Chapter 2

The Evolution of Pentameric Ligand-Gated Ion Channels

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Abstract

Fast, ionotropic neurotransmission mediated by ligand-gated ion channels is essential for timely behavioral responses in multicellular organisms. Metazoa employ more ionotropic neurotransmitters in more types of synapses, inhibitory or excitatory, than is generally appreciated. It is becoming increasingly clear that the adaptability of a single neurotransmitter receptor superfamily, the pentameric ligand-gated ion channels (pLGICs), makes the diversity in ionotropic neurotransmission possible. Modification of a common pLGIC structure generates channels that are gated by ligands as different as protons, histamine or zinc and that pair common neurotransmitters with both cation and anion permeability. A phylogeny of the pLGIC gene family from representative metazoa suggests that pLGIC diversity is ancient and evolution of contemporary phyla was characterized by a surprising loss of pLGIC diversity. The pLGIC superfamily reveals aspects of early metazoan evolution, may help us identify novel neurotransmitters and can inform our exploration of structure/function relationships.

Introduction

With the cloning of the alpha subunit of the nicotinic acetylcholine receptor (nAChR), the nAChR became the exemplar of a functionally diverse superfamily of neurotransmitter receptors, the pentameric ligand-gated ion channels (pLGICs).[1] It also marked the beginning of the molecular analysis of neurotransmitter receptors and of our ability to understand channel structure, function and evolution in a new and enlightening dimension.[2,3] More than 25 years later, the pLGIC superfamily continues to expand with the discovery of new invertebrate and even bacterial pLGICs. Here I briefly summarize what is known about the underlying pLGIC structure and its many functional adaptations, I discuss some features of the pLGIC superfamily's evolution and finally I speculate on the opportunities that such a large and varied family of proteins presents for understanding metazoan evolution, neurochemistry and protein structure/function relationships.

Structure: pLGICs Share an Underlying Structure

One expects members of a protein superfamily to share structural motifs. The pLGIC superfamily combines a seemingly paradoxical lack of conservation at the level of sequence with a highly conserved underlying structure. The pLGIC channels are pentamers formed by five homologous subunits (Fig. 1). Channels can be homomeric or heteromeric with up to five different subunits

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required to form a functional channel.[4] The amino acid sequence and predicted topology of the pLGIC subunits has traditionally defined their inclusion in a superfamily.[5-7] Even though the more divergent subunits show as little as 10-15% amino acid identity across a ~400-500 amino acid peptide, in multiple sequence alignments several residues are identical across almost the entire protein superfamily and scores of other residues show a high degree of conservation.[7,8] These conserved residues are scattered about the polypeptide, which facilitates sequence alignments and suggests that a characteristic structure underlies all pLGICs. That said, our criteria for what defines a pLGIC continue to be challenged as new pLGICs are characterized. For instance, a pair of what had appeared to be absolutely conserved cysteines that form a disulfide bond in the extracellular domain of pLGICs—and the reason metazoan pLGICs are referred to as "cys-loop" channels—turn out not to be present in bacterial pLGICs nor in at least one nematode pLGIC[7] (J. Dent. unpublished).

Conservation is not uniform throughout the subunit sequence. Based on topology and hydrophobicity, subunits can be divided into domains: a signal sequence, an amino-terminal extracellular ligand-binding domain, four transmembrane domains that form the transmembrane pore and, between the third and fourth transmembrane domains, a large cytoplasmic loop (Fig. 1A). The transmembrane domains are the most conserved, followed by the extracellular ligand-binding domain. The intracellular loops, even among closely related subunit types, typically show very little conservation.

In the past two decades we have been treated to increasingly detailed images that reveal the underlying pLGIC structure implied by the sequence conservation. These consist primarily of the electron diffraction images of quasicrystalline nAChRs from the electric ray *Torpedo californica*,[9-13] X-ray crystallographic data from the homopentameric acetylcholine binding protein (AChBP)—essentially a soluble pentameric extracellular domain without the transmembrane domains—from the snail *Lymnea stagnalis*,[14] crystallographic data of the extracellular domain of a mouse α1 nAChR subunit[15] and crystallographic data from the complete pentameric channels at 2.9-3.3 Å resolution from two bacterial pLGICs.[16-18] In these images the subunits traverse the membrane perpendicularly and form a ring that defines the transmembrane pore (Fig. 1B). When viewed from above, the channels appear much like a simple rose window or oculus. The extracellular domain is globular, extends ~30 Å above the lipid bilayer and consists primarily of beta sheets in the form of a beta sandwich. The extracellular domains sit atop the four transmembrane domains that are largely embedded in the membrane (Fig. 1C). The transmembrane domains of each channel subunit are alpha helices that span the lipid bilayer roughly perpendicular to the membrane surface. The M2 domains of each subunit line the pore with a stripe of polar amino acids on the M2 alpha helix facing the pore lumen. The M2 domains are circumscribed by a ring of alternating M1 and M3 domains, with the M4 domains deployed on the outside facing the lipid. The pore, starting at the lip of the extracellular domains, is funnel-shaped with the spout at the cytoplasmic end of the transmembrane domains. The short loop between the M2 and M3 domains on the extracellular side interacts with the disulfide ("cys") loop of the extracellular domains. In low resolution images of the nAChRs, the large intracellular loop, absent in bacterial pLGICs, forms a cytoplasmic vestibule through which ions must pass after escaping the transmembrane pore (Fig. 1C).[11]

The action of pLGICs begins when ligand-binding initiates a series of allosteric changes that propagate through the protein resulting in movement of the transmembranes domains and opening of the pore. Neurotransmitter ligands bind the extracellular domain, which is the determinant of ligand specificity.[19] We do not yet have a high-resolution structure of a pLGIC bound to its native ligand, but AChBP structures with bound agonists[20] confirm affinity labeling[21-26] and site-directed mutagenesis[27-31] studies showing that the ligand binding site sits at the interface between two subunits near the outer surface of the channel. A channel can have up to five ligand-binding sites depending on the how many adjacent subunit pairs bind ligand. The

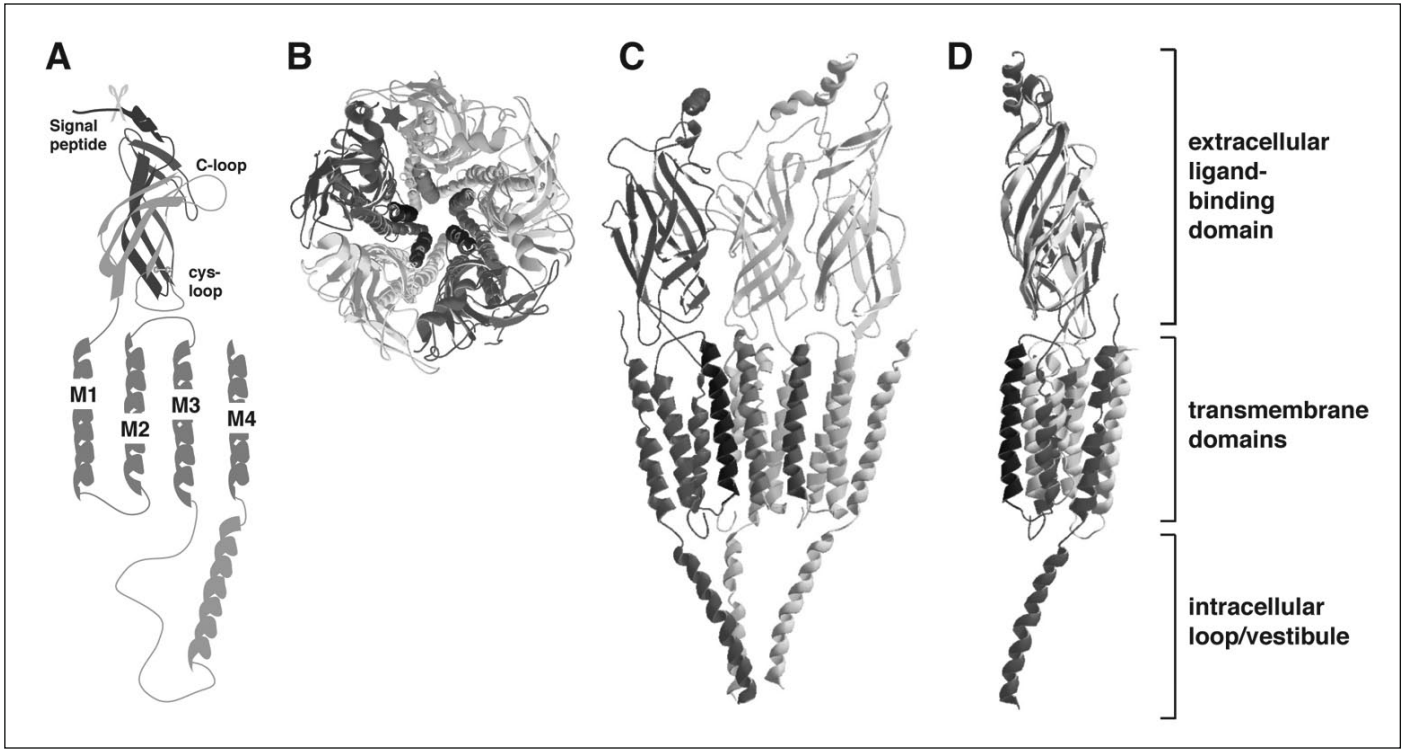

Figure 1. pLGIC structure. A) A cartoon showing the topology of a pLGIC subunit. B) A view from the extracellular side of the *Torpedo* nAChR looking down through the pore. Each subunit is rendered in a different shade. The M2 transmembrane domains that line the pore are darkened. The star indicates the location of a ligand binding site. C) A side view of B with two subunits removed to reveal the pore. Only part of the intracellular vestibule is resolved. D) One subunit from C (dark blue) overlain with the *Gloeobactor violaceus* pH-sensitive channel (GLIC, light lavender). Note the high degree of structural conservation despite low (~20%) sequence identity. A color version of this image is available at www.landesbioscience.com/curie.

ligand-binding pocket is a cage formed by aromatic side chains. The aromatic residues that form the pocket reside on four loops: the A, B and C loops on the (+) face—arbitrarily defined—of one subunit and the D loop on the (–) face of an adjacent subunit. Additional loops (E and F) on the (–) face also contribute to the binding site. In addition to delimiting the binding pocket, residues of the aromatic cage form cation-π interactions with the ligand, residues on different loops forming the cation-π interactions in different channels.[32-35] Apart from predictions based on homology models[27,36,37] and the demonstration that a phenylalanine to tyrosine change in the B-loop increases the sensitivity of glycine receptors to GABA,[38] little else is known about how pLGICs discriminate among ligands.

The transmembrane domains form the channel gate and the ion selective filter. The location of the gate and how the transmembrane domains move to open and close it remain controversial. Proposed gate locations on the M2 domain include: near the extracellular side[18] in the middle[9,39,40] or near the cytoplasmic side of the pore.[41] During gating the M2 domains may tilt or rotate.[9,17,18] In contrast, structural determinants of ion selectivity are well understood. pLGICs are typically selective for monovalent anions or cations. Rings of charged amino acids at the cytoplasmic side of the M2 domain, where it links to the M1 domain, determine the ion selectivity of the open pore. Changing the ion selectivity of a channel from anion to cation or vice versa is as simple as changing a few, or in some cases a single amino acid in the M2 domain.[42-45]

There has been significant progress tracing the allosteric steps between ligand-binding and gating of the pore. Results of mutagenesis experiments agree with predictions from crystal structures that the point of allosteric contact between the extracellular domain and the transmembrane domains occurs at the interface of the disulfide loop on the extracellular domain and the M2-M3 loop.[46-48] Using site-directed mutagenesis and single-channel kinetics, Auerbach and colleagues proposed a Brownian wave model of channel opening that involves sequential movement of four blocks of amino acids.[49,50] Thus, in a plausible model of channel gating, ligand-induced twisting of the beta sandwich nudges the M3-M4 loop tilting the M2 domains and opening the channel.

Even with only a handful of crystals, the conservation of the underlying pLGIC structure is striking. The representative extracellular domains from vertebrate, mollusk (AChBP) and bacterial subunits correspond almost perfectly at the level of secondary and tertiary structure despite the differences in function (pH response in bacteria[51] versus acetylcholine binding in metazoans) and scant primary sequence homology (20% amino acid identity in Fig. 1D). Presumably, all members of the pLGIC superfamily will adhere closely to the paradigm established by the known structures; differences in pLGIC properties will reflect modest adaptations of that paradigm.

Function: pLGICs Can Mediate Many Types of Ionotropic Neurotransmission

In metazoa, the biological function of the characterized pLGICs is to link neurotransmitter release by a presynaptic cell to ion permeability in a postsynaptic cell, thus converting a chemical signal to an electrical signal. However, the neurotransmitters and permeable ions can vary greatly. The ionotropic neurotransmitters in vertebrates are acetylcholine, GABA, glutamate, glycine, serotonin and adenosine triphosphate (ATP). Vertebrate pLGICs mediate neurotransmission by all but glutamate and ATP: glutamate is mediated by the structurally distinct tetrameric AMPA/NMDA-type receptors and ATP is mediated by trimeric P2X receptors. Vertebrate acetylcholine and serotonin receptors are cation-selective and thus excitatory. The GABA and glycine receptors are anion selective and typically inhibitory, although under certain conditions—early in development or after injury—the reversal potential of chloride, the primary permeable anion, can be high enough that GABA and glycine are excitatory.[52,53]

Compared to vertebrates, invertebrates employ a greatly expanded repertoire of neurotransmitter-ion combinations. In addition to inhibitory GABA receptors and excitatory nAChRs, there is

evidence for: glutamate-gated chloride channels in insect muscle and in mollusk and crustacean neurons,[54-57] excitatory GABA currents in crustacean, mollusk and nematode muscle,[58-61] inhibitory histamine currents in the insect visual system,[62,63] inhibitory acetylcholine and dopamine currents as well as both excitatory and inhibitory histamine currents in mollusk neurons.[64-68] Although pharmacological evidence suggested that some of these currents are mediated by pLGICs,[69] proof that pLGICs are diverse enough to mediate all of these currents awaited cloning of the receptor subunits and examination of their primary sequence.

The extent of the corresponding pLGIC diversity is increasingly apparent, largely as a result of the sequencing of invertebrate genomes and the discovery of scores of predicted pLGIC subunits that are not obvious orthologs of the known vertebrate receptors. Characterization of these orphan pLGIC receptors led to the discovery of nematode acetylcholine-gated chloride channels,[70] insect cation-selective GABA receptors,[71] arthropod pH-gated chloride channels,[72,73] insect histamine gated chloride channels,[74,75] the mollusk acetylcholine-gated chloride channel[76] and a vertebrate Zn^{+}-gated cation channel.[77] Other channels found in directed searches for known currents include nematode and insect glutamate-gated chloride channels.[78,79] In the nematode *C. elegans*, new pLGICs have turned up in genetic screens for mutants affecting neurotransmission. These include the glutamate-gated chloride channels,[80,81] the GABA-gated cation channel,[82] the serotonin-gated chloride channel[83] and the pH-gated cation channel.[84]

Because of convergent evolution, a list of channel types based on ligand-ion combinations in fact under-represents the full extent of pLGIC diversity. Several channels share neurotransmitter-sensitivity and ion-selectivity but their subunits are clearly not orthologous. Examples include the glutamate-gated chloride channels from mollusks and ecdysozoa[85] and the acetylcholine-gated chloride channels from mollusks and nematodes[70,76] (Fig. 2). The mollusk acetylcholine-gated chloride channel appears to be a typical, if more divergent, nAChR and even shares with nAChRs a sensitivity to bungarotoxin.[76] However, its ion selectivity motif is that of an anion channel. In contrast, the sequence of the nematode acetylcholine-gated chloride channel subunit is more similar to other anion-selective (GABA-type) pLGIC subunits and it appears to have independently evolved the ability to bind acetylcholine. In fact, the ligand-binding site of nematode acetylcholine-gated chloride channels and nAChRs are not any more similar than any other pair of binding sites, an indication that acetylcholine-gated chloride channels evolved a unique structural solution to the problem of binding acetylcholine.

The emphasis here has been on pLGIC diversity yet there is significant overlap in the spectrum of pLGICs found in the various phyla. pLGIC subunit types that appear to be universal to bilateria include two types of nAChRs, the α1- and α7-types, as well as GABA-gated chloride channels composed of both α- and β-type subunits (Fig. 2). An obvious question is whether it is possible to infer the conservation of a particular circuit or behavior based on the conservation of pLGIC types. So far it appears not. α1-type nAChRs act at cholinergic neuromuscular junctions in vertebrates and nematodes whereas glutamate is the neuromuscular transmitter in insects.[86] α7-type nAChRs act in the vertebrate central nervous system and in macrophages[87] but can act at neuromuscular junctions in nematodes.[88] Thus, the context in which a given pLGIC type is used is highly adaptable.

Although the characterized metazoan pLGICs mediate neurotransmission, the discovery of pLGICs in prokaryotes hints at other possible roles. The GLIC (Gloebacter violaceus Ligand-gated Ion Channel) and ELIC (Erwinia chrysanthemi Ligand-gated Ion Channel) pLGICs are found in Gram-negative bacteria, which regulate pH and ion concentrations in the periplasmic space between the inner and outer membrane.[89] A reasonable but untested role for the bacterial pLGICs would be to maintain periplasmic ion homeostasis. If periplasmic channels are chemosensory, responding to cues in the environment, they may represent a first step on the path to the evolution of neurotransmitter receptors.[8,90] Some metazoan pLGICs may also play homeostatic or chemosensory roles that reflect the ancestral pLGIC function.[91,92]

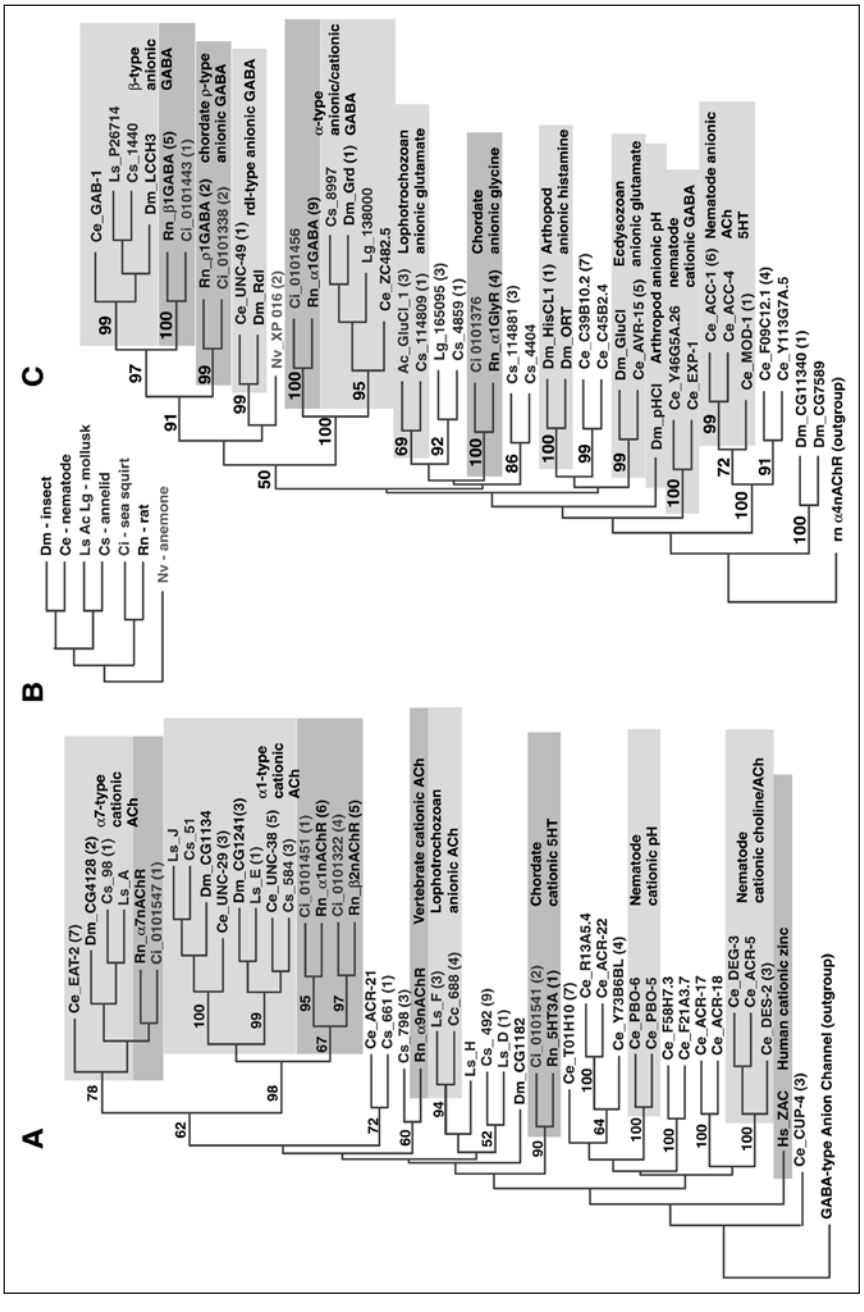

Figure 2. Please see legend on following page.

Figure 2, veiwed on previous page. Evolution of pLGIC subunits. A) A phylogenetic tree of representative nAChR-like channel subunits constructed using maximum likelihood. B) A phylogenetic tree showing evolutionary relationships of the species used in A and C. Ecdysozoa are represented by the clade that includes arthropods (insects) and nematodes, Lophotrochozoa by the clade that includes mollusks and annelids and chordates by the clade that includes rat and *Ciona*. C) A phylogenetic tree of representative GABA-like channel subunits. Subunits are from: *Aplysia californica* (Ac, mollusk), *Caenorhabditis elegans* (Ce, nematode), *Ciona intestinalis* (Ci, primitive chordate), *Capitella sp I* (Cs, annelid), *Drosophila melanogaster* (Dm, insect), *Homo sapiens* (Hs, vertebrate), *Lymnea stagnalis* (Ls, mollusk), *Lottia gigantea* (Lg, mollusk), *Nematostella vectensis* (Nv, anemone), *Rattus norvegicus* (Rn, vertebrate). Numbers on the branch indicate bootstrap values out of 100. Numbers in parentheses indicate subunits in that clade from that organism not shown in the tree. Subunits for which the ligand is known are shaded. Note that the list of subunits from *Ciona,* mollusks, annelids and *Nematostella* is incomplete.

Evolution: pLGIC Diversity Appears To Be Ancient

The diversity of pLGICs in metazoa begs the question how a protein superfamily of such scope evolved. Conspicuous and in need of explanation is the distinct spectrum of channels found in individual phyla.[8,85] Vertebrates have glycine receptors but ecdysozoa (nematodes and arthropods) and mollusks apparently lack them. Mollusks and ecdysozoa have glutamate-gated chloride channels but these are not present vertebrates. Even within the ecdysozoa, insects have histamine receptors but nematodes apparently do not, whereas nematodes have acetylcholine-gated chloride channels but insects do not. If these phylum- and superphylum-specific channels were not present in the common ancestor, when did they evolve? If they were in the common ancestor, when were they lost?

A phylogenetic analysis of the metazoan pLGIC superfamily suggests that almost all of the metazoan receptor types were likely present in the common ancestor of bilateria (protostomes and deuterostomes) and possibly in the common ancestor of the bilateria and cnidaria (jellyfish and anemones) as well[8,85,93] (Fig. 2). The evidence consists of the following observation and argument: all studied bilateria have pLGIC GABA receptors that are more closely related to each other than to any other channel type, i.e., they form a clade. Thus, the common ancestor of bilateria expressed a GABA receptor subunit and the divergence of the GABA receptor subunits reflects the radiation of the phyla that express them. Moreover, the GABA receptor subunit gene in the common ancestor of bilateria had already diverged from genes encoding other pLGIC subunit types. It follows that all of the other (nonGABA) pLGIC types found in any bilaterian were present in the common ancestor. The first cnidarian genome sequence, that of *Nematostella vectensis*, also appears to encode GABA receptors that diverged from the bilaterian GABA subunits after the various subunit types diverged,[85] which would push the origin of pLGIC diversity back to the common ancestor of the bilateria and cnidaria, an organism that existed more than 600 million years ago.[94,95]

The cation channels show a similar evolutionary pattern. There are several distinct types of nAChRs. The vertebrate α1- and β-type nAChR subunits typically combine to form obligate heteromeric channels.[96] In arthropods and nematodes there are two paralogous nAChR-like clades whose subunits also form heteromers.[4,97] Together, these four clades (two vertebrate and two invertebrate) form a larger clade (here referred to as the α1nAChR-like clade), meaning that the common ancestor of bilateria had at least one α1-type nAChR subunit.[8,98] A separate clade includes the vertebrate α7nAChR subunits as well as subunits from arthropods, nematodes, mollusks and annelids. Thus, the α7nAChR-like subunits also share an ancestral gene that predated the divergence of the bilateria. Beyond these shared clades are a number of phylum-specific clades: $5HT_3$ (cationic serotonin) receptors in vertebrates and several clades in invertebrates—so far primarily in the nematodes but also including the mollusk acetylcholine-gated chloride channel.[76] The nematode clades are little characterized but among them are the proton-gated

cation channel[84] and a channel that responds most strongly to choline.[91] Whether the invertebrate cation channel clades are as diverse in ligand specificity as the anion channels remains to be seen, but in any case the tree topology supports a model in which the α1nAChR-like and α7nAChR-like subunits as well as the other cation channel subunit clades were present in the common ancestor of the bilateria.

The argument for a diverse pLGIC superfamily in the common ancestor of bilateria is subject to two caveats. The lesser caveat concerns the great phylogenetic/time span covered by trees that encompass all bilateria. Phylogenies measure distance in units of sequence change and unequal rates of change distort the distances between sequences. Nematodes evolve more rapidly than vertebrates[99] and therefore rate differences will distort the metazoan pLGIC phylogenies. But the argument for early divergence of subunit types depends on the topology of the tree, which does not seem to be affected. For instance, GABA receptors from nematodes and vertebrates form a clade, as do the nematode and vertebrate α7-type nAChRs, which they would not do if the apparently ancient origin of invertebrate-specific channels were an artifact of differences in the overall rate of evolution.[8] The second caveat is that the observed tree topologies could be explained by a recent (<500 myr) horizontal transfer of subunit genes. That explanation is difficult to support given the apparent rarity of horizontal gene transfer in metazoan.[100]

If the common ancestor of the bilateria had a more diverse complement of pLGICs than extant metazoa, gene loss must explain the difference. Primitive chordates such as *Ciona intestinalis* encode the same spectrum of pLGICs as vertebrates, consistent with the loss of invertebrate pLGICs before the divergence of the chordates. On the other hand, the modest overlap in nematode and arthropod pLGICs may indicate substantial gene loss since the divergence of the rapidly evolving ecdysozoa. Culling of pLGIC subunit genes from the various metazoan genomes has also not been monotonic. Certain subunit families have expanded substantially in some phyla and not others.[8,98] The vertebrate α1-type nAChRs appear to have expanded since the vertebrate-invertebrate split and even since the divergence of vertebrates from other chordates. The complement of GABA receptor subunits expanded substantially in vertebrates; where *C. elegans* and *Drosophila* have, respectively, two and three α- and β-type GABA receptor subunits, mammals have 19. Conversely, vertebrates encode a single α7nAChR subunit where *Drosophila* encodes three and *C. elegans* encodes nine. It seems the pLGIC superfamily is in constant flux. What drives the selective expansion and contraction of pLGIC subunit families is an intriguing and unexplored question.

What Good Is pLGIC Diversity?

The pLGIC superfamily is both a resource and an experimental system in which to explore questions of protein and nervous system evolution. There are three areas where I see the diversity of the pLGICs being exploited:

Pushing Back the Origin of Metazoan pLGICs

The sequencing of additional metazoan genomes raises the prospect of the being able to approximately reconstruct the genome of the common ancestor of the metazoa, including the component of the genome that encoded the pLGICs. The question is how far back we can trace the evolution of the existing channels before the obscuring effects of sequence divergence prevent any further deductions. Cnidaria apparently have both fast cholinergic and GABAergic neurotransmission and the next step will be to determine whether this neurotransmission is mediated by orthologs of the corresponding bilaterian pLGICs.[101] The genome sequence of *N. vectensis* will greatly facilitate that task. The next step would be to determine whether nAChRs and ionotropic GABA receptors are also present in a basal metazoan, a position proposed for the comb jellies.[102,103]

Identifying Novel Transmitters

With the advent of complete genome sequences it has been possible to catalog and characterize all of the pLGICs in an organism. The result has been the identification of pLGICs that respond to many neurotransmitters that were not previously thought to act on pLGICs. It has also resulted in the identification of pLGICs subunits that do not, at least in heterologous systems, respond to any known neurotransmitter (J. Dent unpublished observation). Many of these will be subunits that form obligate heteromeric channels and whose partner subunits have not been identified. However, where entire clades do not respond to known neurotransmitters,[8] we must consider the possibility that the in vivo ligand for these channels is not among the usual suspects. The recent discovery that protons mediate ionotropic neurotransmission via pLGICs demonstrates that characterization of orphan pLGIC receptors will lead us to new neurotransmitters.[84] One systematic approach to identify new neurotransmitters would be to use orphan pLGICs in a bioassay to screen candidate compounds.[104]

The Evolution of Ligand Specificity

The pLGIC superfamily represents at least half a billion years of evolutionary trial and error with the effect of identifying a large number of functional and physiologically useful neurotransmitter receptors from among the vastly larger number of useless pLGIC structures. The subunits that survive the evolutionary filter contain information about functional constraints on sequence and structure that can, in principle, be extracted using covariance techniques.[105,106] A rudimentary covariance approach was used to identify the residues that confer ion selectivity.[44] To identify subtler and more variable properties, such as ligand specificity, will require large sequence alignments from >100 functionally characterized channels, but we are rapidly approaching that number. Before us is the exciting prospect of combining correlative and crystallographic data to trace the path of amino acid changes that allowed the evolution of novel ligand specificities. Learning how to change ligand specificity is likely to be much more difficult than changing ion selectivity but also potentially more rewarding as it promises to enhance our understanding of the allosteric changes involved in gating. Ultimately, the test of our mastery of pLGIC structure, function and evolution will be to engineer channels with ligand specificities not found in nature for use as experimental probes, biosensors and medical therapies.

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