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New insights into the structural bases of activation of Cys-loop receptors

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ABSTRACT

Neurotransmitter receptors of the Cys-loop superfamily mediate rapid synaptic transmission throughout the nervous system, and include receptors activated by ACh, GABA, glycine and serotonin. They are involved in physiological processes, including learning and memory, and in neurological disorders, and they are targets for clinically relevant drugs. Cys-loop receptors assemble either from five copies of one type of subunit, giving rise to homomeric receptors, or from several types of subunits, giving rise to heteromeric receptors. Homomeric receptors are invaluable models for probing fundamental relationships between structure and function. Receptors contain a large extracellular domain that carries the binding sites and a transmembrane region that forms the ion pore. How the structural changes elicited by agonist binding are propagated through a distance of 50 Å to the ion channel gate is central to understanding receptor function. Depending on the receptor subtype, occupancy of either two, as in the prototype muscle nicotinic receptor, or three binding sites, as in homomeric receptors, is required for full activation. The conformational changes initiated at the binding sites are propagated to the gate through the interface between the extracellular and transmembrane domains. This region forms a network that relays structural changes from the binding site towards the pore, and also contributes to open channel lifetime and rate of desensitization. Thus, this coupling region controls the beginning and duration of a synaptic response. Here we review recent advances in the molecular mechanism by which Cys-loop receptors are activated with particular emphasis on homomeric receptors.

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1. Introduction

The human brain is a vast and complicated network, where billions of nerve cells use signals to communicate with each other. Chemical synaptic transmission is the main process by which nerve cells signal one another. It offers the advantages of signal amplification, reversal of polarity and great potential for modulation, all important properties for higher brain function. At chemical synapses, the neurotransmitter is released into a narrow synaptic gap after depolarization of the presynaptic terminal and binds to a postsynaptic receptor. Neurotransmitter-gated ion channels are a family of synaptic receptors that convert the chemical signal into an electrical one by rapidly opening a channel that allows the flux of ions through the membrane. Just as important, the channel closes within a few milliseconds as the transmitter dissociates to terminate the synaptic event. Thus, moment-to-moment communication relies on rapid on and off responses of synaptic receptors.

Neurotransmitter-gated ion channels of the Cys-loop superfamily play key roles in chemical synapses throughout the nervous system, and include receptors activated by acetylcholine (ACh), γ-aminobutyric acid (GABA), glycine, and serotonin (5-HT) (Le and Changeux, 2001; Lester et al., 2004; Sine and Engel, 2006; Bartos et al., 2009a). They are known as Cys-loop receptors because all family subunits contain a pair of disulfide-bonded cysteines separated by 13 residues which form a loop located at the interface between extracellular and transmembrane domains. Their vital role in converting chemical recognition into an electrical impulse makes these receptors prime loci for learning, memory and disease processes, as well as targets for clinically relevant drugs. Cys-loop receptors are targets of widely prescribed drugs, such as neuromuscular blockers, barbiturates and benzodiazepines. In the last years, an ever increasing number of human and animal diseases has been found to be caused by defective function of Cys-loop receptors, such as Alzheimer's and Parkinson's disease, schizophrenia, hereditary epilepsies, attention-deficit, hyperactivity disorder, autoimmune autonomic neuropathy, autism, myasthenia gravis, and congenital myasthenic syndromes (Kalamida et al., 2007).

In vertebrates, Cys-loop receptors can be cation-selective, as nicotinic ACh (nAChRs) and 5-hydroxytryptamine type 3 (5-HT_3) receptors, or anion-selective channels, as $GABA_A$, $GABA_C$ and glycine receptors. The selectivity of the channels for cations or

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anions governs the sign of the current, and in most of the cases, the type of response: inhibitory, for anionic channels because they hyperpolarize the cell, and excitatory, for cationic channels because they induce membrane depolarization by allowing a net influx of Na^+ ions into the cell.

Nicotinic receptors have been object of attention since Claude Bernard investigated the action of the Central American arrow poison, curare. The muscle nAChR was the first to be identified and purified, and the first to be characterized biochemically and electrophysiologically. The nAChR is widely distributed throughout the animal kingdom, from nematodes to human (Le and Changeux, 1995). It is expressed in many regions of the central and peripheral nervous system and plays a major role in neuromuscular transmission. This receptor is the target of competitive blockers, such as curare, and other muscle relaxants used in surgery and it is modulated by a great variety of compounds (Arias et al., 2006). nAChRs are also present in various non-neuronal tissues, such as glia, blood cells (De Rosa et al., 2005), keratinocytes (Maus et al., 1998), endothelial cells (Macklin et al., 1998), multiple cell types of the digestive system and lung cells (Wessler et al., 2003) .

5-HT_3 receptors are found in the central and peripheral nervous system. They are involved in sensory processing, nociception, emesis, cardiovascular regulation, and gut function (Thompson and Lummis, 2007). Selective antagonists are used as antiemetic agents during antineoplasic therapy. To date, five different subunits are known in human, and all subunits show splice variants (A–E) (Niesler et al., 2003, 2007).

$GABA_A$ and glycine receptors are mainly involved in inhibition in the central nervous system, with the $GABA_A$ receptor distributed throughout the central nervous system and the glycine receptor found predominantly in the brainstem and spinal cord. The activity of GABA receptors is allosterically enhanced by benzodiazepines, barbiturates, intravenous anesthetics, alcohols, steroids and volatile anesthetics; and it is blocked by picrotoxin (Steinbach and Akk, 2001; Akk et al., 2007; Hanson et al., 2008; Olsen and Sieghart, 2009). Glycine receptors are targets of the plant alkaloid strychnine which acts as a competitive antagonist, leading to agitation, muscle spasms, and convulsions. The development of therapeutic agents against these receptors may therefore have significant utility as muscle relaxants and analgesic agents (Connolly and Wafford, 2004).

The essential function of Cys-loop receptors is to couple the binding of the agonist to the opening of the ion channel. Given that this process governs synaptic transmission, elucidation of its mechanism and the structures involved has been a long-standing challenge.

2. Overall structure

Cys-loop receptors are composed of five identical (homopentamers) or different (heteropentamers) polypeptide chains arranged around an axis perpendicular to the membrane (Fig. 1a). A wide number of subunits have been cloned for all members of the superfamily (Ligand-gated ion channel database, http://www.ebi.ac.uk/compneur-srv/LGICdb/cys-loop.php). In nAChRs, subunits are classified in two types, α and non-α, with the α-type subunits containing a disulfide bridge in the binding site. Present day homomeric Cys-loop receptors likely descended from a homomeric bacterial counterpart (Tasneem et al., 2005). They are the simplest structural class of receptors of the superfamily, and they therefore represent a model system to examine structural and mechanistic aspects of channel activation. Homomeric receptors include the neuronal α7 nAChR that is involved in a range of neurological and psychiatric disorders, including Alzheimer's disease, attention deficit hyperactivity disorder and schizophrenia (Kalamida et al., 2007).

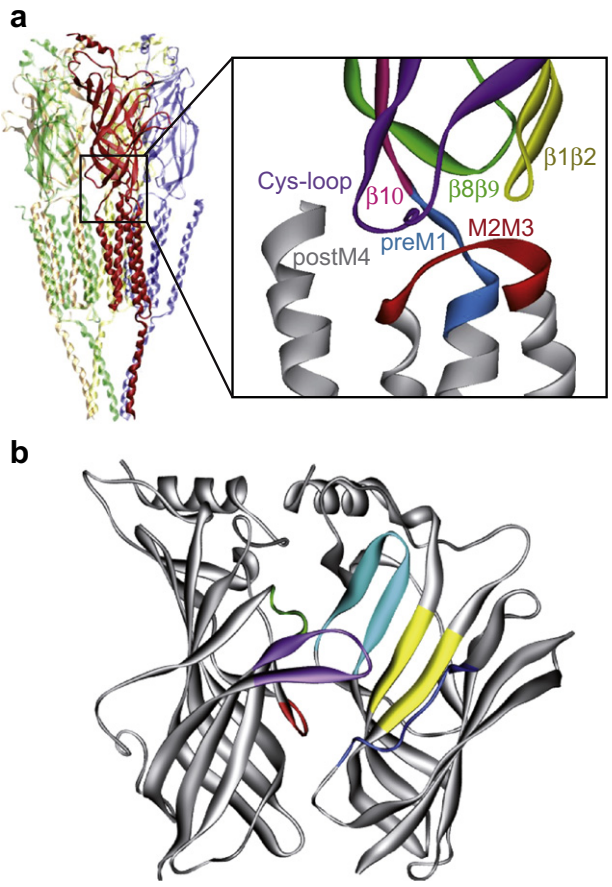

Fig. 1. Structure of the nAChR. (a) Cartoon diagram for the *Torpedo* nAChR (PDB 2BG9) and view of the structures at the interface between extracellular and transmembrane domains: β1β2 in yellow, Cys-loop in purple, β8β9 in green, β10 in pink, pre-M1 in light blue, and M2–M3 linker in red. (b) Extracellular domain showing the loops at the principal (Loop A in red, Loop B in green, and Loop C in pink) and complementary faces of the binding site (Loop D in yellow, Loop E in light blue, and Loop F in blue) (AChBP, PDB: 1UW6).

In serotonin-activated receptors, only 5-HT_3A subunits are able to form functional homomeric channels in heterologous expression systems and probably in native cells (Hussy et al., 1994; Holbrook et al., 2009).

All subunits share a basic scaffold composed of: (1) a large N-terminal extracellular domain of ~200 amino acids; (2) three transmembrane domains (TM) separated by short loops; (3) a cytoplasmic loop of variable size and amino acid sequence; and (4) a fourth transmembrane domain with a relatively short and variable extracellular COOH-terminal sequence (Bartos et al., 2009a).

Recent structural studies have provided an insight into the three dimensional structure of nAChRs and all members of the superfamily. In particular, a high resolution structural model (4 Å) of the nAChR from the marine ray *Torpedo* (Unwin, 2005) has been invaluable in the interpretation of functional and pharmacological data (PDB code 2BG9, Fig. 1a). This refined 4 Å resolution electron microscopy structure shows that the N-terminal extracellular portion is built around a β-sandwich core consisting mainly of ten β-strands from each subunit, resulting in a whole domain that contains two binding sites for ACh. The membrane-spanning portion, composed of the four α-helical segments from each subunit, is joined covalently to the extracellular domain at the N-terminal end of M1 (Fig. 1a). Thus, Cys-loop receptors are built on a modular basis, with the extracellular domain containing the agonist binding sites, and the transmembrane domain containing the pore and the channel gate (Fig. 1a).

In addition to this structural model, an atomic resolution (1.94 Å) structure of the extracellular domain of the nAChR α1 subunit has been determined (Dellisanti et al., 2007), and also high resolution structural information has become available from studies of proteins which show close sequence similarity to nAChRs, namely: (i) the high-resolution structures of soluble ACh binding proteins (AChBP) from the fresh-water snail *Lymnaea stagnalis* (2.7 Å; PDB 1I9B (Brejc et al., 2002)), the sea snail *Aplysia californica* (1.96–3.4 Å; PDB 2BYN (Hansen et al., 2005)), and the freshwater snail *Bulinus truncatus* (2.0 Å; PDB 2BJ0 (Celie et al., 2005)); and (ii) X-ray structures of prokaryotic pentameric ligand-gated ion channels from the bacterium *Erwinia chrysanthemi* (ELIC, 3.3 Å; PDB 2VL0 (Hilf and Dutzler, 2008)) and *Gloeobacter violaceus* (GLIC, 3.1 Å; PDB 3EHZ (Hilf and Dutzler, 2009a); 2.9 Å; PDB 3EAM (Bocquet et al., 2009)). Since GLIC structure was determined at low pH, a condition where the open probability of the channel is high, it depicts the receptor in a potentially open state. Thus, the comparison of ELIC (closed state) and GLIC (open state) structures has provided insights into structural changes associated with channel opening.

More recently, the first three-dimensional structure of an eukaryotic Cys-loop Receptor, the homopentameric *Caenorhabditis elegans* glutamate-gated chloride channel α (GluCl), was determined (Hibbs and Gouaux, 2011). GluClα is most similar to the α1 glycine receptor, with which it shares 34% amino acid sequence identity. The X-ray structure of the GluCl–Fab complex was determined with the allosteric agonist ivermectin (PDB 3RHW), which binds in the transmembrane domain and stabilizes the receptor in the open conformation, and in additional structures with L-glutamate (PDB 3RIF) and the open-channel blocker picrotoxin (PDB 3RI5). The overall architecture of the extracellular domain is similar to that found in the bacterial pentameric receptor orthologues. The transmembrane helices adopt a fold like the bacterial receptors and nAChR, with the five M2 segments lining the pore and adopting an open channel conformation, similar to the conformation visualized in the GLIC structure.

3. The extracellular domain contains the agonist binding sites

Our knowledge of the structure of the extracellular domain of Cys-loop receptors took a giant step forward with the solution of the high-resolution structure of the AChBP from *L. stagnalis* (Brejc et al., 2001). This soluble protein is produced and stored in glial cells and is released in an ACh-dependent manner in the synaptic cleft where it regulates synaptic transmission. AChBP lacks the transmembrane region but contains many of the structural cornerstones that give nAChRs their unique signature and has therefore become a functional and structural model of the extracellular domain of Cys-loop receptors (Cromer et al., 2002; Reeves et al., 2003). It contains 210 amino acids and shares ~15–24% sequence identity to aligned sequences of the amino-terminal, extracellular halves of Cys-loop receptor subunits. Each AChBP monomer consists of an N-terminal α-helix, two short 3_{10} helices, and a core of 10 β-strands that form a β-sandwich structure. The inner β-sheet is formed by β1, β2, β3, β5, β6 and β8, and the outer β-sheet by β4, β7, β9 and β10. The N- and C-terminals are located at top and bottom of the pentamer, respectively. In Cys-loop receptors, the end of β10 connects to the start of M1. Located at the bottom of the subunit, the linker between β6 and β7 strands is the signature Cys-loop found in all members of the superfamily.

Each agonist binding site is found in a cavity at an interface between two adjacent subunits (Fig. 1b, reviewed in Sine, 2002). One interface, called the principal or "positive" face, contributes three loops that span β strands and harbor predominantly key aromatic residues; these regions have been named as Loop A (which corresponds to the β4β5 loop), Loop B (β7β8 loop), Loop C (β9β10 loop). The adjacent subunit, which forms the complementary or "negative" face, contributes three β strands with residues clustered in segments called Loops D–F. Thus, key residues from the principal face come from Loop A (W86 and Y93), Loop B (W149 and G153) and Loop C (Y190, C192, C193 and Y198) (residues correspond to *Torpedo* α subunit). The complementary face is formed by residues from Loop D (W55 and D57), E (L109, R111, T117 and L119) and F (D174 and E176) (residues from δ or γ *Torpedo* subunits) (Brejc et al., 2001; Sine, 2002) (Fig. 1b). In the heteromeric muscle nAChR, the principal faces of both binding sites are provided by the two α1 subunits whereas the complementary faces, by the ε or γ and δ subunits. In homomeric receptors, such as α7 nAChRs, both faces of the five binding sites are contributed by α7 subunits.

4. The transmembrane domain comprises the ion pore and the gate

Structural and electrophysiological studies have shown that the ion channel is largely lined by the M2 domains of the five subunits. The model of the closed pore of *Torpedo* nAChR at 4 Å confirmed the long-held view that the pore is shaped by an inner ring of five α-helices (M2 segments). An outer ring of fifteen α-helices (M1, M3 and M4 segments) shields the inner ring from the lipids (Miyazawa et al., 2003; Unwin, 2005). Since the complete model was obtained in the absence of agonist, it is considered to depict the closed resting state although this remains a matter of controversy. The pore is maximally constricted in the middle of the membrane due to side-to-side interactions between hydrophobic residues of neighboring helices (positions 9′ and 13′). This tight hydrophobic girdle creates an energetic barrier to ions across the membrane, and it probably corresponds to the gate (Miyazawa et al., 2003; Unwin, 2005). The ion pore contains the filter selectivity, which is the structure that determines which types of ions are able to pass through the channel (Villarroel and Sakmann, 1992). Several regions contribute to ion selectivity in Cys-loop receptors through a series of charged rings all along the ion translocation pathway (Sine et al., 2010). Insights from the GluCl structure suggest that minimal determinants of selectivity are given by residues located at the cytoplasmic end of M2 (-1′A and -2′P for anions and -1′E for cations) (Hibbs and Gouaux, 2011).

The long intracellular region between M3 and M4 contains a short α-helix. It is thought to be associated with cytoskeletal proteins that allow the clustering of the receptors at appropriate regions of the membrane (Feng et al., 1998). This region contains phosphorylation sites, and it has been demonstrated that phosphorylation modulates expression, upregulation, desensitization, and interaction with cytoskeleton proteins of nAChRs (Huganir et al., 1986; Swope et al., 1995). This intracellular region has been shown to contribute to channel conductance in 5-HT_3A receptors (Kelley et al., 2003; Peters et al., 2004). Elegant studies have shown that the replacement of three arginine residues at this domain (R432, R436, and R440) of the 5-HT_3A receptor by those found at equivalent positions in the 5-HT3B subunit (glutamine, aspartic acid, and alanine) increases about 40-fold the single channel conductance of the homopentameric 5-HT_3A receptor and allows detection of single channel activity (Kelley et al., 2003; Peters et al., 2004; Bouzat et al., 2004; Corradi et al., 2009).

5. Structural changes at the binding site and the pore during channel activation

The gating reaction couples local structural changes at the binding sites with changes in the ion channel that allow the increase in

conductance. The identification of residues that transduce neurotransmitter binding into channel gating is now possible by combining the information of residue locations within functionally crucial regions of the receptors from the high-resolution structures with electrophysiological, pharmacological, and computational studies.

Binding of the agonist and the resulting conformational changes have been well studied in nAChR. Stabilization forces of the agonist at the binding site include cation–π, dipole–cation, hydrogen bonding and van der Waals interactions (Dougherty, 2007; Xiu et al., 2009). In Cys-loop receptors, a cation–π interaction between the natural agonist and one of the aromatic residues at the principal face of the binding pocket is always seen, although its precise location varies (Xiu et al., 2009; Thompson et al., 2010). This interaction is important for binding affinity and activation. W149 of Loop B of the α subunit is responsible for this interaction in α4β2 and muscle AChRs (Xiu et al., 2009), and Y93 of Loop A, in α7 (Puskar et al., 2011).

How are the conformational changes initiated by agonist binding translated into channel opening? There is an increasing consensus that agonists affect the mobility of C and F loops at the binding site and elicit closure of the binding cavity *via* capping by the C-loop, movement that seems to be the initial conformational change underlying channel activation (Mukhtasimova et al., 2005, 2009; Bartos et al., 2009a; Wang et al., 2009). In this regard, the superposition of crystal structures of AChBP with a variety of agonists and antagonists shows that Loop C from the principal face is in an "open" conformation in the resting state of the receptor and in the presence of the agonist, it caps the entrance to the binding cavity, trapping the agonist (Ulens et al., 2006). Molecular dynamics simulation also revealed a time-dependent change of Loop C from uncapped or open to a capped or closed conformation (Law et al., 2005; Gao et al., 2005). Loop C is connected directly with M1 via β10, and therefore it might propagate conformational changes occurring after agonist binding to the interface between the extracellular and transmembrane domains. During closure of Loop C, the conserved tyrosine (Y190 in nAChR) is drawn closer to K145 in β7 strand, breaking or weakening a previous interaction between this lysine and D200 in β10 strand at the extracellular-transmembrane interface (Mukhtasimova et al., 2005; Sine and Engel, 2006). Mutagenesis combined with electrophysiological studies has shown that residues of other loops are also involved in binding and gating. For example, W55 in loop D is involved in channel gating and desensitization of muscle and α7 nAChRs (Akk, 2002; Gay et al., 2008). In the same loop, Q57 contributes to the high potency of morantel to activate α7 nAChRs (Bartos et al., 2009b). Position 153 (Loop B) has been shown to be associated with a slow-channel syndrome (Sine et al., 1995), to be involved in the high efficacy of activation of nematode nAChRs by anthelmintic drugs (Rayes et al., 2004), to affect gating by interacting with Loop C in neuronal nAChRs (Grutter et al., 2003), and to affect agonist efficacy of muscle nAChRs (Purohit and Auerbach, 2011). Recent studies suggest that activation may also involve the movement of Loop B and that flexibility of this loop is critical for normal function of the nAChR (Purohit and Auerbach, 2011).

The endpoint of the activation process is the transient removal of the barrier to ion flow. Various molecular rearrangements have been proposed to underlie channel opening but the fundamental motion of M2 that opens the pore remains unknown. It was first proposed that channel opening proceeds by a rotation of the pore-lining helices around their helix axis (Miyazawa et al., 2003; Law et al., 2005) whereas subsequent studies suggested rigid-body tilting of M2 (Paas et al., 2005), a subtle rearrangement of transmembrane segments (Cymes et al., 2005; Cymes and Grosman, 2008), or a mixed picture. A subtle movement is fully consistent with the concept of "hydrophobic gating" proposed for the nAChR (Miyazawa et al., 2003; Beckstein and Sansom, 2006; Ivanov et al., 2007). Here, the large hydrophobic residues located at positions 9′, 13′ and 17′ of M2 may act as a desolvation barrier for ions instead of a steric one.

Comparison of the structures of prokaryotic pentameric ligand-gated ion channels that depict different conducting states, closed in ELIC and open channel in GLIC, has provided insights into the channel opening mechanism. The transmembrane pore of ELIC is constricted on its extracellular side: Two hydrophobic rings carry bulky side chains that interrupt the water-filled channel. Similar to the prediction for the nAChR, these residues thus probably serve as gates that prevent ion conduction in the closed conformation of the protein. In contrast, the equivalent region of GLIC shows a funnel-shaped opening with a linearly decreasing diameter that places its narrowest part at the intracellular entry of the channel. This shows a clear difference between what could be a closed or an open state and suggests that pore opening proceeds by a change of the tilt of the pore-forming helices (Hilf and Dutzler, 2009b). Although the nAChR structure was attributed to represent a closed state of the channel, it shows a continuous aqueous pore with an overall conformation of the helices that resembles the respective orientation in GLIC. Its exact relationship with the two prokaryotic structures at higher resolution is thus still unclear. In the GluCl structure in the presence of the allosteric agonist ivermectin the transmembrane helices adopt a fold like the bacterial receptors and nAChR. Binding of ivermectin at subunit interfaces spreads apart M1 and M3 domains, and stabilizes apical portion of M2 in an orientation tilted away from the pore, similarly to the open conformation visualized in the GLIC structure (Hibbs and Gouaux, 2011).

6. Number and locations of agonist binding sites required to activate homomeric Cys-loop receptors: the electrical fingerprinting strategy

The ancestral Cys-loop receptor was likely homomeric and contained five identical binding sites, similarly to present day homomeric receptors, such as α7 and 5-HT_3A receptors (Le et al., 2002; Tasneem et al., 2005). Evolution led to the appearance of new subunits which lost the ability to form agonist binding sites, giving rise to heteromeric receptors with fewer than five binding sites. The prototypic heteromeric receptors, muscle nAChR and $GABA_A$, contain only two agonist binding sites. Because homomeric receptors contain five identical transmitter binding sites, a long standing question has been how many of these sites are required to be occupied by the agonist to activate the receptor.

Direct evidence of the relationship between agonist occupancy and activation was obtained by applying an electrical fingerprinting strategy in a model of homomeric Cys-loop receptors, the α7-5HT_3A chimeric receptor (Rayes et al., 2009). The chimeric α7-5HT_{3A} receptor, which has served as a prototype for investigating the pharmacology of neuronal α7 nAChRs (Eisele et al., 1993; Rayes et al., 2005), shows unique properties for applying the electrical fingerprinting strategy. It has a very low conductance due to the 5-HT_3A pore. However, mutations of the three arginine residues in the M3–M4 linker (Kelley et al., 2003) lead to a high conductance or QDA form. The mutations increase the unitary current amplitude from undetectable to about 10 pA at a membrane potential of −120 mV (Bouzat et al., 2004; Rayes et al., 2005, Fig. 2a). Further, the mutations that affect conductance have the advantage that they do not alter the intrinsic kinetics of channel gating (Rayes et al., 2005; Bouzat et al., 2008).

After co-expressing high and low conductance forms of the α7-5HT_{3A} chimera in BOSC 23 cells, a saturating concentration of ACh elicits channel opening events with five discrete and equally

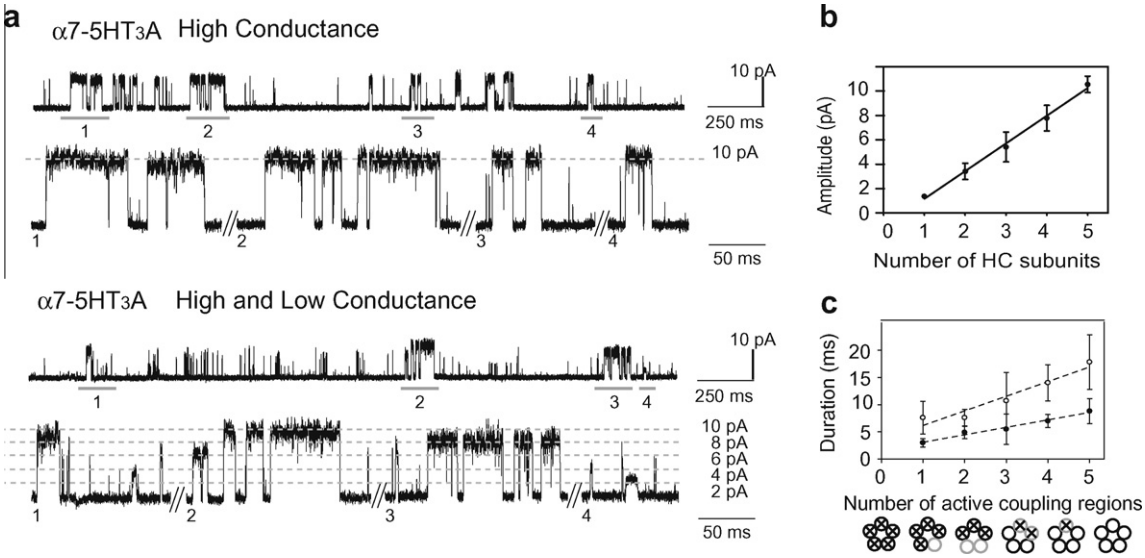

Fig. 2. Electrical fingerprinting strategy: Co-expression of high- and low-conductance forms of the α7-5HT$_3$A subunit. Single channel recordings were obtained in the presence of 1 mM ACh at a membrane potential of −120 mV. (a) Cells were transfected with the high conductance form alone (top) or together with the low conductance form (bottom panel) of the α7-5HT$_3$A subunit. Channel traces are shown at two different time scales. Segments from the 250-ms traces (marked with numbers) are shown below at higher time resolution (50 ms scale). (b) Plot of mean current amplitude (±SD) against number of high conductance subunits (HC) obtained from the analysis of channel amplitudes of pentameric receptors containing different number of HC subunits. (c) Channel lifetime (●) and burst duration (○) as a function of the number of active coupling regions. The crosses in the subunit schemes indicate inactive coupling regions.

spaced current amplitudes, one for each possible number of high-conductance subunits per pentameric receptor (Fig. 2a). The observation of equally spaced current amplitudes indicates that the number of mutant subunits, but not the location of the subunits within the pentamer, governs the unitary current amplitude (Fig. 2b). In addition, the channel open duration time does not differ among amplitude classes (Rayes et al., 2009).

Another requirement to unequivocally determine the duration of receptors with different number of functional binding sites by applying the fingerprinting strategy is to disable the agonist binding site to which the reporter subunit contributes, while allowing ACh to occupy the remaining unaltered binding sites. Structure–function studies of muscle and neuronal nAChRs established that Y190 (Sine et al., 1994) at the principal face and W55 (Chiara et al., 1998) at the complementary face are essential for agonist binding. The mutations of these residues to threonine inhibit completely the ability of ACh to elicit single channel and macroscopic currents in α7 nAChRs (Rayes et al., 2009). Thus, by combining high- and low-conductance forms of subunits carrying or not-carrying mutations at the binding site, and monitoring single channel current amplitude, the number of functional binding sites in the receptor that elicited each channel opening event can be determined (Rayes et al., 2009).

The results revealed that agonist occupancy of only one site allows channel activation but the open channel lifetime is very brief. Occupancy of two nonconsecutive agonist binding sites allows proper activation, but only when three agonist molecules are bound to the receptor in a nonconsecutive array is channel lifetime maximal. Thus, the third site resembles the positive allosteric site found in heteromeric receptors located at noncanonical subunit interfaces, such as the site for benzodiazepines in GABA$_A$ (Hanson and Czajkowski, 2008), and for morantel (Seo et al., 2009) and zinc (Hsiao et al., 2008) in neuronal nAChRs. Moreover, it has been suggested that homology between the two types of binding sites can be traced to a gene duplication event resulting in a modified subunit that contributes to a modulatory site (Smith and Olsen, 1995). Thus, it is possible that positive allosteric modulators, which have emerged as a therapeutic strategy for disorders associated with receptor deficit (Gotti et al., 2006), evolved from a site for the natural neurotransmitter. Kinetic modeling studies for 5-HT$_{3A}$ receptors (Solt et al., 2007; Corradi et al., 2009) and glycine receptors (Beato et al., 2004; Lape et al., 2008) suggest that three binding sites are required for optimal activation, in agreement with direct evidence from the electrical fingerprinting strategy in the α7-5HT$_3$A chimera.

Macroscopic recording studies reveal that in homomeric receptors the availability of more binding sites than those required for maximal activation (three) enhances agonist sensitivity (Rayes et al., 2009). The enhanced sensitivity, together with the benefit of a single self-assembling gene product, could have been important as far back as prokaryotes (Tasneem et al., 2005) and before the appearance of structurally efficient synapes. In present day homomeric receptors, high agonist sensitivity could be important in the function of extra- and pre-synaptic receptors (Jones and Wonnacott, 2004).

7. The coupling region: the extracellular-transmembrane interface is involved in coupling agonist binding to channel gating

The agonist binding site projects into the synaptic cleft, whereas the region that gates ion flow localizes within the membrane (Unwin, 2005). Communication over the 50 Å separating the two regions is thus essential to the function of Cys-loop receptors. The extracellular-transmembrane interface has attracted attention because it is a structural transition zone where β-sheets from the binding domain merge with α-helices from the pore (Fig. 1a). Within this zone several regions form a network that relays structural changes from the binding site towards the pore. Structures at the interface include: β1β2 loop, Cys-loop, β8β9 loop, and the end of β10, all from the extracellular region, and the pre-M1 region, M2–M3 linker, and the C-terminal end of M4 from the transmembrane region (Fig. 1a).

A structural interplay between loops at the interface required for coupling agonist binding to channel gating was demonstrated by generating a chimeric receptor composed of the AChBP protein,

which presumably evolved without the constraint of functional coupling to an ion pore, and the pore domain from the 5-HT_3A receptor (Brejc et al., 2001; Bouzat et al., 2004). Although the chimeric receptor expresses on the cell surface and shows high affinity for ACh, it is not functional. However, if amino-acid sequences of three loops (β1β2, Cys- and β8β9) in AChBP are changed to their 5-HT_3A counterparts, ACh binds with low affinity characteristic of activatable receptors. Moreover, ACh is capable of triggering opening of the ion pore. Since the efficacy for channel opening is low, it is possible that additional loops at the interface are required to be further exchanged to achieve high efficacious activation. Nevertheless, the findings reveal that this region mediates a bi-directional allosteric interaction between the binding sites and the pore domain and that the functional coupling process is mediated by a network of loops from both domains (Bouzat et al., 2004).

Further insights into the role of the interface in synaptic responses mediated by homomeric Cys-loop receptors emerged from studies of the chimeric α7-5HT_3A receptor. The interface of the starting α7-5HT_3A chimera carries α7 sequences in β1β2 (whose sequence is identical to that of 5-HT_3A), Cys-loop, β8β9, and 5-HT_3A sequences in the M2–M3 linker, β10 and pre-M1 region. The comparison of the kinetics of the chimera with that of the parent receptors has provided further insights into the contribution of the interface loops to channel function (Bouzat et al., 2008). Macroscopic currents of wild-type α7 channels decay very rapidly supporting extremely fast desensitization (<1 ms), and channel activation occurs mainly as isolated brief events (∼300 μs; Fig. 3). In contrast, the decay rate of 5-HT_3A currents is slow (∼1 s), and activation of the high-conductance 5-HT_3A receptor occurs in many long openings (∼100 ms) grouped in bursts, which in turn, coalesce in long clusters (Fig. 3). The decay rate of currents from the chimeric α7-5HT_3A receptor, which shows structures of both α7 and 5-HT_3A receptors at the interface (Fig. 3), is fast but not as fast that of α7 (∼10 ms), channels show intermediate open durations (∼6 ms) and activation occurs mainly in bursts of few openings, and not in long clusters as in 5HT_3A or single openings as in α7. Thus, the chimera shows an intermediate kinetic profile between that of the parent receptors. The contribution of the mixed α7/5-HT3A interface to this intermediate profile was studied by generating two additional chimeras starting from the α7-5HT_3A chimera: an all-5HT_3A chimera in which major loops within the binding-pore interface contain residues solely from the 5-HT_3A receptor (all-5HT_3A), and an all-α7 chimera in which these loops contain residues solely from the α7 receptor. Macroscopic and single channel currents from the all-5HT_3A chimera approach those of the 5-HT_3A receptor (Fig. 3), exhibiting slow desensitization and clusters of many long single-channel openings. Analogously for the all-α7 chimera, the kinetics of macroscopic and single-channel current approaches those of the α7 receptor, exhibiting fast desensitization and brief channel openings (Fig. 3). Thus, substitution of residues from the parent receptors into the extracellular-transmembrane interface of the α7-5HT_3A chimera reconstitutes the fundamental activation and desensitization properties of the parent homomeric receptors. The replacement of individual loops one at a time revealed that kinetics depends on the interplay between all loops, giving further support to the idea of the interface as a complex network of loops which couples conformational changes at the binding site to those at the ion pore (Bouzat et al., 2008).

The finding that the extracellular-transmembrane interface is involved in the rate of fast desensitization is of significance. Desensitization seems not to affect normal muscular transmission through nAChR, but it has a role in synaptic transmission in pathologies underlying gain-of-function mutations of the muscle nAChR (Elenes et al., 2006); it contributes to the termination of responses mediated by α7 and 5-HT_3A receptors (Solt et al., 2007; Bouzat et al., 2008; Corradi et al., 2009), and it may be important under the presence of drugs, endogenous ligands or phosphorylation (Gumilar et al., 2003; Arias et al., 2006). Despite its relevance in controlling synaptic efficacy, understanding the structural movements underlying desensitization has lagged behind. This is due to the fact that the extent of desensitization results not only from its onset rate but also from the kinetics of recovery from desensitization and gating. Also, the accurate measurement of desensitization rate can be achieved only under optimal time-resolution systems, and finally, desensitization probably involves many different conformational states.

Taken together, the emerging picture shows that the extracellular-transmembrane interface is a key element for coupling agonist binding to channel opening, and also for determining open channel lifetime and rate of desensitization. Therefore, this region is involved in the beginning, duration and refractory period of a synaptic response.

7.1. Key residues at the extracellular-transmembrane interface

The important role of the interface loops, β1β2, Cys, β8β9, the end of β10, pre-M1 region, M2–M3 linker and post-M4, in coupling agonist binding to channel gating has been shown in numerous studies for all members of the Cys-loop superfamily (Reviewed in Sine and Engel, 2006; Bartos et al., 2009a; Taly et al., 2009; Thompson et al., 2010).

A great number of different reports have shown that the β1β2 loop is essential for gating in different family members (Kash et al., 2003; Xiu et al., 2005; Lee and Sine, 2005). The Cys-loop, which is the signature of the family, inserts between the pre-M1 region (close to its C-terminal half) and the M2–M3 domain (close to its N-terminal half). It is essential for nAChR assembly (Fu and Sine, 1996; Green and Wanamaker, 1997). Its crucial role in channel gating has been widely demonstrated for all family members (Shen et al., 2003; Kash et al., 2003; Chakrapani et al., 2004; Xiu et al., 2005; Grutter et al., 2005). The role of β8β9 loop is less understood, but computational and experimental evidence confirm that it is involve in channel gating (Bouzat et al., 2004, 2008). This loop is long and relatively unstructured, and it is the region of most sequence variation among family members. A glutamate residue in β8β9 loop was found to undergo agonist-dependent movements during receptor activation (Lyford et al., 2003; Sine and Engel, 2006). By using fluorescence anisotropy decay to study the segmental motion of side chains in AChBP, Hibbs et al. (2006) demonstrated that agonists (but not antagonists) induce changes in conformational dynamics in the β8β9 linker. Simulations of a homology model of α7 showed that β8β9 moves inward toward its subunit. This motion occurs in all of the subunits, but it occurs to the greatest degree in the subunits adjacent to those whose C-loops move out the most (Law et al., 2005). The pre-M1 region, which connects β10 to M1, contains several cationic residues, including arginines that are conserved in several Cys-loop receptors. One of the arginine residues, R209, which is present in all family members, has been shown to have a fundamental role in gating of the human muscle nAChR (Lee and Sine, 2005). The adjacent arginine residue in 5-HT_3 receptors (R222) has also been implicated in channel gating (Hu et al., 2003). Several lines of experimental evidence reveal that the M2–M3 linker and flanking regions play a key role in channel gating in all Cys-loop receptors (Grosman et al., 2000; Kash et al., 2004; Grutter et al., 2005; Castillo et al., 2006; Jha et al., 2007). The importance of this region is also supported by the identification of mutations that lead to human diseases (Shiang et al., 1995; Sine and Engel, 2006). The M4 domain is the least conserved among the transmembrane domains, is the most hydrophobic, and has been extensively labeled by hydrophobic probes (Blanton and Cohen, 1992). The C-terminal region of M4 is located at the interface (Fig. 1a).

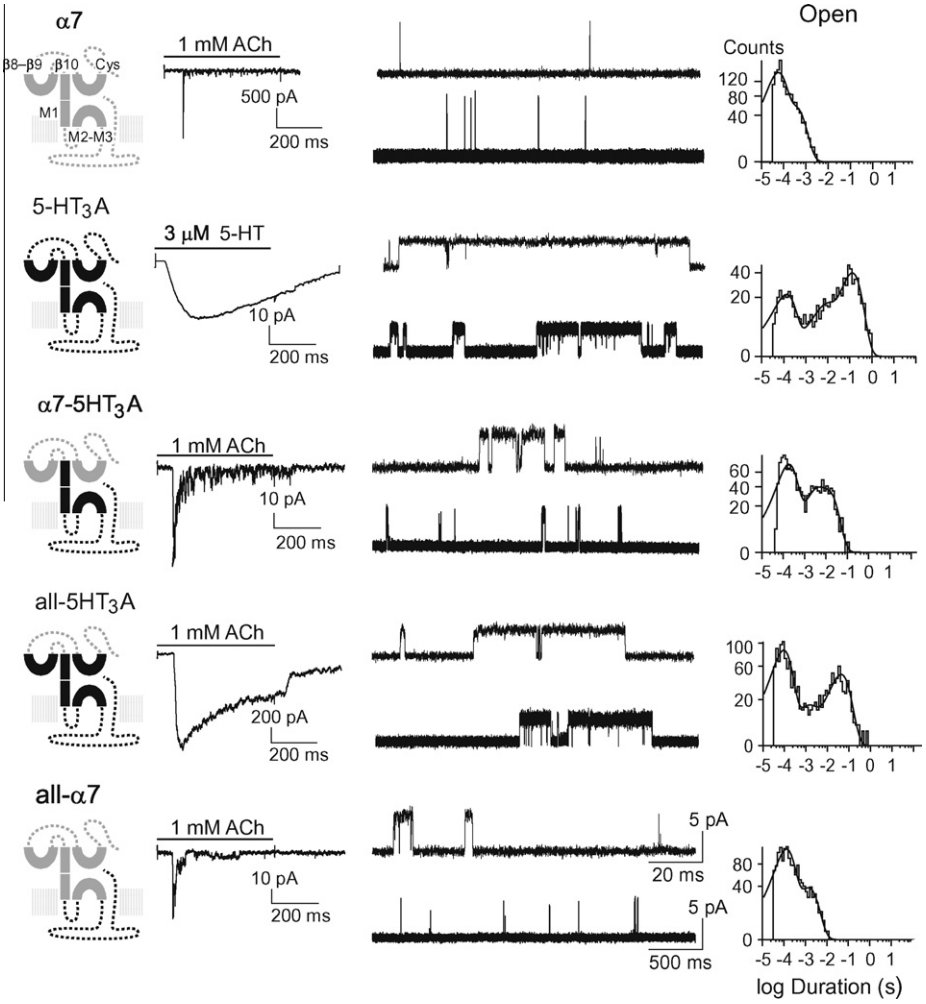

Fig. 3. Macroscopic and single-channel currents from α7-5HT$_3$A chimeric receptors. Left: Schematic diagrams representing one subunit of each homomeric receptor with α7 sequences in gray and 5-HT$_3$A in black. Loops located at the interface between extracellular and transmembrane domains are labeled. Middle: Macroscopic currents from outside-out patches elicited by rapid perfusion of the agonist at the indicated concentration. Membrane potential: −50 mV, filter: 5 kHz. Right: Single-channel traces, shown at two different temporal scales, obtained in the cell-attached configuration with the corresponding open time histograms. Single channels were activated by 100 μM ACh (α7), 3 μM 5-HT (5-HT$_3$A), and 500 μM ACh (α7-5HT$_3$A and all-5HT$_3$A). For the all-α7 chimera single-channel events were recorded in the outside-out patch configuration activated by 1 mM of ACh. Openings are represented as upward deflections. Membrane potential: −70 mV, filter: 9 kHz.

Potential interactions between residues in this portion of M4 and residues in the extracellular domain, including the Cys-loop, have been determined by computational studies of α7 (Taly et al., 2005), in agreement with large experimental evidence showing that this segment contributes to gating kinetics (Bouzat et al., 2000, 2002) and that it moves during channel gating (Mitra et al., 2004).

7.2. Crosstalk among loops at the extracellular-transmembrane interface associated with channel gating

After the identification of key residues, efforts have continued to identify key pairwise interactions involved in the structural changes that occur during gating.

Based on the structure of the *Torpedo* nAChR, Miyazawa et al. (2003) first hypothesized that gating involves a "pin-into-socket" interaction between αV46 at the tip of β1β2 loop and the M2–M3 linker. Further studies show that although these loops are essential for channel gating, the mechanism is more complex and it involves more than a single pairwise interaction (Kash et al., 2004; Reeves et al., 2005). Lee and Sine (Lee and Sine, 2005) identified a transduction pathway in which the pre-M1 domain is coupled to the M2–M3 linker through the β1β2 loop in the human muscle nAChR. Agonist binding leads to the disruption of a salt bridge between the arginine located at the end of β10 in the pre-M1 region (R209) and a glutamate residue (E45) in β1β2 of the α1 subunit (Fig. 1a). The glutamate and flanking valine (V46) residues energetically couple to conserved proline (P272) and serine residues (S269) at the top of M2, and this may be a main point at which the binding domain triggers opening of the channel. The positioning of key elements of this pathway, such as the buried salt bridge formed by R209 and E45, has been later verified by high-resolution structures of the isolated α-subunit extracellular domain (Dellisanti et al., 2007) and the bacterial channel GLIC (Hilf and Dutzler, 2008). The equivalent salt bridge has also been found to be important in GABA$_A$ (Wang et al., 2007) and GABA$_C$ receptors but it does not appear to exist in 5-HT$_3$A receptors (Price et al., 2007). Another pathway in which the pre-M1 region is coupled also to the M2–M3 linker through the Cys-loop was further identified in the human muscle nAChR (Lee et al., 2009). The studies reveal energetic coupling among αL210 from the pre-M1 region, αF135 and αF137 from the Cys-loop and αL273 from the M2–M3 linker. A highly conserved proline is found in the M2–M3 loop of nAChR (P272) and 5-HT$_3$ receptors but not in the anionic GABA$_A$ and glycine receptors. The key role of this proline in channel gating was first studied by Lummis et al. (2005) in 5-HT$_3$A receptors,

proposing that isomerization of this proline into the *cis* conformation may bend the M2–M3 loop, which may in turn move M2 and allow channel opening (Lummis et al., 2005). Proline 272 has also an important role in channel gating of the muscle nAChR, where it functionally couples to the flanking valine residues from β1β2 (V46) and Cys-loop (V132), serving as an anchor that joins the hydrophobic residues from both loops (Lee et al., 2009).

The comparison of ELIC and GLIC structures has provided insights into the movements of structures at the interface during channel activation (Hilf and Dutzler, 2009b). In GLIC the side chains of negatively charged residues in the β1β2 loop and the Cys-loop form salt bridges with the conserved arginine at the end of β10. Since neither the end of β10 nor the Cys-loop shows pronounced differences between ELIC and GLIC, this interaction might stabilize the local structure in both conformations of the channel. In contrast, the β1β2 loop differs in both structures. While in GLIC its tip packs against a conserved proline residue in the M2–M3 linker, both residues are moved apart in ELIC. Thus, channel opening may be elicited by a local change in the β1β2 loop that makes the main contacts to the moving elements of the pore domain via interactions to the M2–M3 linker. The formation of novel interactions promotes channel opening that proceeds by a tilt of the M2 and M3 helices, which move like a rigid unit to open the extracellular part of the transmembrane pore to a waterfilled funnel-shaped pore (Hilf and Dutzler, 2009b).

Summing up, there is now a large body of experimental evidence showing that several fundamental structures are conserved throughout the family, which, in turn, suggests a gating mechanism conserved throughout the family. Although the roles of loops at the extracellular-transmembrane interface seem to be conserved in all Cys-loop receptors, specific inter-residue couplings vary among subtypes. The overall findings reveal that it is unlikely that the entire responsibility of gating rests on only a few amino acids. A variety of functional and computational evidence over the last years suggests that movements around the binding site propagate through the β-strands to cause rearrangements of the interface. The emerging view indicates that β1β2 loop, Cys-loop, M2–M3 linker and pre-M1 region act jointly to allow the increase in ion conductance that follows the binding of the agonist. It is less known how other interface regions, such as the post-M4 region and β8β9 loop, participate in this mechanism.

By applying normal mode analysis, Taly and coworkers (Taly et al., 2005, 2006; Taly, 2007) explored protein flexibility on a three dimensional model of α7 and proposed the quaternary twist model for channel gating. The model describes that channel opening occurs mainly due to a symmetrical reorganization of the quaternary structure of the entire protein complex with opposing rotations of the extracellular and transmembrane domains. This global twisting motion has been also proposed by the comparison of ELIC (representing the closed state) and GLIC (potentially open state) structures (Bocquet et al., 2009).

A one-microsecond-long molecular dynamics simulation of channel closing elicited by a pH change was performed in GLIC (Nury et al., 2010). The simulation revealed an early closure of the channel followed by a concerted—yet still partial—transition, with two adjacent subunits following the same scenario, in a progressive manner, leading to a fully closed channel. The closing transition involves an overall twist movement where the extracellular and transmembrane domains move in opposite directions, similar to that suggested by the comparison of GLIC and ELIC structures (Bocquet et al., 2009) and normal mode analysis (Taly et al., 2005). The authors proposed a hypothetical, qualitative model of the full conformational transition that would progress from one subunit to the neighboring one, through a "domino-like" process, and involve at least two steps: large fluctuations at the top of the M2 helix, followed by a more global tertiary rearrangement of the whole subunit (Nury et al., 2010).

7.3. Relationship between the number of coupling regions in homomeric receptors and channel activation

The extracellular-transmembrane interface (coupling region) of the whole receptor plays a key role in channel gating and controls open channel lifetime. Because homomeric receptors contain five identical extracellular-transmembrane interfaces, one from each subunit, the electrical fingerprinting strategy was applied to determine the relationship between the number of individual interfaces and channel activation in the receptor model, α7-5HT$_3$A.

For this strategy, a receptor subunit (α7-5HT$_3$A) with both a disabled coupling region and, again, reporter mutations that alter unitary conductance was used (Andersen et al., 2011). Studies on α7-5HT$_{3A}$ chimeras showed that substitutions in the pre-M1 region, changing the 5-HT$_{3A}$ sequence PLFYAVS to the α7 sequence TLYYGLN, prevent agonist-evoked currents without affecting expression on the cell surface (Bouzat et al., 2008), indicating that the TLYYGLN mutant chimera contains an inactive coupling region. Co-expression of two different α7-5HT$_3$A subunits, one carrying a functional coupling region and the other carrying the inactive coupling region (both of which are identified by the reporter mutations), shows single-channel openings whose amplitudes can be quantified according to the number of reporter mutant subunits per receptor, allowing correlation of the number of functional coupling regions with mean open time. A plot of mean open duration against the number of coupling regions is linear and indicates each coupling region contributes ~1.4 ms to open channel lifetime (Fig. 2c). Similarly a plot of mean burst duration against the number of active coupling regions is approximately linear, with each coupling region contributing ~2.7 ms to burst duration. The linear relationships further show that each coupling region contributes independently and symmetrically to open and burst durations (Fig. 2c).

The overall findings show that whereas the agonist binding sites contribute inter-dependently and asymmetrically to open channel stability, the coupling regions contribute independently and symmetrically.

The simultaneous alteration of the numbers and locations of active coupling regions and binding sites showed that a coupling region in a subunit flanked by inactive binding sites can still stabilize the open channel, and allowed the determination of minimal requirements for channel opening regardless of stability: Channel opening can occur in a receptor with only one active coupling region flanked by functional binding sites, or with one active binding site flanked by functional coupling regions (Andersen et al., 2011).

These minimal requirements for channel opening suggest that either movement of fewer than five M2 domains is enough to fully open the channel, or more likely, that movement of fewer than five M2 domains induces a regenerating movement of the remaining M2s.

Over the past several years structural and computational studies favor one of these two possibilities. In the apparently closed state of the *Torpedo* nAChR, the narrowest region of the channel is wide enough to accommodate a hydrated sodium ion (Unwin, 2005). However, hydrophobicity of the channel severely limits the ingress of water molecules producing a negligible rate of ion translocation (Beckstein and Sansom, 2006; Wang et al., 2008). In the open state, the increased channel diameter increases water occupancy and consequently ion conduction. Although studies of model hydrophobic nanotubes suggest that small changes in channel diameter can produce substantial increases in water occupancy (Beckstein and Sansom, 2004), it seems unlikely that movement of only one coupling region and its associated M2 would allow ingress of water sufficient for full ion conduction. Instead movement of one coupling region and its connected M2 segment may promote a domino-like movement of the other four M2s that produces

a fully conducting channel. However, the M2 segments associated with an inactive coupling region, although enabling full ion conductance, may be unable to stabilize the open channel and thus reduce open channel lifetime.

8. Conclusions

Cys-loop receptors behave as chemical to electrical converters. The essence of their function relies on coupling neurotransmitter binding at the extracellular domain to ion channel opening. By the combination of affinity labeling, mutagenesis, electrophysiology, kinetic modeling, electron microscopy, and crystal structure analysis, the elucidation of the allosteric activation mechanism is beginning to emerge. Homomeric receptors are the simplest structural class of receptors of this superfamily and they therefore represent a model system to examine structural and mechanistic aspects of channel activation. After agonist binding, the closure of Loop C at the binding pocket may trigger movements that are propagated to the interfacial region between extracellular and transmembrane domains, named as the coupling region. Within this region, several loops converge to form a network of interdependent residues that relay structural changes from the agonist binding site towards the pore. This network also controls channel lifetime and rate of desensitization; thus, it is involved in the beginning, duration and refractory period of a synaptic response. In homomeric receptors, occupancy of only one binding site allows channel opening, but occupancy of three is required for maximal open-channel duration. In this type of receptors, the coupling regions contribute independently and symmetrically to open channel stability, properties that mirror those of the connecting M2 transmembrane domains, which form the walls of the ion pore. M2 domains associated with inactive coupling regions, although enabling full ion conductance, are unable to stabilize the open channel and consequently reduce open channel lifetime.

The complex gating mechanism still awaits further investigation with functional analysis combined with real time monitoring of conformational changes during channel activation guided with high resolution crystal structures of Cys-loop receptors.

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