Evolutionary history of the ligand-gated ion-channel superfamily of receptors

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The fast-acting ligand-gated ion channels (LGICs) constitute a group that encompasses nicotinic ACh, 5-HT_3, $GABA_A$ and glycine receptors. Undoubtedly, they all share a common evolutionary ancestor, and the group can therefore be considered to be a gene superfamily. Because the members of the superfamily are all receptors, it is reasonable to suppose that their common ancestor must also have been some type of receptor, and because the receptors are made of similar subunits, the ancestor was probably homo-oligomeric. Although we failed to find a group of proteins that are related evolutionarily to this superfamily, the analysis of the evolutionary relationships within the superfamily is possible and can give rise to information about the evolution of the structure and function of present-day receptors and indeed of the nervous system itself.

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THE LIGAND-GATED ion channel (LGIC) group is the best known of the receptor families mainly as a result of the extensive characterization of one of its members, the nicotinic ACh receptor (nAChR), which is used as a paradigm for the whole LGIC superfamily[1]. The nAChR and the 5-HT_3 receptors are selective for cations (and hence excitatory), whereas GABA receptors and glycine receptors are selective for anions (and are thus inhibitory). Members of the family have a high degree of amino-acid sequence similarity, and have some highly characteristic sequence motifs, for example, a 15-residue cys-loop[2] that is in the N-terminus domain of all members of the family. They are all oligomers, most probably pentamers, and sequence information also reveals that for any given member of the family, the subunits are themselves homologous*. Biochemical, mutational and hydropathy analyses (for review, see Ref. 3) show that each of these subunits has an N-terminus extracellular domain that is thought to contain the ligand-binding site, four putative transmembrane regions (M1–M4), and a short extracellular C terminus (Fig. 1). The second of the transmembrane regions (M2) is the main contributor to the ion channel[5–8]. Traditionally, these four transmembrane regions have been considered to be α helices, but more recent data from electron microscopy[4], Fourier-transform infrared spectroscopy analysis[9] and molecular modelling[10] indicate that, with the exception of M2, this might not be so. The ionotropic glutamate receptors have also been proposed as candidates for membership of this superfamily. Their transmembrane domain is at least analogous to that of LGICs, and they share similar channel properties but the extracellular domain is much bigger, and has no obvious sequence identity with the LGICs (Ref. 11). The lack of information about the origin of the LGIC superfamily is a major hindrance to the application of any sort of homology modelling[12] to elucidate the tertiary structure of these receptors, and their membrane-bound nature has, so far, frustrated all attempts to obtain suitable crystals for high resolution X-ray analysis.

One of the areas that has contributed much in recent years to our knowledge of LGICs is molecular biology. The sequencing of more than one hundred genes has revealed, on the one hand, the presence of closely related receptor families and, on the other hand, an unexpected degree of receptor diversity.

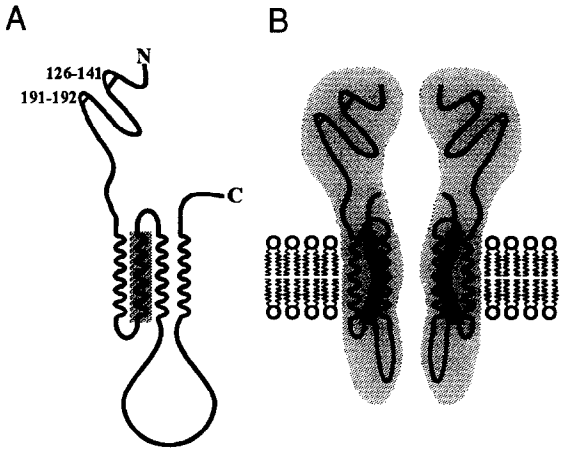

Fig. 1. ***Cartoon depicting the current view of the ligand-gated ion channels, topology.*** **(A)** *Each ligand-gated ion channel (LGIC) subunit has an extracellular N-terminus domain, containing the characteristic 15 residue cys-loop (sequence 126–141 of* Torpedo *α subunit). The adjacent cysteines (191–192) are unique to the α subunits of nicotinic ACh receptor. The M2 region, which is generally accepted as the 'lining' of the ion channel, is shaded. (***B***) Proposed positioning of the subunits (only two of the five are depicted) in the lipid bilayer; note the kink in the M2 regions that provides a degree of restriction in the lumen of the channel (see Ref. 4 for discussion). The general shading indicates the probable overall shape of the receptor.*

**In evolutionary terms, homology refers to entities that have a common, genetic origin, although they might look very different. On the contrary, analogy refers to entities that look similar, but have independent origins. The common example is the wings of bats and birds. As vertebrate forelimbs they are homologous, but as wings they are analogous, because birds and bats had independent origins.*

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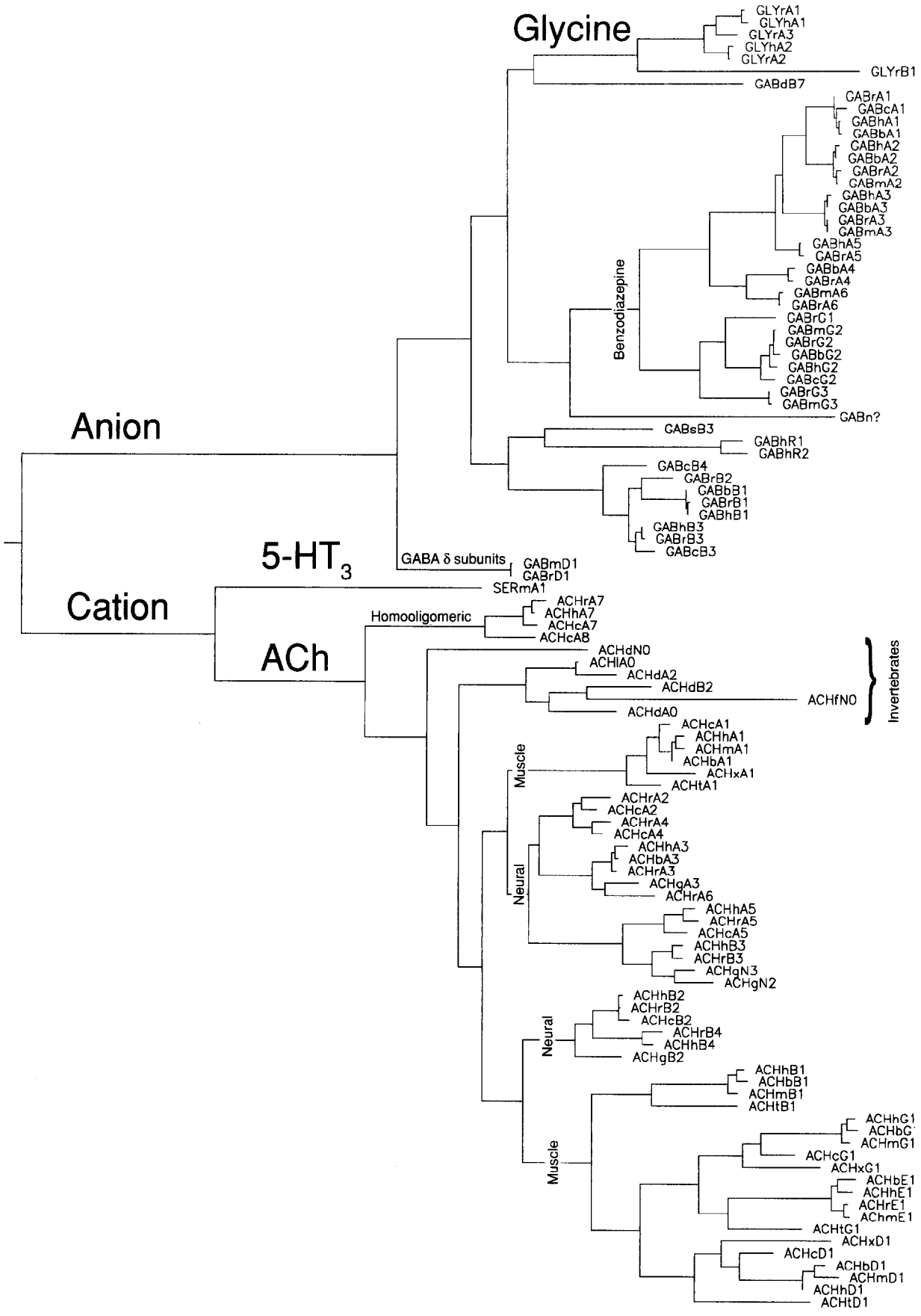

The receptors are not only widely distributed in phylogenetic terms, they occur from nematodes and insects to vertebrates and mammals, but there is also a great variability of receptor subunits. It is this variability that enables the study of the receptors from an evolutionary point of view.

Construction of an evolutionary tree of the LGIC superfamily

An alignment of 106 sequences of amino acids of LGIC receptors obtained from the Daresbury Laboratory database, UK, was the starting point for the construction of the evolutionary tree shown in

Fig. 2. (Left.) ***Evolutionary tree of the ligand-gated ion channel superfamily.*** *The method used to build the tree was the maximum likelihood approach[13,14], using the J. Felsenstein Phylip 3.5 package[15]. This statistical method looks for the tree that maximizes the probability of the data under the assumption of a given model of DNA evolution. Because there were 106 sequences, it was impractical (for reasons of computational time) to make a thorough search of topologies and to calculate for each the likelihood. Instead, a matrix of maximum likelihood distances was calculated and the neighbour-joining[16] and Fitch[17] methods were applied to it. Also, and only to look for alternative topologies, a maximum parsimony algorithm[18] was applied to the original data. A further step was to use one sequence only of each clade that was composed constantly of the same subunits in the previous analyses. In this case, and because the number of sequences was reduced to 25, a more comprehensive topology search was carried out using the maximum likelihood method. After this, the tree was expanded to all the sequences. From all the tree topologies found, the one with the highest likelihood was chosen as the best. Finally, to this tree a local rearrangement search for a better tree was applied. Because we have no idea about the ancestral states of the ligand-gated ion channel (LGIC) superfamily, rooting the tree is rather arbitrary. Our choice was to root it at the middle point. This choice appears to be appropriate as it separates anionic and cationic receptors. Two other topologies were tested against the best found here. Cockcroft* et al.*[19] placed the rat and chicken β2 subunits (the only non-α neuronal subunits available at that time) as a sister branch of the α neuronal subunits. So we calculated the likelihood of the best tree changing the position of the neuronal β2–β4 group, placing it as a sister branch of the neuronal α-subunit clade. The likelihood of this modified tree was lower than the original best, and hence was no longer considered. The second topology tested was positioning the glycine receptors as a sister branch of GABA, and as before, the tree obtained had a lower likelihood than the original best. The same analyses were made, excluding the transmembrane regions, and the same results were obtained. Transmembrane regions are more restricted in their amino acid compositions because hydrophobic residues are better suited for the lipid environment, and this could be the source of convergences (that is, the same amino acid in a particular position but originating from a different mutation) that can produce wrong tree topologies. However, it is also true that in the extracellular domain, there are positions that are functionally or structurally restricted in their amino acid composition and, thus, probably the extracellular and transmembrane domains are equally informative. Symbols used: six character names of receptors relate to the following nomenclature: RRRsS#, where RRR indicates the type of receptor, s the organism, S the subunit type and # the subunit number (where 0 is undetermined). Type of receptor, RRR: ACH, acetylcholine receptor; GAB, GABA receptor; GLY, glycine receptor; and SER, 5-HT$_3$ receptor. Organism, s: b, bovine; c, chicken; d,* Drosophila*; f, filaria; g, goldfish; h, human; l, locust; m, mouse; n, nematode; r, rat; s, snail; t,* Torpedo*; and x,* Xenopus*. Subunit type, S: A, alpha; B, beta; G, gamma; D, delta; E, epsilon; R, rho; N, non-alpha; and ?, undetermined.*
Database accession numbers for the sequences are:
ACHbA1:P02709; ACHbA3:X57032; ACHbB1:P04758; ACHbD1:P04759; ACHbE1:P02715; ACHbG1:P13536; ACHcA1:P09479; ACHcA2:P09480; ACHcA4:P09482; ACHcA5:B39218; ACHcA7:P22770; ACHcA8:JH0173; ACHcB2:P09484; ACHcD1:P02717; ACHcG1:P02713; ACHdA0:P09478; ACHdA2:P17644; ACHdA4:S12899; ACHdN0:P04755; ACHfN0:L12543; ACHgA3:P18845; ACHgB2:P19370; ACHgN2:P13908; ACHgN3:P18257; ACHhA1:P02708; ACHhA3:A37040; ACHhA5:P30532; ACHhA7:L25827; ACHhB1:P11230; ACHhB2:P17787; ACHhB3:S25587; ACHhB4:S27274; ACHhD1:X55019; X53091-516; ACHhE1:X66403; ACHhG1:P07510; ACHIA0:P23414; ACHmA1:P04756; ACHmB1:P09690; ACHmD1:P02716; ACHmE1:P20782; ACHmG1:M30514; ACHrA2:P12389; ACHrA3:P04757; ACHrA4:P09483; ACHrA5:P20420; ACHrA6:L08227; ACHrA7:M85273; ACHrB2:P12390; ACHrB3:P12391; ACHrB4:P12392; ACHrE1:P09660; ACHtA1:P02710; ACHtB1:P02712; ACHtD1:P02718; ACHtG1:P02714; ACHxA1:P22456; ACHxD1:P09628; ACHxG1:P05376; GABbA1:P08219; GABbA2:P10063; GABbA3:P10064; GABbA4:P20237; GABbB1:P08220; GABbG2:P22300; GABcA1:P19150; GABcB3:P19019; GABcB4:P24045; GABcG2:P21548; GABdB7:P25123; GABhA1:P14867; GABhA2:S62907; GABhA3:S62908; GABhA5:L08485; GABhB1:P18505; GABhB3:P28472; GABhG2:P18507; GABhR1:P24046; GABhR2:P28476; GABmA2:P26048; GABmA3:P26049; GABmA6:P16305; GABmD1:P22933; GABmG2:P22723; GABmG3:P27681; GABn?:X73584; GABrA1:P18504; GABrA2:P23576; GABrA3:P20236; GABrA4:P28471; GABrA5:P19969; GABrA6:P30191; GABrB1:P15431; GABrB2:P15432; GABrB3:P15433; GABrD1:M35162; GABrG1:P23574; GABrG2:P18508; GABrG3:P28473; GABsB3:P26714; GLYhA1:P23415; GLYhA2:P23416; GLYrA1:P07727; GLYrA2:P22771; GLYrA3:P24524; GLYrB1:P20781; and SERmA1:P23979.

Fig. 2. The initial protein alignment was used as a template for the alignment of the corresponding DNA sequences. Whenever possible, DNA rather than amino acid information is used for molecular evolutionary analysis because detailed models of the way the former evolves exist but we have no such models for the latter[20–22]. This in turn enables evolutionary trees that are based on more realistic or less arbitrary assumptions to be constructed. Taking this into consideration, only positions shared by all the sequences were considered, as there is no useful evolutionary model for deletions and insertions. Hence, DNA information of only 270 shared codons was used. This eliminated the initial section of each protein, the cytoplasmic loop between the third and fourth transmembrane regions and several other short sections, that is, regions where there is no obvious sequence identity in the superfamily. The nature of the genetic code is such that mutations at the third codon position are usually silent (that is, do not change the amino acid) and, consequently, have a higher mutation rate. Sequence divergence between some types of LGIC receptors is not small and, hence, there exists the possibility that in the third codon position, superimposed mutations have accumulated with time. This might lead to inconsistencies in the evolutionary analysis and, thus, only the first two codon positions were used.

The tree in Fig. 3 has the same topology as the tree in Fig. 2, but branch lengths were recalculated assuming a molecular clock (see Box 1). A fairly simple statistical test, suggested by J. Felsenstein[15] to judge the appropriateness of the molecular-clock assumption, was applied. It is based on a likelihood ratio between trees constructed with and without the assumption of a molecular clock. The result of the test suggests that the molecular-clock hypothesis might not be fully applicable in this case but such tests are probabilistic and therefore not infallible. Comparing trees from Figs 2 and 3 and assuming that the root is placed correctly, it seems that much of the deviation from the hypothetical evolutionary regularity of the molecular clock comes from the 5-HT$_3$, nicotinic α7–8 (and some sequences from invertebrates) and GABA δ subunits that seem to have evolved less (slower) than the remainder. However, we have to take into account that the constancy of evolution advocated by the molecular-clock hypothesis refers to a given protein, and in this case, although the receptors are all very similar proteins, each subunit is coded by a different gene and, thus, is truly different. Moreover, the recognized problem of different rates of molecular evolution in

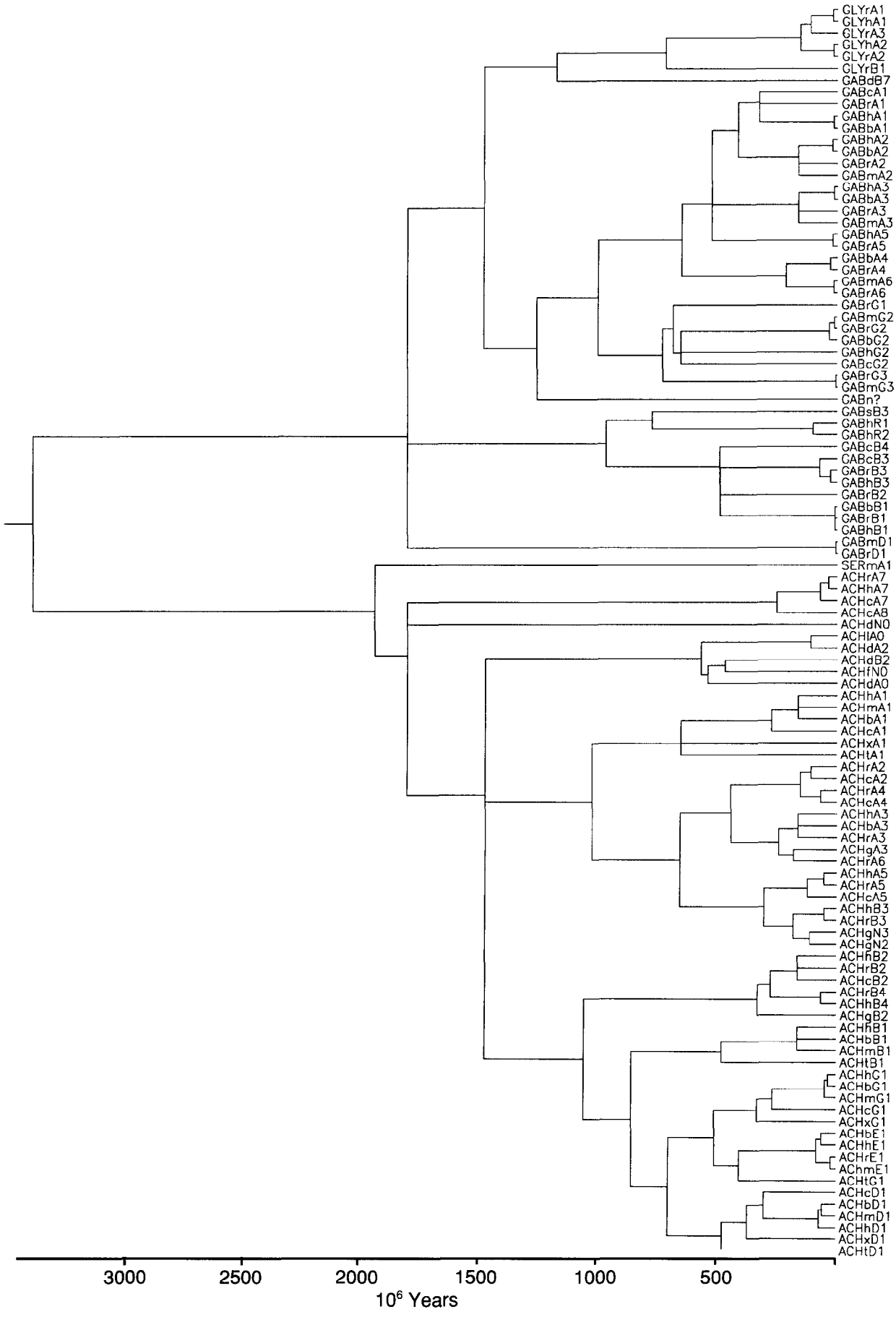

Fig. 3. ***Evolutionary tree of the ligand-gated ion channel superfamily, assuming a molecular clock.*** *The topology is the same as in Fig. 2 but the distance from the ancestor to any tip (present-day receptor) is the same or, in other words, all subunits have evolved at the same rate (see Box 1). For symbol explanation see Fig. 2. Calibration from relative to absolute timescale was based on the fossil record for the time of divergence of the lineages leading to mammals and birds (approximately 300 million years ago), mammals, birds and amphibians (approximately 350 million years ago), and fish and the remaining vertebrates (approximately 430 million years ago)[23]. The naming of the main branches is as in Fig. 2.*

Box 1. The molecular clock

The early comparative studies of protein amino acid sequences carried out on haemoglobin[a,b] and cytochrome c (Ref. c) showed a constancy in the rate of amino acid substitutions among several mammalian lineages. Consequently, Zuckerkandl and Pauling[b] suggested that the rate of molecular evolution of a given protein is almost constant over time, hence the term 'molecular clock'.

If this is true, proteins could be used to date divergence times of species (or genes) in a similar way that isotopes are used. It is worth noting that a molecular clock is not needed for reconstructing phylogenetic trees, and as long as backward and parallel mutations are uncommon, these can now be estimated quite effortlessly. To estimate divergence times using molecular information, a known timescale is needed to convert relative molecular distances to real time. Fossil information is used for this purpose, although it might not be precise.

Opponents of this hypothesis, however, suggested that rates of evolution can be higher after gene duplication, either because of positive selection[d] (advantageous mutations) or a much more relaxed selection pressure (purifying selection)[e]. Another cause of deviation from the constancy rate of evolution advocated by the molecular-clock hypothesis is the generation time. There is evidence that rodents have a higher rate of nucleotide substitution than primates[f], and that within primates, monkeys have a higher rate than humans[g]. This is because inheritable mutations are established during formation of gametes, and thus, organisms with a lower generational period, and hence more germ-line DNA replications per year, have higher apparent rates of mutation. For example, mice and rats with similar number of generations per year have similar substitution rates[g].

Although we now know that substitution rates are not rigorously constant and that they might differ from species to species, they could be nearly uniform if a long enough evolutionary period is considered (especially if it goes beyond the time of divergence of most lineages), and they can give rough estimations of divergence times[h]. For a complete treatment of molecular-evolution analysis, see Refs i and j.

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different species (see Box 1) might also be present, given that our analysis covers sequences from nematodes and insects to rodents and primates. An example of this might be the case of the nAChR from filaria, ACHfNO, that seems to have evolved much faster than the remainder of its group. As stated above, however, the test is not infallible and we might be missing important information if we ignore the molecular clock.

Origin of the LGIC superfamily

By rooting the tree in the middle of its length, the LGICs' ancestor is placed between cationic and anionic receptors. Assuming a molecular clock, the date estimated for this ancestor is at least 2500 million years ago. This date is in agreement with that obtained in an earlier study[19] that was based on 30, mainly nicotinic, sequences only. The first impression might well be that this origin, probably before the first eukaryotes[24], is surprisingly remote for a neuronal receptor. However, this seems not to be an isolated case because, despite the lack of sequence similarity, G-protein-coupled receptors, another major group of cell-surface signalling proteins, are known to have a tertiary structure that is similar to bacteriorhodopsin[25] and, hence, are probably homologous to what is clearly a prokaryotic protein. Such considerations suggest that these very important surface-signalling molecules that are associated with present day nervous systems were readily available well before this novel signalling function made its appearance during evolution. The earlier study referred to above[19] suggested that LGIC-like proteins might be widely represented in a variety of organisms, and the ancestral role of primitive LGIC receptors was discussed in the context of osmotic regulation and nutrient seeking, both of which might involve transmembrane ion fluxes and some degree of ligand recognition. Nutrient seeking in particular might relate to the present function of the LGICs, since their extracellular region is a complex molecular-recognition system that would not be needed for osmotic regulation, a function that might more probably relate to that of voltage-dependent ion channels. The phenomenon of desensitization[26] at the molecular level (in contrast to the rather confusing and diverse concept of cellular desensitization) can also be considered in the context of these ancient putative origins of the LGICs. Desensitization would have provided a mechanism to prevent a long-term opening of the channel that could be not only harmful but futile to the primitive cell. It would be interesting to know if the concentration of ACh or other ligands in the synapse is high enough to provoke receptor desensitization, that is, is desensitization a normal and useful part of synaptic transmission? If it is not, there would have been no selection advantage to keep this feature, and this would indicate that desensitization is inherent intrinsically in the basic structure of these receptors and was probably strongly selected for before the LGICs' present function in the nervous system was selected.

So far, there is no evidence of an evolutionary relationship between LGICs and any other protein. Given the estimated divergence time from the common ancestor of at least 2500 million years, this might be surprising. We have, nevertheless, used the information of the evolutionary tree to determine

the origins of these receptors, and have tried to estimate the hypothetical state(s) of the LGIC common ancestor for each of the 810 nucleotide positions analysed. This was accomplished by using the topology of the tree, and a maximum parsimony estimation of the ancestral states. This ancestral sequence, which obviously has several redundancies, was submitted to the Daresbury Laboratory DNA database for a search of similarities. Interestingly, within the 300 best scores, only 5-HT_3 and GABA δ-1 subunits were selected from the LGIC superfamily, suggesting an 'early' nature for these two receptors. In this analysis, we considered only those sequences that had optimized scores higher than the receptor sequence that scored worst. All of them, except one, coded for highly repetitive proteins or repetitive DNA (which by chance alone can have approximately 25% identity with the probe). The exception was a nonrepetitive protein whose reading frame matched that of the probe. This protein is a beta-ketoacyl synthase from *Streptomyces glaucescens* (accession code ×15312). However, the amino acid sequence alignment with the 5-HT_3 and GABA δ1 subunits is not significant[27] (percentage identity between 11% and 12%); although a very conserved feature of all LGICs, the 15 amino acid cys-loop, seems to be present. In the LGICs, there has been much speculation about the role of this unusual structural motif, and it might be that careful analysis of the structure–function relationships of the beta-ketoacyl synthase could provide some insights into the role of the loop in the LGICs.

Although it can be argued that the failure to find a LGIC related protein might be due to the incompleteness of the database, it is more probable that either the sequence signal is no longer recognizable or that LGICs are made of two or more unrelated proteins that are joined by exon shuffling. For example, Unwin[4] noted a probable analogy between the structure of the transmembrane region of LGICs and two related pentameric bacterial toxins, heat-labile enterotoxin[28] and verotoxin[29]. These two toxins have an obvious similarity in their tertiary structure but not at the sequence level[29]. Hence, it is feasible that the transmembrane region of LGICs is derived from a toxin-like related protein, whereas the extracellular domain has a different origin. In this way, we might explain the similarity between LGICs and glutamate receptors that might be homologous in their putative transmembrane regions only.

The lack of rationale in assigning any grade of primitiveness to receptors belonging to 'primitive' organisms should be emphasized. It can be said that an organism is primitive in terms of the moment in the evolutionary timescale at which it appeared. Bacteria are primitive organisms in the sense that all the characteristics that make and constrain them genetically and thermodynamically to remain as bacteria appeared very early. Similarly, an LGIC receptor is a very primitive protein; it has been a receptor for perhaps 2500 million years. A vertebrate nicotinic receptor can, however, be considered more 'primitive' than an insect nicotinic receptor if it shares more attributes with the original protein, and that is dependent on both selection pressures and on chance (genetic drift). Present-day receptors from 'primitive organisms' have evolved over the same period of time as those from mammalian brain. 5-Hydroxytryptamine receptors or GABA δ subunits are probably the most primitive of all receptors as they are more similar to the hypothetical ancestor, regardless of whether they belong to vertebrates or insects.

Evolution of cationic receptors

Within the cationic receptors, the 5-HT_3 and ACh receptors diverge very early. As seen from the tree in Fig. 2, and as mentioned in the previous section, the divergence of 5-HT_3 receptors with respect to the cationic ancestor is the lowest, indicating that they could be the most similar to the primitive cationic receptor.

Among ACh receptors, the most primitive (considering the length of their branch), and the ones that split early from the remainder, are the α7 and α8 subunits, and these are capable of forming homo-oligomeric receptors in *Xenopus* oocytes[30]. After the α7 split, there followed some α or α-like subunits that have been cloned from invertebrates (this does not mean that they cannot be present in vertebrates). It is known that at least one of these subunits (ACHIAO) can also form homo-oligomeric receptors[31] in the *Xenopus* expression system. Given the primitiveness of the α7 subunit, its closeness to 5-HT_3 receptors which are also homo-oligomeric, and the probable fact that there were no other subunits when the α7 appeared, it is highly likely that these receptors might be truly homo-oligomeric in nature.

Nicotinic-receptor α subunits are defined by the possession of a pair of adjacent cysteines in the N-terminus domain that are believed to be involved in binding of ligand. Indeed, binding of ligand in nicotinic receptors is generally thought to be restricted to the α subunits[3]. As seen from the trees, non-α or structural subunits (lacking the pair of cysteines) have derived from α subunits at four independent times. First, there are two independent non-α subunits within the 'invertebrate' group of receptors. Second, there is a main division between α and non-α. Third, within the last α group, and as a sister branch of the α5, the β3 subunits (including the goldfish N3 and N0) appeared.

From Figs 2 and 3, it seems that at approximately the same time, α and β subunits (with the exception of the β3 subunit that has an independent origin) diversified into their neural and muscle subtypes. This could have been caused by the separation of both tissues. According to Fig. 3, the minimum date for this event is between 800 and 1400 million years ago. In a previous study[19], where only 28 ACh, one glycine and four GABA receptors were studied, the neural β2 subunit (the only neural β sequence available at that time) was included within a neural α clade, and this contradicts what we now find with more sequences. In both cases (α and non-α), the neural subunits seem to be more similar to their respective ancestors than their muscle counterparts. This means that until the gene duplication and subsequent specialization of the muscle subunits, neural and muscle receptors were probably the same.

Within the non-α group, the neural type had only a minor bifurcation (β2 and β4), although the goldfish β2 receptors seem to be something different. However, the muscle types split several times giving

rise to very different subunits. The β subunits separated early, and also are the most similar to the ancestor. The other branch split into the δ and γ and ε subunits. What is called a γ subunit in *Torpedo* is, in fact, more closely related to the ε of other vertebrates. In the case of mammals, the ε subunit replaces the γ in the adult.

Evolution of anionic receptors

Within anionic receptors, the most significant fact our analysis suggests is that glycine receptors are derived from GABA receptors. This was surprising given the fact that GABA is a rather complex molecule compared with the simple and common amino acid glycine. It is often assumed that during evolution readily available molecules, such as glycine, were the first to be used as transmitters[11]. However, it is also possible that in the primitive environment, with less complex topologies or compartmentalization, such a very common molecule carried no unique information. The most primitive of the anionic receptors is probably the GABA δ1 subunit. An important feature of GABA receptors is the presence of several modulatory sites which, in response to a variety of ligands, can interact allosterically with the GABA-binding site[32]. In the late 1970s, there was a widely held view that the major modulatory site for the benzodiazepines was restricted to the vertebrates, and that such modulation was a relatively recent acquisition[33]. This proposal has since been refuted by biochemical, pharmacological and electrophysiological experiments[34–37]. It is generally agreed that the recognition of GABA is associated mostly with the β subunits, and β subunits have been observed in both invertebrates and mammals. Within the GABA-receptor family, those subunits that are considered to be primarily concerned with the benzodiazepine responses, α and γ, belong to the sister group of the β class of subunit but they are certainly not temporally linked to the appearance of the vertebrates. The presence of non-β subunits in the invertebrates is not yet well established.

Concluding remarks

The evolutionary analysis of the LGICs, although speculative, has proved to be a useful tool in the study of these receptors from a non-evolutionary perspective, providing several new insights into their possible structure and function. Also, the knowledge of the phylogenetic relationships between the receptor genes could be an invaluable aid in the design of mutations, or chimeras, or in extrapolating known functional characteristics of a particular subunit to those to which it is evolutionarily related. For example, it is sometimes difficult or impossible to produce functional receptors from a cloned gene, but it might be feasible to infer its properties from the sequence. Usually, receptor subunits are named on the basis of sequence similarity. However, a simple inspection of the aligned sequences is sometimes not sufficient because some regions are more similar to one subunit and other regions to another. Even the information given by the percentage identities is often confusing or not definitive. Although not infallible, DNA-sequence phylogenetic analysis can give the most accurate and complete representation of the relationships between subunits.

Phylogenetic analysis of DNA sequences also gives information about the tempo and modes of evolution that can be used in the study of the nervous system as a whole. It would be very interesting to compare the evolution of all receptor families on a temporal scale, and to look for correlations between the different phylogenetic trees in terms of novel functions and subunit diversification.

Molecular biology has given us the means to have an overall view of the phylogenetic relationships within the LGIC superfamily but the picture is far from complete and could change markedly with the appearance of new data for the currently rather small glycine and 5-HT_3 families.

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