3 and GLR3.6 (Toyota et al., 2018). Here, apoplastic **Glu acts as a classical transmitter and systemic wound injury signal over long distances** triggering defense responses. Conceptually, this situation is very similar to nociceptive signaling in vertebrates, molluscs (Moroz, 2009; Moroz et al., 2021; Walters, 2018; Walters and Moroz, 2009; Walters and Williams, 2019) or nematodes.

Paracrine or hormone-type signaling could be the most ancestral type of chemical communications, with the notion that the first neural systems were non-synaptic (Jekely, 2021; Moroz, 2009, 2021; Moroz et al., 2021). We also propose that glutamatergic synapses were among the first in early animals, where Glu might act together with ATP, protons, and small peptides as stress/injury signals (Moroz, 2009). Indeed, extended glutamate signaling was shown for ctenophores (Moroz et al., 2014), which might have evolved synapses independently from the rest of the animals (Moroz and Kohn, 2016).

Most of the major components of synapses (the secretory/exocytosis modules and receptive machinery) are highly conserved among eukaryotes and present in choanoflagellates – the sister lineage to all metazoans (King et al., 2008). Furthermore, it appears that only one gene is required to make a glutamatergic cell. The ectopic expression of a vesicular Glu transporter (vGluT) can convert a non-neuronal cell into a glutamatergic cell, fully capable of the vesicular release of glutamate (Takamori et al., 2000).

The SCL17 family of vesicular anionic transporters includes Glu, phosphate, nucleotide transporters, and sialins (Fig. 13 and (Reimer, 2013)). Sialins are a sister protein subfamily capable of Glu and aspartate transport (Miyaji et al., 2008). The recruitment of a single sialin/vGluT transporter in the eukaryotic machinery might be a one-step way to evolve glutamatergic cells in the animal lineage. Such events might occur more than once, and glutamatergic neurons might also evolve independently. Early in evolution, glutamatergic cells (not necessarily neurons) might possess capabilities of Ca-dependent (i.e., highly regulated) Glu secretion. This hypothesis is supported by the widespread distribution of elaborated Glu signaling in all studied early-branching metazoan lineages.

### 8.3. Glu signaling in nerveless animals

The common ancestor of all animals was nerveless. Sponges and

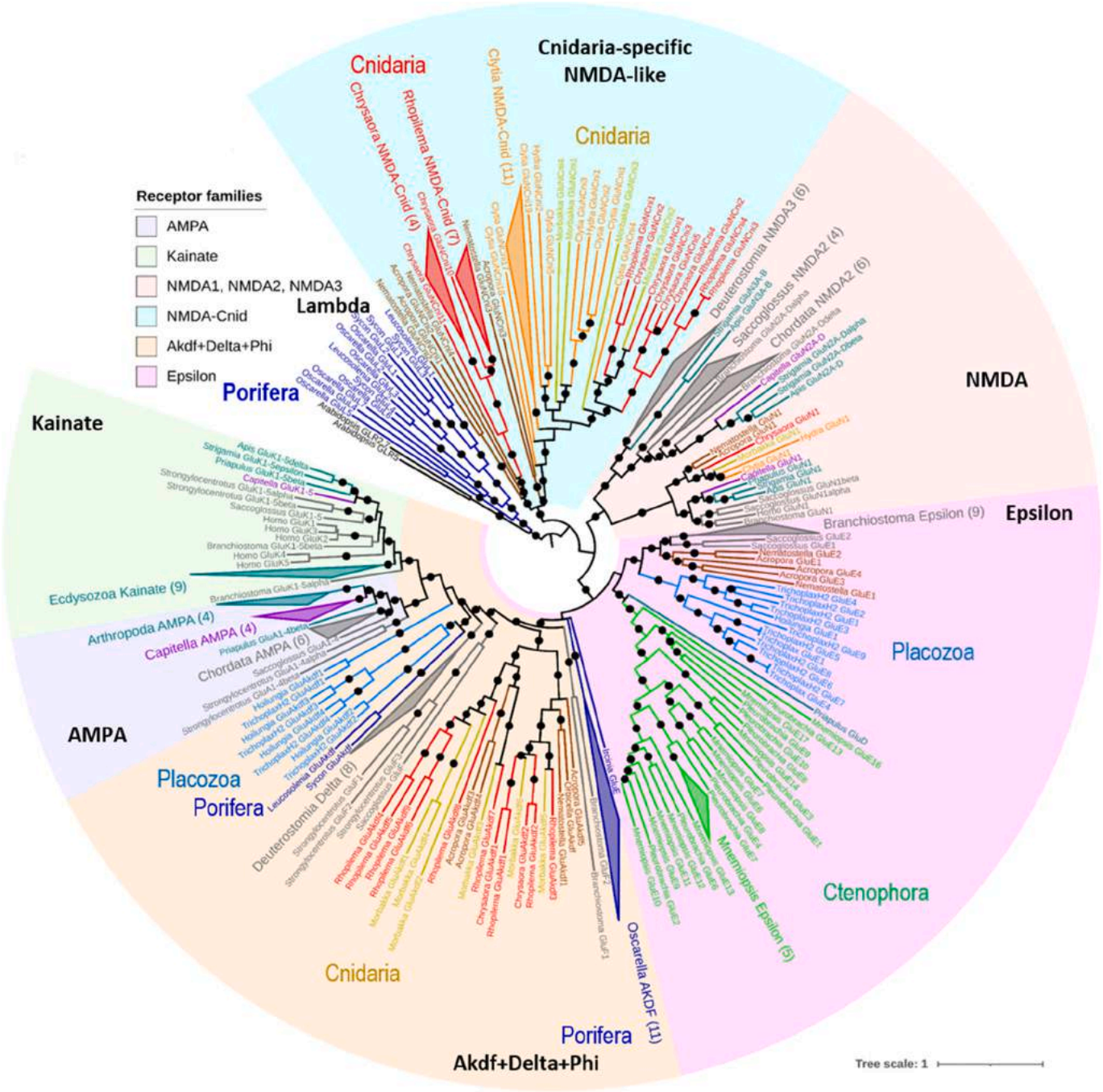


**Fig. 11.** Basal metazoan clades are highly divergent in their iGluR family composition. Ctenophora, Porifera, Placozoa, and Cnidaria are metazoans that diverged before bilaterians. This analysis includes four medusas, two ctenophores, and three placozoans. Phylogenetic tree of iGluRs across Metazoa consists of 25 species, 10 phyla, 258 sequences (Maximum Likelihood). Black dots indicate support values above 70 %. Lineage-specific duplications are shown as colored triangles. Supplemental Fig. 2 and Tables S1 and S2 show a whole tree with uncollapsed nodes and numerical bootstrap support values. The classes of iGluRs such as Lambda, NMDA, Epsilon, Akdf, and Delta correspond to the classification proposed elsewhere (Ramos-Vicente et al., 2018). Ctenophores have only the Epsilon family, placozoans – Epsilon and AKDF, anthozoans – Epsilon, AKDF and NMDA-Cnid, other cnidarians – AKDF, and highly expanded cnidarian-specific NMDA-Cnid. Demosponge *Amphimedon* has no iGluR at all, while calcareous and homoscleromorph sponges have Lambda and AKDF family iGluRs. See also Supplementary Fig. S2.

placozoans are two animal groups with the primary absence of neural and muscular systems. In other words, their ecology and relatively complex behaviors are coordinated by alternative integrative systems, including Glu signaling (Moroz et al., 2021).

In desmosponges, Glu induced rhythmic contractions and coordinated flow of water and nutrients during feeding (Elliott and Leys, 2010; Ellwanger et al., 2007; Ellwanger and Nickel, 2006). Metabotropic receptors might mediate this type of response because these species (and the model species *Amphimedon*) lost iGluRs from the common animal ancestor (Fig. 11). One known exception is the marine desmosponge *Ircinia*, where we found one iGluR (Fig. 11). We also do not know the sources of endogenous Glu release in sponges, if any. There is also a possibility that sponges might sense exogenous Glu or other metabolites derived from food sources (e.g., bacteria), endosymbionts, or the environment.

In contrast, other sponge classes possess a diversity of iGluRs (Fig. 11, Supplementary Tables 1 and 2). There is an expansion of iGluRs in calcareous sponges (e.g., *Sycon* and *Leucosolenia,* 5 and 3 iGluRs, respectively) and Homoscleromorpha (*Oscarella*, 18 iGluRs). Most sponge iGluRs belong to the sponge-specific Lambda family, and a broad Epsilon + Akdf + Delta + Phi supercluster, shared with most metazoans (Figs. 11 and 12). The analysis of motif organization suggests that sponge iGluRs might be gated by glycine or other amino acids, in addition to Glu. Future studies, especially on calcareous sponges and Homoscleromorpha, will be critical in deciphering the origin and evolution of Glu signaling in animals.

Placozoa is the second group of nerveless basal metazoans. These are the simplest known free-living animals with a relatively small number of cell types (Eitel et al., 2018; Mayorova et al., 2019; Romanova, 2019; Romanova et al., 2021; Smith and Mayorova, 2019; Smith et al., 2014, 2021) but with complex and well-coordinated behaviors (Armon et al., 2018; Smith et al., 2015, 2019; Varoqueaux et al., 2018; Zuccolotto-Arellano and Cuervo-Gonzalez, 2020), including social behaviors (Fortunato and Aktipis, 2019) and diverse systems of voltage-gated ion channels (Romanova et al., 2020b; Senatore et al., 2016, 2017) and receptors (Moroz et al., 2020a, 2021). We identified at least 13 iGluRs in *Trichoplax* sp. (H2 haplotype), which belong to two classes (9 Epsilon and 4 Akdf) according to the recent classification (Ramos-Vicente et al., 2018, 2021). The other placozoan genus *Hoilungia* has 4 AKDF and only one Epsilon-type receptor (Fig. 11), suggesting a loss of ancestral diversity of iGluRs in *Hoilungia*. Both Glu and Gly can be ligands for the different classes of placozoan iGluRs (Moroz et al., 2020b; Romanova et al., 2020a).

Glu transmitter functions are unknown in placozoans, but Gly has a distinct physiological action controlling locomotion and contractivity and can be a chemoattractant (Romanova et al., 2020a). Surprisingly, we also found genes encoding putative vGluTs in placozoans (Fig. 13). vGluTs might be a critical placozoan innovation suggesting the presence of 'true' glutamatergic cells with more localized and regulated Ca-dependent Glu secretion. Canonical vGluTs are absent in sponges and ctenophores, where sialins or kin might perform these functions. However, the statistical support of the branches separating sialins and vGluTs is not high. Future experimental validations of predicted vesicular glutamate transporters and sialins from these early-branching metazoans would be necessary.

### 8.4. Glu signaling in ctenophores

Ctenophores are viewed as the earliest branching animal lineage with possible independent origins of neurons, synapses, muscles, and mesoderm (Moroz et al., 2014; Moroz and Kohn, 2015, 2016). Multiple origins of neurons from different secretory cells is a plausible scenario (Moroz, 2021), but Glu release (vesicular or non-vesicular via transporters) from virtually any cell type is one of the most versatile intercellular signaling pathways regardless of neuronal or synaptic genealogy (Moroz and Romanova, 2021). Glu might act as the transmitter for muscles (Moroz, 2015b; Moroz and Kohn, 2016). The muscle cells in *Pleurobrachia* and *Mnemiopsis* are highly sensitive to both L- and D-glutamate. Both enantiomers induced action potentials in muscles, increased intracellular $Ca^{2+}$, and contractions (Moroz et al., 2014). L-Glu was found to be a more potent agonist than D-glutamate or L-/D-aspartate. Interestingly, D-glutamate has also been detected as an endogenous metabolite (Moroz et al., 2014, 2020b), and it might act as a signaling molecule in ctenophores.

Two investigated ctenophore species have *only* Epsilon-type iGluRs (Figs. 11 and 12). Numerous iGluRs duplication events occurred after the divergence of *Pleurobrachia* and *Mnemiopsis,* implying extensive parallel evolution of Glu signaling.

Unfortunately, we know nothing about the neural circuits in ctenophores and sources of Glu release. Endogenous Glu might be released from neurons, muscles themselves, and other cell populations. Ctenophores have a remarkable lineage-specific expansion of SLC17 transporters (Fig. 13), which correlate with a dramatic increase of their cell and tissue type complexity as well as behaviors.

In summary, the diversity of GluRs in ctenophores is incredible. Many iGluRs have differential expression in sensory structures such as

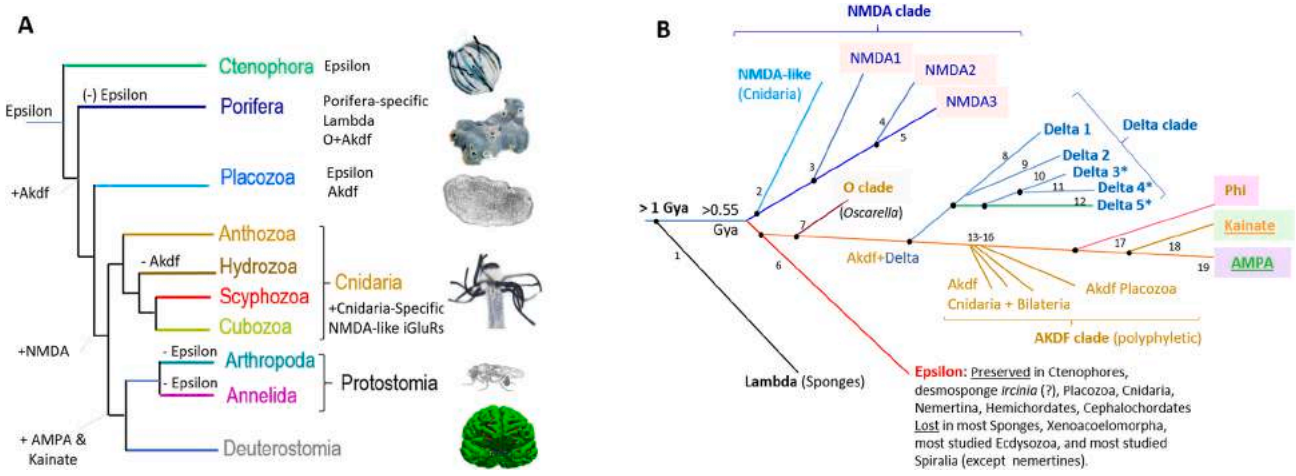


**Fig. 12.** Parallel evolution of iGluRs animals with emphasis on basal metazoans. A. Schematic phylogenetic relationships among representative animal clades with gain and loss of different classes of iGluRs. The iGluR classes were named according to (Ramos-Vicente et al., 2018), and reconstruction of gene gain/loss was derived from the phylogenetic trees (Figs. 9, 11, 15 and 16, Supplementary Figs. S2–S5). **B.** The proposed genealogy and diversification of classes and subclasses of iGluRs in Metazoa. The overall phylogenetic classification of iGluRs will continue to be developed with growing comparative information. See text for detail.

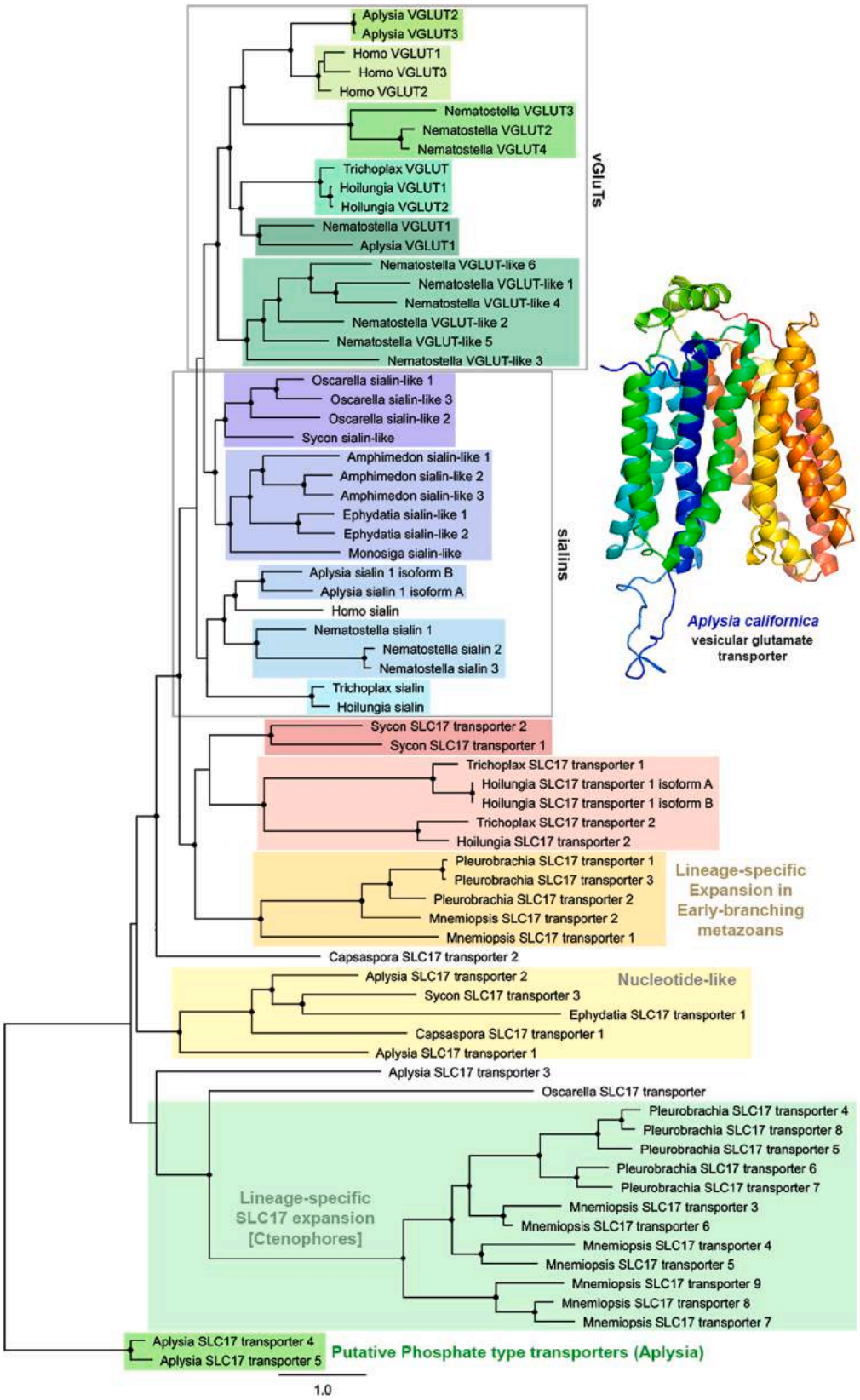


**Fig. 13.** Phylogenetic relationships among different classes of anionic vesicular transporters: Vesicular glutamate transporters (VGluT) **vs. Sialins**. The figure shows Maximum Likelihood phylogenetic tree of SLC17 family transporters (vGluTs, sialins, phosphate, and nucleoside transporters) across Metazoa (13 species, 8 phyla, 71 sequences, Supplemental Table 2). VGluTs were found in Placozoa, Cnidaria, and Bilateria but not in sponges or ctenophores. Two clades contain well-characterized vGluTs and sialins from *Homo* and *Aplysia*. Several proteins from *Nematostella* and placozoans occupy basal positions in these clades and are putative orthologs of VGluTs and sialins in basal metazoans. Ctenophore and sponge SLC17 proteins form their lineage-specific clades without orthology to bilaterian SLC17 family transporters. *Nematostella* and placozoans also have some unique SLC17 transporters without orthologs in Bilateria. Sialin-like protein from the unicellular choanoflagellate *Monosiga* is located between metazoan sialin and vGluT clades, suggesting earlier divergence of these transporter clades.

polar fields and aboral organs (Moroz et al., 2014). There are 10–15 iGluRs per species, and all of them belong to the epsilon class (known to be lost in vertebrates). Crystallography and expression of some of these receptors have been performed, suggesting that glycine (instead of Glu) can be the ligand for some of them (Alberstein et al., 2015; Yu et al., 2016). The systemic functions of Gly signaling in ctenophores are unknown.

### 8.5. Glu signaling in cnidaria

The elaborated diversity of iGluRs has been revealed in Cnidaria – the sister lineage of bilaterians and the principal reference taxon in all studies regarding the origins of neuronal organization in Bilateria. In cnidarians, we can recognize all metazoan classes of iGluRs (except the sponge-specific lambda) with significant variability across species (Fig. 11).

Cnidaria is the first animal lineage with both 'true' NMDA and cnidarian-specific NMDA-like receptors (Ramos-Vicente et al., 2018). These cnidarian-specific NMDA family present in Anthozoa and splits into two branches. One branch is Scyphozoa-specific, and members of the second branch occur in all three lineages of Medusozoa (Hydrozoa, Cubozoa, and Scyphozoa). NMDARs are duplicated in the hydrozoan *Clytia* (20) and investigated scyphomedusae (11 each). From the position of the branches, *Hydra* appears to have lost most of the NMDA-Cnid receptors that it shared with *Clytia*. Highly poisonous cubomedusae have fewer receptors (4 AKDF and 4 NMDA-Cnid) than Scyphozoa and Hydrozoa. Finally, the epsilon-type receptors are only found in Anthozoa and lost in other classes (Figs. 11 and 12). The scyphomedusa *Rhopilema* has highly duplicated AKDF receptors (9, Figs. 11 and 12).

The observed diversification of iGluRs and multiple vGluT-type genes (Fig. 13) imply both existences of different classes of glutamatergic cells and a remarkable variety of Glu/Gly signaling in Cnidaria. Regrettably, there are very few, often controversial, studies about the functions of Glu in cnidarians. Most attempts to map glutamatergic cells suggest their non-neuronal localization (Anctil and Carette, 1994; Oren et al., 2014). The available literature data indicate that Glu is not a widespread *neuro*transmitter in studied cnidarians. Some early authors concluded: "it is difficult to suggest that glutamic acid may be involved as a neurotransmitter in the sea anemone" (Carlyle, 1974). In some way, this statement is similar to the original skepticism about the role of Glu as the excitatory neurotransmitter in the mammalian brain (see the history section).

The only reported neuro-specific immunolabeling of glutamate was described in the neuronal plexus of the sea anemone *Phymactis papilosa* (Delgado et al., 2010). Delgado et al. showed Glu immunogold labeling of dense-core vesicles associated with the nerve plexus in this species. However, the localization of Glu in synaptic vesicles remains to be demonstrated. Most of the glutamate immunoreactivity was detected in cells within the ectodermal layer (elongated zymogenic gland cells) but was absent from mesoglea and endoderm (Delgado et al., 2010). In a related sea anemone, *Metridium senile*, specific Glu-immunoreactivity was also found in *ectodermal* non-neuronal cells, ectodermal muscles, and nematocysts (Anctil and Carette, 1994), suggesting that Glu can act as an osmolyte precursor (see also below).

*In situ* hybridization with probes for vGluT again revealed the non-neuronal nature of glutamatergic cells in another anthozoan, *Nematostella vectensis*. In this species, putative glutamatergic cells were expressed in the endodermal cell layer surrounding the pharynx and testis and in the *endoderm* of the body wall surrounding the head (Oren et al., 2014). The authors conclude that glutamatergic cell markers (together with some other low molecular weight transmitters) are expressed in tissues, which are "not part of the nervous system" (Oren et al., 2014). There are anecdotal reports that Glu might be accumulated in sensory-type neurons associated with nematocysts and in interneurons of complex eyes of Cubozoa (Pierobon, 2012). But neither Glu nor Gly are fast neurotransmitters in motor networks of *Cyanea* (Scyphozoa), where taurine and β-alanine are likely transmitter candidates (Anderson and Trapido-Rosenthal, 2009). More careful and more comparative mapping studies of neuronal vs. non-neuronal glutamatergic cells in Cnidaria are needed.

Glu is a prominent signal molecule with systemic integrative functions in cnidarians, regardless of its endogenous or exogenous sources. Glu controls chemoreception, nematocyst discharges, endogenous ectodermal and endodermal pacemaker activities in body column and tentacles, and muscle contractions (Goldner et al., 1969; Kass-Simon et al., 2003; Kass-Simon and Pierobon, 2007; Kay and Kass-Simon, 2009; Pierobon, 2012; Pierobon et al., 2004). As a coordinator of the major effector subsystems in *Hydra,* the systemic functions of Glu include modulation of three endogenous pacemaker systems and possible other electrogenic ensembles with close interactions between neural and, primarily, non-neuronal conductive systems (Mackie, 1990). It appears that most of the reported Glu cellular targets contribute to the feeding and defense behaviors.

Glu suppressed the stereotypic glutathione-induced feeding response in *Hydra* (Lenhoff and Bovaird, 1961). Similarly, Glu reversible inhibited the contractions of isolated circular muscles, promoting mouth opening in the sea anemone. Glu can be released by electrical stimulation (Carlyle, 1974) and act on various Glu receptors. Carlyle proposed that nematocysts and mucus-secreting cells may be potential sources of Glu release (Carlyle, 1974).

Single-cell RNA-seq data on cnidarians also confirm that most putative glutamatergic cells are not neurons. For example, by reanalyzing scRNA-seq data from *Hydra* ((Siebert et al., 2019) and Supplement 1), we find the predominant localization of vGluT in nematocytes and their precursors - nematoblasts (Fig. 14). Nematocytes are the populations of 'sting' cells, which release toxins in potential prey. They are also referred to as cnidocytes, which give the name of the phylum Cnidaria. Nematocytes can share their early development with neurons (see Fig. 14) and might be considered as highly specialized sensory-effector cells (Bosch et al., 2017).

Nematocytes contain extraordinarily high concentrations of poly-gamma-glutamate. Furthermore, the calculated intracapsular concentration of Glu monomer itself can reach 2 M (Weber, 1990). It might be possible that the observed high expression of vGluT (Fig. 14) is associated with poly-glutamate synthesis and Glu accumulation. Poly-gamma-glutamate is a unique polymer (Nair et al., 2021), initially discovered in bacteria as a part of their defense and immunity adaptations (Candela and Fouet, 2006). Interestingly, the poly-glutamate synthase gene is directly acquired by cnidarians from bacteria using horizontal gene transfer mechanisms (Denker et al., 2008). Exogenous Glu is also reported to increase stenotele discharge in *Hydra* (Scappaticci et al., 2010; Scappaticci and Kass-Simon, 2008), and positive feedback from Glu released during cnidocyte discharge might also contribute to coordinated feeding/defensive behaviors.

In summary, there is a schism between the enormous lineage-specific diversification of iGluRs, identified in genomic datasets, and fragmental functional and localized studies of Glu signaling in Cnidaria. With reported varieties of Glu-induced responses and behaviors, no convincing data confirmed the neuron-specific release of Glu and its role as an actual neurotransmitter in Cnidaria. At this moment, non-neuronal Glu release might be viewed as the dominant paracrine Glu signaling. In other words, there are no identified glutamatergic neurons yet, although lack of evidence is not the evidence of the absence. We think that this question must be reinvestigated. Cnidarian neurons possess a diversity of iGluRs and metabotropic Glu receptors, but their ligand-specificities (e.g., Glu, Gly, β-alanine, etc.) have to be determined in future studies. The established roles of Glu in osmotic/stress regulation (e.g., nematocyst discharges) and feeding behaviors could be traced to ancestral functions and metabolic usage of this molecule. The predominant non-neuronal Glu functions in four (out of 5) basal metazoan lineages might be considered a prelude to the elaborate expansion of glutamatergic signaling in bilaterians and its more profound recruitment into

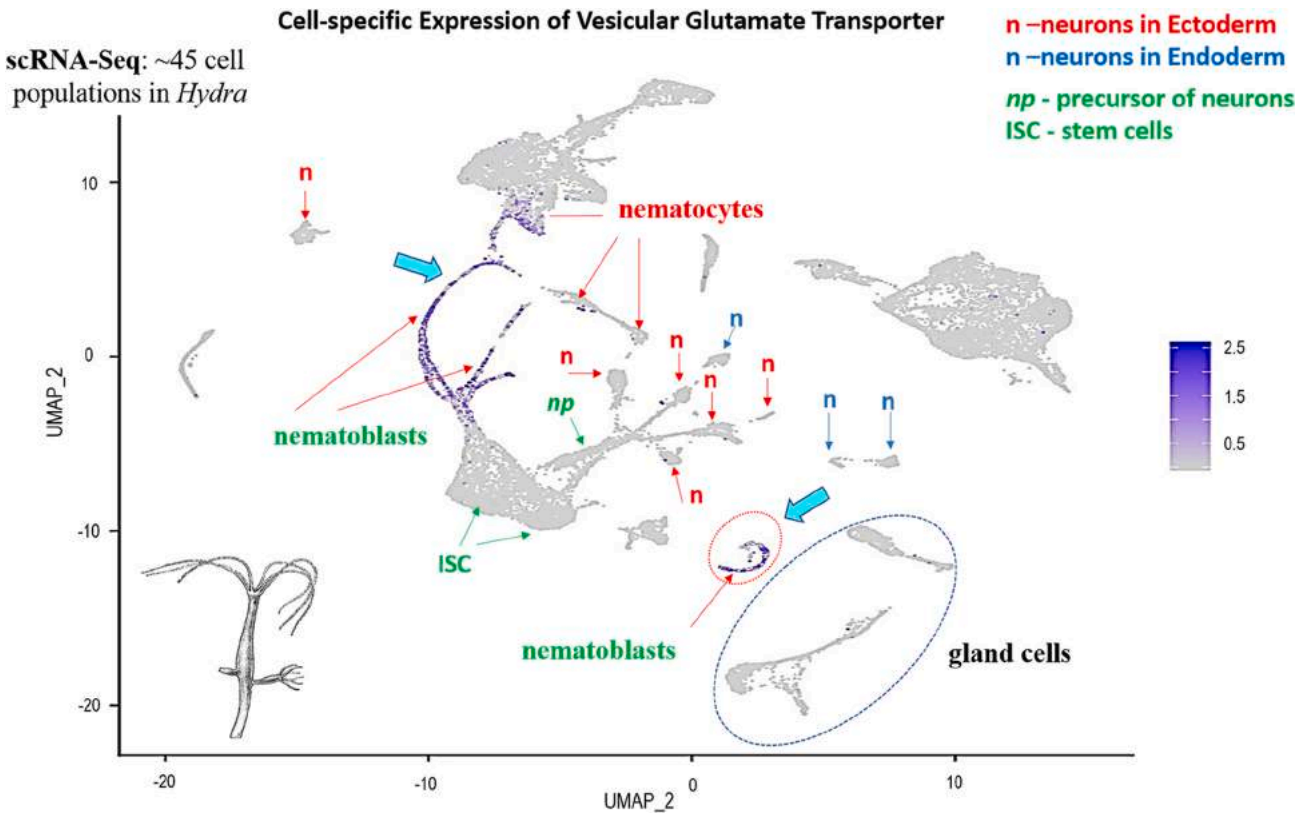


**Fig. 14.** Single-cell deciphering of glutamate signaling in *Hydra*: The majority of glutamatergic cells are non-neuronal (dark blue dots). We used VGluT as a marker of glutamatergic cells. We reanalyzed the original whole body scRNA-seq data from Siebert et al. (Supplementary Methods, Supplement 1), using the reported markers for different cell types (Siebert et al., 2019). There are about 45 cell clusters. In the freshwater polyp, *Hydra* neurons were present both in endodermal (blue 'n' and arrows) and endodermal (red 'n' and arrows) layers. Stem cells (ISC) and precursors of neurons (np) and nematoblasts are marked green. Note that the vesicular glutamate transporter was expressed in developmental nematoblasts, and only a few individual cells were clustered with ectodermal neurons. Nematoblasts accumulate poly-gamma-glutamate, and the observed high differential expression of VGluT likely contributes to Glu uptake required to synthesize this polymer. Nematoblasts and precursors of neurons might derive from the same population of ISC. See text for details.

neural functions.

### 8.6. Glu signaling in bilateria

Apart from vertebrates, Glu signaling is studied in relatively sufficient detail in representatives of just three phyla: *C. elegans* (Nematoda), *Drosophila* (Arthropoda), and *Aplysia* (Mollusca). Each of these neuroscience models highlights different aspects of Glu functions in nervous systems: neuronal identity and circuit organization (nematodes), neuromuscular transmissions (arthropods), learning and memory (gastropod and cephalopod molluscs). For more than 25 phyla, we have no data about Glu functions or even mapping studies. Many gaps in comparative analysis prevent generalizations about glutamatergic systems in bilaterians. Here, we will emphasize five topics and questions relevant to the evolution of neurotransmitter systems.

First, Glu is recruited as a true *neuro*transmitter across all studied bilaterians. However, invertebrate central nervous systems contain ~10 % (or less) glutamatergic neurons (the rest of neurons use other transmitters). It contrasts with the 'overrepresentation' of glutamatergic neurons in the vertebrate-type CNS, especially in the cortex. Such unusual evolutionary selection should be investigated in detail. Glu chemical reactivity, stability, polarity, synthesis, omnipresence, inherent links to bioenergetics, and nitrogen metabolism are all essential parameters contributing to the recruitment of this amino acid into neural circuits. The ancestry of Glu signaling and systemic functions must also be considered to explain both the selection and preservation of Glu as the major excitatory transmitter in our brain (see below).

Second, there is an apparent dichotomy across different animal lineages regarding the position of glutamatergic cells within neural architecture. In vertebrates, nematodes, and molluscs Glu is one of the sensory and interneuronal transmitters. It is the primary low molecular weight transmitter in nociceptive/mechanosensory neurons in *C. elegans* (Maricq et al., 1995; Schafer, 2015) and *Aplysia* (Antzoulatos and Byrne, 2004; Dale and Kandel, 1993; Drake et al., 2005; Jing et al., 2015; Walters et al., 2004). Only small fractions of motor neurons in molluscs are glutamatergic (Di Cosmo et al., 1999, 2006; Fox and Lloyd, 1999). Glu only modulates cholinergic neuromuscular transmission in vertebrates (Colombo and Francolini, 2019). In contrast, Glu is the neuromuscular transmitter in arthropods, including *Drosophila*; and flies use acetylcholine as the mechanosensory transmitter. Could arthropods independently switch to the use of Glu in their neuromuscular transmission? Why? The answers would depend on studies of basal ecdysozoans such as priapulids, and basal bilaterians such as Xenoacoelomorpha, echinoderms, hemichordates, and cephalochordates. Bioenergetic constraints (which might favor glutamate usage during high energy demands) and the reconstruction of the muscular control in the common ancestors of all bilaterians might provide keys.

Third, lineage-specific expansions are also a prominent feature of the evolution of iGluRs within virtually every animal phylum or superclade (Fig. 15). These trends are especially noticeable as independent duplications of iGluRs and Cys-loop receptors in flies and crustaceans (Polinski et al., 2021), which can be explained by the extraordinary development of the chemosensation in these groups. One extra conspicuous innovation in lobsters and shrimps is the developing chimeric ligand-gated receptors with pattern-recognition motifs (Fig. 15). These

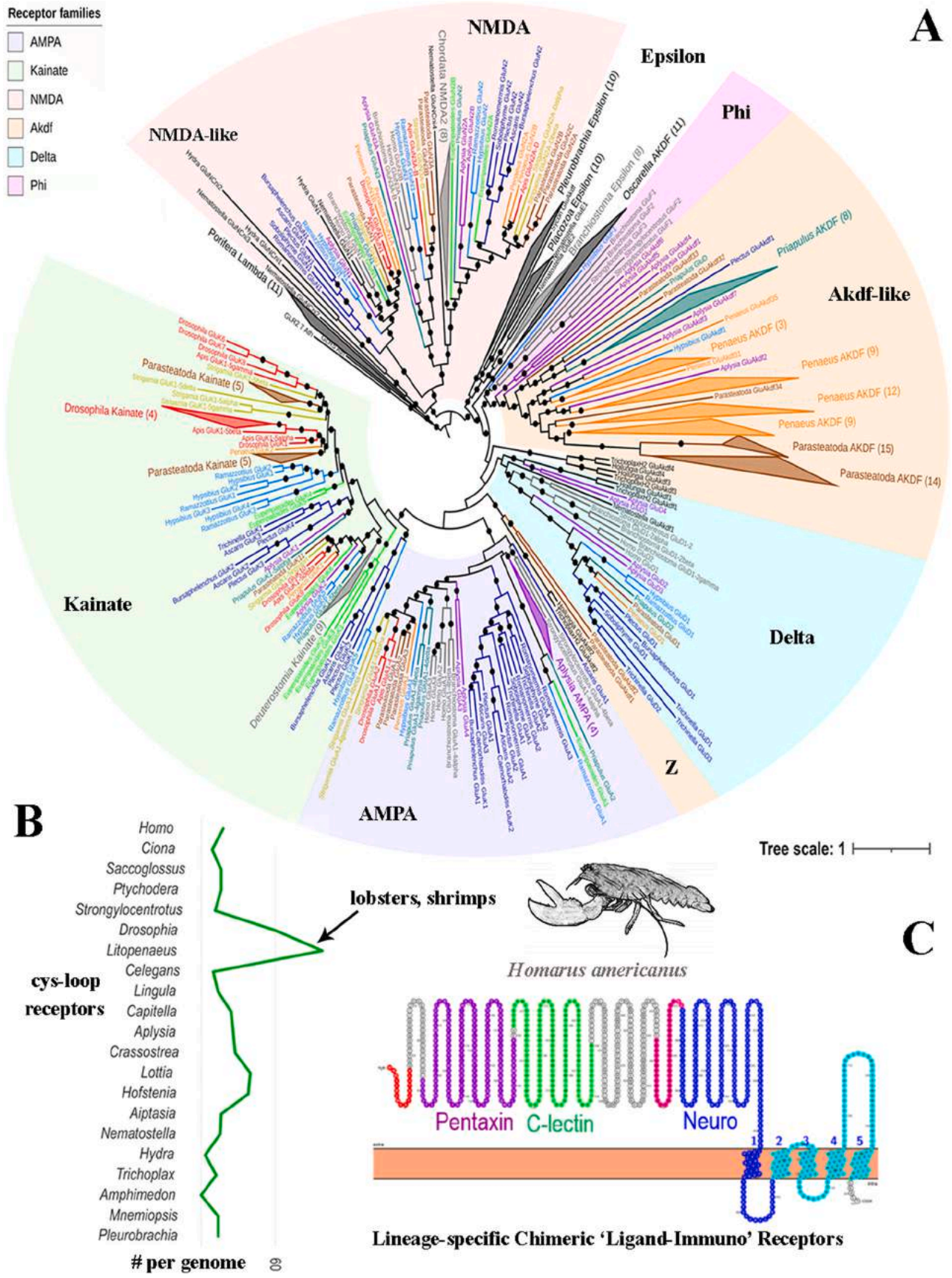


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**Fig. 15.** Parallel evolution of glutamate receptors with an emphasis on Ecdysozoa: Specification and hybrid immune-neuro receptors. **A**. Maximum Likelihood phylogenetic tree of iGluRs (28 species, 11 phyla, 361 sequences, see Supplementary Tables S1 and S2) for sequences). Black dots show support values above 70 %. A whole tree with uncollapsed nodes and numerical bootstrap support values is in Supplemental Fig. 3. Various ecdysozoan lineages show highly divergent duplications of iGluRs. The classes of iGluRs such as Lambda, NMDA, Epsilon, Akdf, and Delta correspond to the classification proposed elsewhere (Ramos-Vicente et al., 2018). Spiders and shrimps have an extremely high diversity of iGluRs due to extensive duplications of AKDF subfamily. On the other hand, nematodes have relatively smaller numbers of iGluRs per their genomes. See text for details. B. The family of pentameric cys-loop Glu receptors also show remarkable lineage-specific diversification in Arthropods, including a novel class of chimeric "ligand-pattern recognition" receptors recently identified in the American lobster (Polinski et al., 2021) and the shrimp *Litopenaeus*. See also Supplementary Fig. S3.

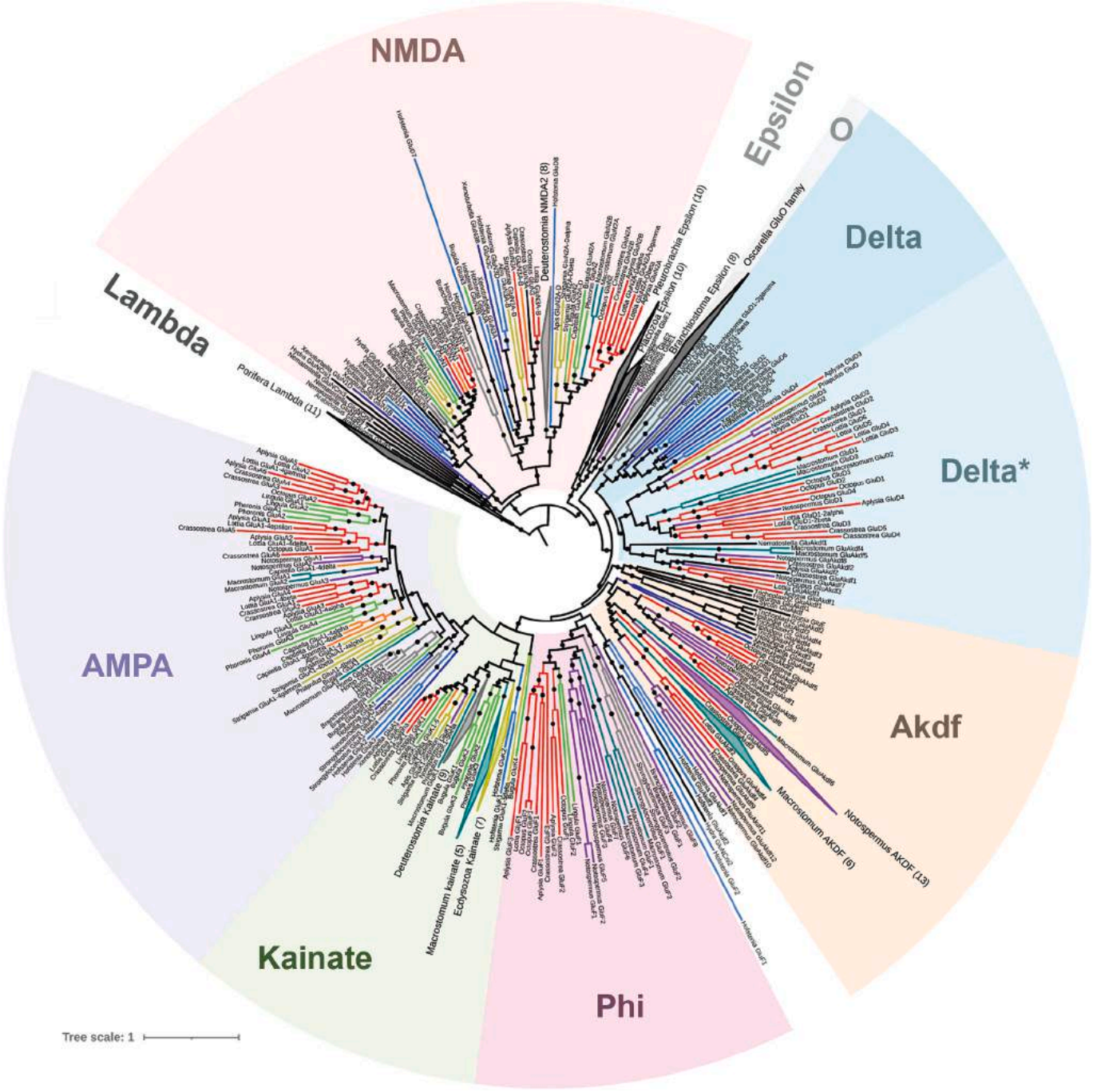


**Fig. 16.** Parallel Evolution of iGluRs in animals with emphasis on Spiralia. Maximum Likelihood phylogenetic tree of iGluRs (27 species, 18 phyla, 382 sequences, see Supplementary Tables S1 and S2). This tree includes seven spiralian phyla and Xenoacoelomorpha (the acoel, *Hofstenia,* and *Xenoturbella*). The diversity of iGluRs does not correlate with the presence of complex nervous systems. One of the largest complements of iGluR genes were found in the nemertine *Notospermus* (44), the oyster *Crassostrea* (29), the flatworm *Macrostomum* (28), and the acoel *Hofstenia* (25). Among molluscs, *Octopus,* with the most complex nervous system in invertebrates, has the fewest number iGluRs (19). Nemertine *Notospermus* is the only member of Spiralia, which retained Epsilon subfamily iGluRs and completely lost the NMDA subfamily. Black dots show support values above 70 %. (see Supplemental Fig. 4 for the tree with uncollapsed nodes and numerical bootstrap support values). See also Supplementary Fig. S4.

receptor types might provide potential mechanical coupling between chemosensory and immune systems (Polinski et al., 2021).

Fourth is the greater molecular secretory complexity of glutamatergic synapses and the assortment of Glu receptors of all types in invertebrates compared to vertebrates. In most studied invertebrates, Glu often acts as an inhibitory transmitter via Cys-loop receptors (Kehoe et al., 2009). Glu-dependent inhibitory signaling pathways were lost in the vertebrate lineage.

The superior molecular diversification of Glu-gated receptors suggests that the pharmacology of glutamatergic synapses in invertebrates is different from the well-studied subsets of agonists and antagonists for Kainate, AMPA, and NMDA receptors (Ha et al., 2006; Moroz et al., 1993). Some invertebrate glutamatergic synapses can be more complex in terms of co-released secretory/neuropeptide molecules than synapses in our cortex. For example, in the *Aplysia* sensory-motor pair (VC-L7), often used as the paradigm for neuroplasticity studies (Dale and Kandel, 1993; Kandel, 2001), we detected 14 different iGluRs and about a dozen predicted neuropeptide co-transmitters (Moroz et al., 2006), including those transported to synapses (Puthanveettil et al., 2013); making it one of the most complex transmissions among studied Glu synapses. Unfortunately, functional roles for most co-released peptides at this synapse are unknown).

Finally, emerging single-cell RNA-seq data suggest that glutamatergic neurons could be more diverse genetically, developmentally,

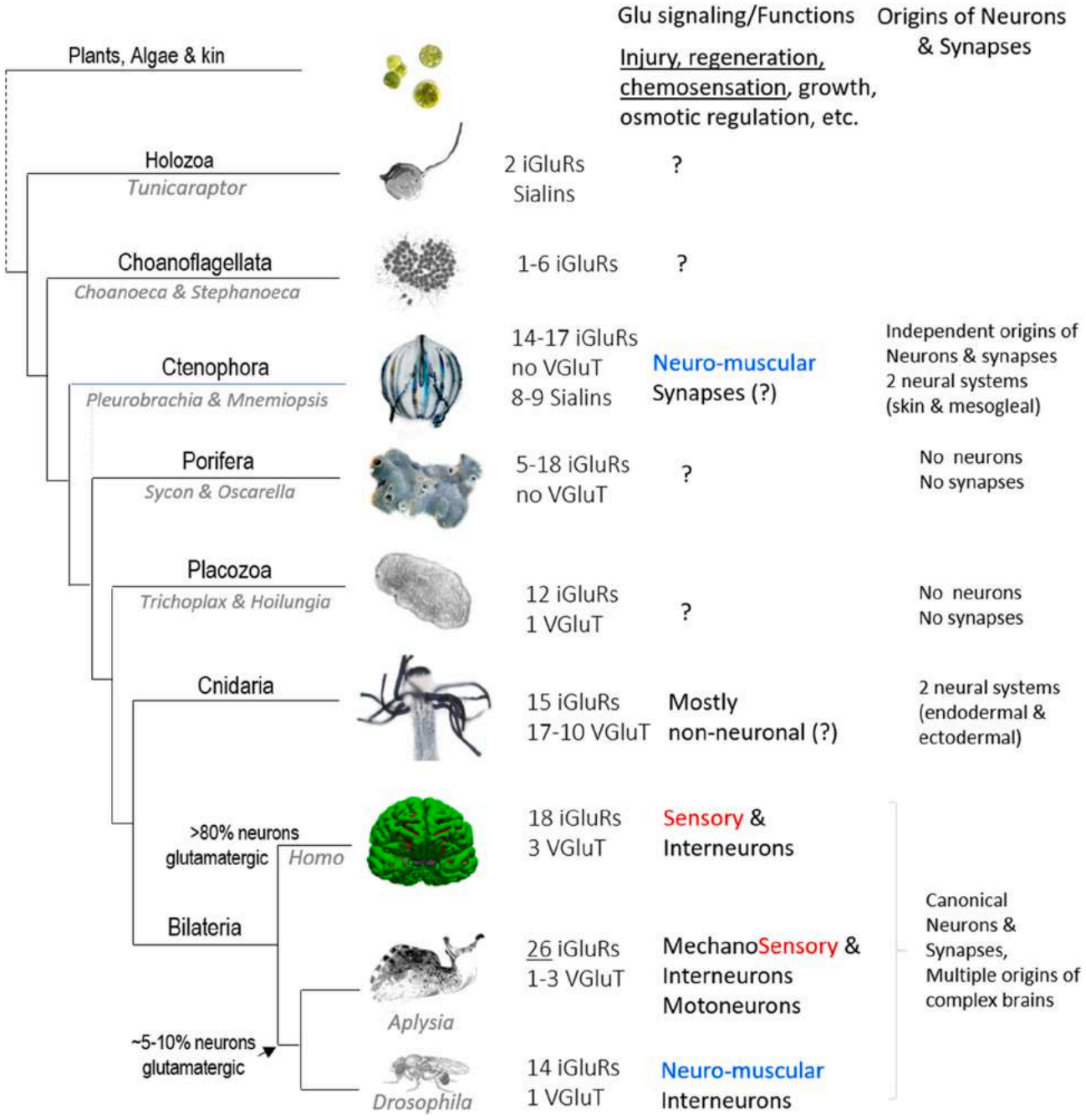


**Fig. 17.** Diversity of glutamatergic systems across Metazoa with three eukaryotic outgroups. The diagram shows the predicted number of vesicular Glu transporters and iGluRs in the sequenced genomes of representative species. Two left columns briefly summarized the functions and position of glutamatergic systems in neural circuits (where such data are available). Of note, every lineage shows multiple duplication events with a potential sub-functional specification of iGluR regardless of apparent neuronal morphological complexity.

and genealogically than GABAergic neurons across brain structures and species (Armand et al., 2021; Bakken et al., 2020; Lake et al., 2016; Luo et al., 2017; Mayer et al., 2018; Tasic et al., 2018; Yao et al., 2021; Zeisel et al., 2015).

### 8.7. Emerging diversity of iGluRs in bilateria

Considering the lack of available data for most bilaterian lineages, it might be relevant in the context of this review to comment on the emerging diversity of iGluRs. Ramos-Vicente and colleagues provided the first large-scale systematic analysis of iGluRs in metazoans (Ramos-Vicente et al., 2018). Figs. 16 and 17 (and Supplementary Figs. 3–5) expand the representation of species and further illuminate the complexity and phylogeny of iGluRs in bilaterians.

There are multiple clusters of nearly every class of iGluRs within the major bilaterian superclades. All bilaterians can be grouped into three superclades: Deuterostomes (echinoderms, hemichordates, and chordates, but see (Kapli et al., 2021; Kapli and Telford, 2020; Philippe et al., 2011)), and two protostome superclades: Ecdysozoa (which unites animals with regularly molting chitin-based cuticles such as arthropods, nematodes, and kin) and Spiralia (with characteristic spiralian development such as molluscs, annelids and flatworms, and kin (Kocot et al., 2011; Laumer et al., 2019)).

First, we have analyzed the iGluR complement in the genomes of 15 species from 4 ecdysozoan phyla (7 nematodes, honey bee, fruit fly, spider, millipede, shrimp, 2 tardigrades, and an onychophoran). Next, we built the phylogenetic tree of their Glu receptors together with a selection of receptors from other animal phyla (Fig. 15; 361 sequences total, Supplement 1 and Supplementary Tables 1 and 2). Here, we would like to stress that the naming of these iGluR families (AMPA, Kainate, NMDA1, NMDA2, Phi, Delta, and AKDF according to Ramos-Vicente et al., 2018) does not always indicate that respective vertebrate agonists and antagonists of particular groups of iGluRs would have the same pharmacological actions (e.g., as shown for molluscan NMDA-type receptors (Ha et al., 2006; Moroz et al., 1993)).

Spiders and shrimps are notable for their large number of iGluRs (58 and 47, respectively). Their receptor abundance is due to independent lineage-specific duplications in the AKDF receptor superfamily (31 and 34 AKDF members, respectively). In spiders, there are also 10 kainate-type receptors. The duplicated clusters of AKDF receptors in shrimps and spiders have the longest branches in the tree, suggesting their fast evolution.

The genome of the nematode *C. elegans* has a relatively small number of iGluRs among the animal kingdom (two NMDA and eight non-NMDA subunits (Brockie et al., 2001)). We also analyzed iGluRs of other nematodes (5 in *Soboliphyme* and 10 in *Plectus*) and showed that these receptors represent the AMPA, Kainate, NMDA1, NMDA2 Phi, and AKDF families.

All Ecdysozoa have NMDA1. We have also confirmed NMDA2, NMDA3, and Phi family receptors in Ecdysozoa (Brockie et al., 2001; Greer et al., 2017; Ramos-Vicente et al., 2018). They are apparently absent from the sequenced centipede, but present in spiders, shrimps, and tardigrades. Only spiders and tardigrades have NMDA3 among Ecdysozoa.

Our trees (Figs. 15 and 16, and Supplementary Figs. 4 and 5) show that kainate receptors were duplicated independently in insect and spider lineages and to a small extent in tardigrades. Centipedes, shrimp, and nematode genomes have approximately the same amount of AMPA and Kainate class receptors.

Second, incorporating additional Spiralian and Acoela lineages (Fig. 16, Supplementary Fig. 4) further illuminates the lineage-specific diversification of metazoan iGluRs and their expanding classification. Kainate and AMPA are two sister classes of metazoan iGluRs. However, AKDF groups are highly heterogenous, potentially - polyphyletic (Fig. 16, Supplementary Fig. 5), suggesting that further comparative analyses would be required to decipher the genealogy of iGluRs in bilaterians and metazoans in general.

Surprisingly, predatory nemertean genomes have many receptor duplications in the AKDF (21) and Phi (5) subfamilies, but all NMDA receptors were lost. However, nemerteans are the only representatives of Spiralia that retain Epsilon receptors, while molluscs, annelids, and others lost them. Among molluscs, the oyster lineage shows the greatest number of lineage-specific duplications of AKDF receptors (Fig. 16). Its genome has 29 glutamate receptors in total - more than *Lottia* (23), *Aplysia* (23), and *Octopus* (19). *Octopus sinensis* almost coincides with *Aplysia* in the set of receptors, except for 5 AKDF duplications. Cephalopods, the primates of the sea, apparently have an extensive system of Glu signaling and iGluRs involved in transmission at the giant synapse, chromatophore muscles, statocysts, and in the CNS (De Santis and Messenger, 1989; Di Cosmo et al., 1999, 2004, 2006).

The free-living flatworm *Macrostomum* genome has duplications of AKDF (14), Kainate (5), and the presence of AMPA, Phi, NMDA1 and NMDA2 classes. Brainless bryozoans, brachiopods, and phoronids (all with a simpler neural organization) have a comparable but small number of iGluRs (14, 9, and 9) and the same set of classes (AMPA, Kainate, AKDF, NMDA1, NMDA2). Moreover, on the phylogenetic tree, iGluRs of these three species are often not grouped. Instead, bryozoan is grouped with nemertean or *Macrostomum*; and the brachiopod *Ligula* is clustered with *Aplysia* or *Capitella*.

Adding iGluRs from Xenoacoelomorpha, one of the most basal bilaterian lineages (including Xenoturbellida and Acoela (Bourlat et al., 2006; Cannon et al., 2016; Gee, 2016; Kapli et al., 2021; Kapli and Telford, 2020; Philippe et al., 2011; Rouse et al., 2016)), improved the phylogenetic clustering of NMDA-type receptors. There is also a distinct cluster of Delta-type receptors in *Xenoturbella* and *Hofstenia*, sister to canonical Delta receptors from chordates (Fig. 16). Despite their very simple bodyplans, both *Xenoturbella* and *Hofstenia* possess a remarkable molecular diversity of NMDA and other classes of iGluRs receptors, except for the apparent loss of Epsilon-type receptors.

In summary, the extended sampling revealed 15–19 distinct phyletic lineages (classes) of animal iGluRs (Figs. 12 and 16), which is significantly greater than described in vertebrates (with just four classes: NMDA, AMPA, Kainate and Delta). We anticipate that adding more animal groups and sister eukaryotic taxa will further expand and clarify the genealogy of iGluRs as well as expand the spectrum of Glu functions in nervous systems and beyond.

Of note, Gly and D-serine alone, or in addition to Glu, can also be natural agonists of iGluRs (Stroebel et al., 2021). The lack of exclusive Glu specificity of iGluRs is evident for many prokaryotic and eukaryotic lineages, and these pharmacological data were discussed above. The hypothesis that glycine might pre-date glutamate as an agonist of iGluRs in metazoans was also proposed (Stroebel and Paoletti, 2020) based on the abundance of predicted Gly-binding motifs in some sequenced iGluRs.

However, there are no data about the basal metazoan lineages such as sponges and ctenophores that Gly is the functional signal molecule in these species. It was suggested that Gly could regulate behaviors of the placozoan *Trichoplax* and be a chemoattractant (Romanova et al., 2020a). The Mayer group also showed that only some iGluRs in ctenophores are gated by Gly (Alberstein et al., 2015; Yu et al., 2016), and Gly-gated iGluRs were also confirmed to be present in the amphioxus (Ramos-Vicente et al., 2018). But in ctenophores, Glu included muscle contractions and elevation of intracellular $Ca^{2+}$ (Moroz et al., 2014). Combined with the enormous diversity of iGluRs encoded in the genomes of basal metazoans (most of them are not characterized pharmacologically), we think that there is not yet conclusive evidence that Gly was the first transmitter or neurotransmitter in metazoans. To be conservative, we proposed the following hypothesis.

Different types of iGluRs in the common ancestor of metazoan (Urmetazoan) could be activated by various ligands, including Gly and Glu, and other amino acids, as it was widely shown in plants and prokaryotes. In other words, ancestral/Urmetazoan iGluRs could be

universal amino acid sensors (the feature still preserved in metazoans). Nevertheless, relatively high concentrations of released and extracellular Glu (compared to other potential agonists) could make these receptors as functional glutamate receptors – similar to iGluRs described in plants. Gly can be both agonist, modulator, and even functional (inhibitory) antagonist here. These various scenarios and the scope of Gly, D-serine, alanine, etc., functions should be further tested experimentally/pharmacologically by expressing different iGluRs from key basal metazoans and their outgroups.

## 9. Perspectives and conclusions

Glu was recruited to arrays of cellular and systemic functions over 3.8 billion years of evolution. And most of these recruitment events were linked to the chemical properties of Glu and its inherent coupling to the cellular metabolic pathways and the TCA cycle. It appears that the many functions of Glu can be derived from the ancient role of Glu as a signal molecule for stress and injury-related adaptations.

Forty years after the famous Watkins and Evans paper (Watkins and Evans, 1981), the progress has been enormous, but deciphering the evolution of Glu signaling is still in its infancy. The natural (evolutionary and hierarchical) classifications of Glu receptors or glutamatergic neurons have not yet been established. Research efforts on basal metazoans, basal bilaterians, and basal chordates such as amphioxus and kin will be essential. We can learn a lot from the astonishing diversity of eukaryotic organisms and animals, viewing them as nature's gifts to neuroscientists.

Without diving into comparative details, we will readdress once more the long-standing question about the neurochemistry design of our brain. Why do glutamatergic neurons dominate cell populations (~80 % of neurons) in the mammalian cortex? Why does glutamate occupy such a central place as an excitatory transmitter in the vertebrate CNS?

Although it is an open-ended question, the answer might be based on bioenergetics constraints and the chemical selection of molecules available in the common ancestor for vertebrates 500+ million years ago.

Glu perfectly fits the place and became the 'winner' over its competitors (e.g., aspartate, glycine, monoamines, and acetylcholine). The 'favorable' features for the eventual glutamate selection, supporting the behavior-driven rise of neuronal complexity, include its universal availability, stability, simplicity of synthesis, release, and uptake, as well as strong inherent links to the TCA cycle and metabolic pathways. Plus, the Glu receptors' broad-spectrum and incredible diversity had been evolutionarily tuned in past eons and available for the natural selection processes at the end of Cambrian and during Ordovician, when vertebrates start to compete with arthropods and cephalopods in ancient seas. Combined, these biochemical features provided one of the most flexible signal molecule candidates for energy-demanding selection of Glu as an interneuronal messenger during the evolution of vertebrates and mammals.

The increase of any neuron connectivity and nervous system complexity are very costly processes (Bordone et al., 2019). The growth of distant neuronal processes, novel synaptic terminals, and wiring dramatically increases the cell surface ratio to cell volume. Together with spiking activity, and the maintenance of neuronal homeostasis, such expansion requires a lot of energy. With about 2 % of body weight, the brain consumes ~20% of energy in adult humans and ~50 % in children. It is estimated that approximately 47 % and 34 % of the total energy usage supports action potentials and postsynaptic effects of cortex neurotransmission in rodents, and perhaps 74–80 % in humans (Attwell and Laughlin, 2001). The maintenance of resting potentials consumes a smaller ATP amount (13 %). Every spike in the human cortex eventually consumes two billion ATP molecules (Lennie, 2003). As a result, the available energy (glucose consumption about 4.6 g per hour or 120 g per day) can only sustain simultaneous spiking (at rate 10 spikes per 200 ms) for about 3 % of cortical neurons at average or 'less than 10 % of cortex can be active at all' (Lennie, 2003).

In contrast, the energy expended on glutamate uptake and glutamine synthesis is only 2–3% of the signaling-related total cost in rodents, and 5 % in humans. But Glu can be the principal energy substrate for the brain, as shown by Krebs (1953) and further expanded in modern studies (Bordone et al., 2019; McKenna, 2013). Glu is a natural choice for its preferential selection in costly neuronal (cortex) computation and wiring. Glu, after its release, can be used as an energy resource (e.g., in astrocytes), helping to sustain more activated neurons or recruit more synapses/cells for multitasking in a complex brain.

Basic calculations provide interesting insights into the Glu potential as an energy source. Only one ATP molecule is required for the uptake of one molecule of glutamate. However, total ATP from the complete oxidation of one molecule of exogenous glutamate via the TCA cycle and pyruvate recycling pathway is 24–27. NET energy yield from uptake and oxidation of one glutamate molecule is 23–26 (McKenna, 2013). It is a perfect usage of the transmitter both for signaling and energy production. Of note, there are about 8000 Glu molecules per synaptic vesicle, and ~130 vesicles can be released from a single synaptic bouton within 10 min of low-frequency stimulation (Wang et al., 2019b). It is equal to the theoretical production of ~20 million ATP molecules per physiological activation of one synapse if all released Glu would be used for this purpose. One cortex neuron can make about 17500 synaptic contacts (Lennie, 2003), considering the average synaptic density of about $7 \times 10^8/mm^2$ in the human cortex. If only about 10 % of synapses released glutamate, the potential yield via this amount of Glu oxidation would be an order of 20 billion ATP molecules. Even considering that a small fraction of synaptically released Glu would be used as the energy source (by astrocytes and eventually neurons), such an event can compensate the cost of EPSP per single spike, which is estimated as one billion ATP molecules. Thus, ***the natural selection of Glu in the mammalian cortex over other transmitters can be an energetically favorable outcome***.

Even more interesting are the findings that the presence of Glu reduced the rate of glucose oxidation by 75 %, sparing it for other energetic needs. The glucose transporter (GLUT1) expression in the human brain is higher than in chimpanzee and macaque brains (Pfefferle et al., 2011), confirming the need to energetically support larger brains.

On the other hand, the initial widespread recruitment of Glu, and its potential toxicity as an excitatory transmitter, also facilitates compartmentalization and more localized chemical transmission together with the modular organization of neuronal ensembles, as well as co-transmission, glial diversity, and metabolic coupling between neurons and glia.

Due to its position in cellular energetics (Fig. 2), GABA is also a perfect 'choice' to balance the potential 'overexcitation'/neurotoxicity induced by Glu. GABA is produced from Glu and can be a conserved evolutionary solution for Glu inactivation or reduction of its concentrations. At the same time, GABA can also fuel the TCA cycle, recovering Glu as a by-product. Metabolically, Glu-GABA coupling is a perfect pair for biologically and chemically differential signaling in neural circuits and other cells and tissues.

In conclusion, it might well be that the primordial recruitment of Glu at the dawn of prebiotic evolution paved the path to elementary cognition and actual human cognition 3.5 billion years after. We can finish this essay about the evolution of Glu signaling with the quote: "Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."– Winston Churchill (November 10, 1942).

## Data availability

The data that support the findings of this study are available in supplement and public databases.

## Author contributions

L.L.M., A.B.K. and D.R. designed the study; M.A.N. involved in phylogenetic analysis; A.B.K. and P.P. involved for scRNA; D.R. and A.B. K. analyzed protein sequences; D.R. performed protein modeling and metabolome visualization; D.R., M.A.N., A.B.K. and L.L.M analyzed the data; L.L.M and D.R. wrote the draft of the paper; and all authors reviewed, commented on, and edited the manuscript.

## Declaration of competing interest

The authors have no conflict of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2021.108740.

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