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Review

Membrane proteins structures: A review on computational modeling tools

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ABSTRACT

Background: Membrane proteins (MPs) play diverse and important functions in living organisms. They constitute 20% to 30% of the known bacterial, archaean and eukaryotic organisms' genomes. In humans, their importance is emphasized as they represent 50% of all known drug targets. Nevertheless, experimental determination of their three-dimensional (3D) structure has proven to be both time consuming and rather expensive, which has led to the development of computational algorithms to complement the available experimental methods and provide valuable insights.
Scope of review: This review highlights the importance of membrane proteins and how computational methods are capable of overcoming challenges associated with their experimental characterization. It covers various MP structural aspects, such as lipid interactions, allostery, and structure prediction, based on methods such as Molecular Dynamics (MD) and Machine-Learning (ML).
Major conclusions: Recent developments in algorithms, tools and hybrid approaches, together with the increase in both computational resources and the amount of available data have resulted in increasingly powerful and trustworthy approaches to model MPs.
General significance: Even though MPs are elementary and important in nature, the determination of their 3D structure has proven to be a challenging endeavor. Computational methods provide a reliable alternative to experimental methods. In this review, we focus on computational techniques to determine the 3D structure of MP and characterize their binding interfaces. We also summarize the most relevant databases and software programs available for the study of MPs.

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1. Introduction

Membrane proteins (MPs) have diverse functional roles, featuring important functions such as ion and molecule transport, immune system molecule recognition and energy transduction [1]. It is therefore fundamental to comprehensively understand their structure and structure-function relationships. 3D structures of various MPs have been characterized in recent years by several experimental methods, such as Nuclear Magnetic Resonance (NMR), X-ray crystallography and cryo-electron microscopy [2]. MPs, unlike soluble proteins, are difficult to analyze in their native environment, due to their insertion in the lipidic membrane [2–3]. They are affected by the membrane and various specific factors, such as cholesterol content [4] and hydrophobic thickness of the lipid bilayer [5], but also influence the membrane structure itself [5a]. All these aspects contribute to the technical experimental difficulties in the structural characterization of MPs, which explains their relatively low number in the Protein Data Bank (PDB) [6], despite their high proportion in the human proteome [7].

The computational prediction of soluble protein structure can be considered a particularly advanced field, both in terms of variety of approaches and the accuracy they can achieve [8]. However, computational prediction of MPs and their interfaces, especially when studying dimers or high-order oligomers, is still in its early days [9]. Current approaches are usually based on a combination of homology modeling [10] or de novo protein structure determination [11] with ML algorithms [12] to predict binding interfaces and/or intermolecular contacts, and MD simulations to refine the models and study their dynamical properties [13].

Some MPs are of particular interest for therapy assessment and drug targeting given their role in physiological processes and biochemical pathways. Among them are G-protein Coupled Receptors (GPCRs), ion channels and transporters. All these cover a wide array of functions while maintaining some common traits among their respective (super)families. Here, we aim at giving a brief overview of MP and the experimental methods for determining their structure, followed by a comprehensive assessment of known computational methods for the prediction of MP structure and structure-related characteristics, such as topology and binding interface prediction. Lastly, we highlight some recent computational studies on key MPs and their main features.

2. Membrane proteins

MPs have been defined as proteins associated to lipid domains, which are involved in communication, regulation and structural coherence. In fact, proteins that entirely or partially span the membrane (intrinsic/Trans membrane (TM) proteins), as well as proteins that are peripherally membrane-bound (peripheral MPs – PMPs), can carry out these functions. Due to the high amount of information and computational methods for MPs, we focused on TM proteins, which will be referred to as MPs. For readers interested in PMPs, specialized reviews can be found covering this class of membrane proteins [14], their interaction with the membrane [15] and the experimental and computational methods for their study [16].

Only a detailed understanding of MP structure-function relationships will allow the understanding of common pathologies at a molecular level and the development of improved pharmacological procedures [17,18,19]. The most functionally relevant intrinsic MPs are typically split into ion channels, membrane receptors and transporters [1a,20]. Ion channels facilitate the diffusion of ions across membranes, bridging the intra- and extracellular environments across the hydrophobic lipid bilayer by allowing hydrophilic molecules and ions to pass through the membrane. Ion channels are structurally modulated by the TM electrochemical potential, the binding of ligands, and mechanical stress and/or changes in the local lipid environment [21]. In some cases, this modulation is required for biological function [22]. Membrane receptors, comprising GPCRs as well as olfactory receptors (ORs) and nuclear receptors [23], play roles in different biochemical and signaling pathways, and in triggering environment, immune, hormonal and neurological responses, which makes them highly interesting targets for therapeutical investigation. They often share common structural traits, allowing for their classification into protein families or superfamilies. Transporters span the cell membrane with recurring specific membrane topologies, energy coupling mechanisms and substrate specificities. They are capable of transporting molecules and ions across the membrane, triggering environment-driven responses, delivering essential nutrients and disposing cellular waste.

MPs as defined in this review consist typically of several domains: extracellular (typically involved in cell-cell signaling and/or interactions), intracellular (performing a wide range of functions such as activating signaling pathways and anchoring cytoskeletal proteins) and intramembrane (such as pores and channels) [24]. TM proteins in general are amphipathic, meaning that they have different electronegativity and hydrophobicity profiles along their structure, allowing them to be both in contact with water (hydrophilic environment) and the membrane (hydrophobic environment). The structure and function of many TM proteins depend on Post Translational Modifications (PTM) such as phosphorylation and glycosylation. The two major recurrent protein structure motifs in MPs are TM α-helices [25], repeatedly crossing the membranes in α-helical bundles and β-strands arranged into super-secondary structures known as β-barrels [26].

3. Experimental structural determination of membrane proteins

Despite their functional importance, only 4.193 structures of membrane proteins (or rather of sub-domains) can be found among the 131.485 determined protein structures deposited at the PDB [7] (statistics from June 29th 2017) (Fig. 1). This means that < 1% of all determined protein structures belong to MP families. This number includes multiple submissions of the same protein under a variety of experimental conditions. In contrast to the limited number of available MP 3D structures, there are 199.322 MP sequence clusters according to UniProt's UniRef (June 29th 2017).

Two major factors can explain this discrepancy: i) difficulties in both expression, which can be done in several organisms [27] but mostly in *Escherichia coli* (*E. coli*) [28] and purification processes; ii) challenges associated with the actual determination of the 3D structure of the
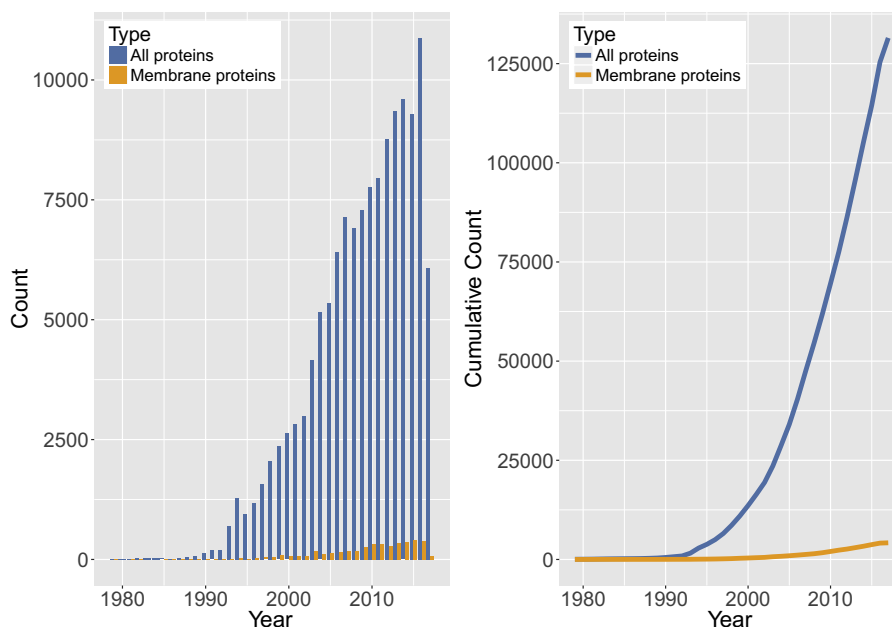
Fig. 1. PDB entries by year of deposition (left) and cumulative number (right). The total number of 3D structures is 131.485 as of June 29th 2017. As is evident in the left plot the number of MP 3D structures (yellow) being deposited every year has plateaued whereas the total number of entries has been steadily increasing since the early 1990s. Source: http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=total&seqid=100

purified MPs through NMR, X-ray crystallography and cryo-electron microscopy (the three most common methods for protein structure determination). Concerning the first point, overexpression of MPs usually leads to cytoplasmic aggregates and changes in the cell metabolism [29]. A few methods have been devised to avoid cytotoxicity, such as using and tuning *E. coli* strains that are not as affected by the protein overexpression (a well-known example being "Walker strains") [30]. Protein extraction and purification can be troublesome as well, since different conditions can lead to different outcomes when it comes to protein stability, state and viability for structure determination [31] (these conditions may come down to something as apparently simple as choosing the right detergent for MP isolation [32]).

3.1. Structure determination

Structures solved by X-ray crystallography are often the result of a high amount of time invested in fine tuning the best possible experimental conditions. After establishing good initial crystallization conditions, further optimization is required [33], namely detergent addition, use of different 3D continuous lipid phases (allowing the protein to freely flow) [34] or antibody fragments to stabilize the protein structure [35]. The latter often results in more stable crystals, but the MP conformation might differ from its native state due to the additional interactions with the antibody fragments. Data collection and analysis can also be problematic, as the variability of crystals and their conditions (i. e. hydrophobic protein regions camouflaged by hydrophobic solvent, making it difficult to assess the transmembrane MP structure) might prevent automated and stable data acquisition and processing [33]. Three noteworthy examples to illustrate the challenges associated with the experimental structure determination of MPs using X-ray crystallography are the aspartate protease, which required 160.000 different conditions in order to achieve good, analyzable crystals [36], an engineered human β_2-adrenergic GPCR, which took 15 years to be solved [37], and the 13 year-long structure determination of the membrane-integral diacylglycerol kinase [38], as noted by Leman et al. in their 2015 review paper [9].

Structure determination by solution NMR spectroscopy has come a long way as well, but some major drawbacks can still be identified: The low sensitivity, the size limitation and the intrinsic motions of the

system under investigation. When it comes specifically to MPs, more problems can be identified, such as sample preparation and spectral crowding [39]. Nonetheless, NMR has proven useful to study the dynamics (e.g. relative population and conformation of different states, exchange rates, internal motions) of MPs undergoing conformational changes, such as channels, transporters and receptors [40]. Recently, new techniques such as solid state NMR (ssNMR) have provided much better results when compared to liquid phase NMR, as there is no molecular weight cap. However, this does not prevent spectral crowding. Compared to X-ray crystallography, NMR, and in particular ssNMR, has the great advantage that is allows to study MP in an actual membrane environment and not in a "detergent simulation" of a membrane [41]. MP structure determination has also been conducted using paramagnetic tags, a technique focused on labelling MPs so that they can later be analyzed with NMR [42] and/or Electron Paramagnetic resonance (EPR) [43]. Recently, it has even been demonstrated that MPs can be studied by ss NMR in their native cellular environment [44].

Cryo-electron microscopy (Cryo-EM) is a technique that has recently gained a lot of popularity among structural biologists. Its main aspect is the imaging of radiation-sensitive entities – cells, viruses and macromolecules – under cryogenic conditions using a transmission electron microscope [45]. It offers great advantages over X-ray crystallography as it does not require crystallization. Its main drawback is the relatively low resolution for membrane proteins when compared to X-ray structures. Some recent examples of MP structures solved by cryo-EM are the transient receptor potential channel 1 at 3.4 Å [46] and the chloride conducting (CLC) ion channel at 3.7 Å [47]. A unified database for protein structures determined through cryo-EM – EMDataBank – is publicly available at http://emdatabank.org/index.html [48].

3.2. Interaction with the lipid environment

When considering MPs, the lipid environment is essential in defining their structure and function, often significantly changing the proteins' properties. While most MP structures are not easily determined, it is useful to note that some MPs can retain their structure and function while in soluble form, which can be tested through their expression as fusion proteins [49]. When this is not possible, detergents can be used to solubilize the expressed proteins [50] by extracting them from the

membrane, ideally without affecting their structure. Distinct detergents, with different hydrophobicity properties, can be used depending on the protein's properties [51]. The choice of the detergents can be time and resource consuming, with no guaranteed results [51–52]. The use of detergents leads to micelle-like structure formation, which is not an accurate representation of the bilayer environment and can result into deformations in structure. Some approaches to overcome these problems include the inclusion of MPs in nanodiscs – detergent free membrane-like structures stabilized by polymers or proteins, which allow for liquid-state NMR studies [53] – and the lipid cubic phase method [54]. The latter works by isolating a biological membrane with the target protein and solubilizing it with detergent. The resulting micelle is purified and homogenized with monoacylglycerol, and contains a bilayer with the target protein [55].

Membrane domains, such as lipid rafts, can change significantly the structure and function of some proteins as these domains have different properties (i.e. high glycosphingolipids content) [5b,56]. In lipid rafts, solvent extraction can be less effective, since these are more effective at retaining MPs than other lipid membrane domains. This works either by surrounding the protein with a tighter and more ordered lipid packing, or by other mechanisms, such as anchoring [5c]. Furthermore, even when not considering lipid rafts or lipid raft-like domains, other lipid structures and molecular organizations (depending on factors such as temperature, pressure, lipid composition and other proteins) can influence the membrane structure, which, in turn, can affect membrane-inserted proteins. This is usually referred to as lipid polymorphism, to which distinct lipid phases are associated, and which has been observed to play a role in G-protein structure and function [5d].

Some intrinsic protein properties such as hydrophobicity, van der Waals (vdW) interactions, prosthetic groups, among others, can play a major role in the interaction between the protein and the membrane. Hydrophobic mismatch, for instance, occurs when the thickness of the bilayer's hydrophobic section does not correlate with the length of the hydrophobic residues of the membrane, generating a mismatch, as characterized for example by calorimetry [57], NMR [58] and fluorimetry techniques [5a,59]. Further changes in the membrane can occur upon insertion and formation of dimers or even high-order oligomers, for example, which contributes toward the complexity of MP-membrane interactions. Other relevant changes are the insertion of peripheral groups (adding a step to the usual two step model considered for MPs' inclusion and dimerization/oligomerization) such as prosthetic groups, more elaborate protein folding, generation of new binding surfaces or portioning of space away from the lipid. This can be studied through a combination of kinetic analysis and NMR [5a,59b,60].

3.3. Oligomerization

Membrane proteins tend to form dimers (homo- or hetero-dimers) or higher-order oligomers by establishing contacts between specific TM domains. TM Helix Association has been proposed to occur in two steps (although, as mentioned above, other models have been proposed that consider additional steps [60]): first the insertion of helices in the membrane, then their association [61]. Methods like Analytical Ultracentrifugation (AUC) [59a], Thiol-Sulfide Exchange [62], Forster Resonance Energy Transfer (FRET) [5h], Steric Trapping [5e] and Genetic Systems [63] can be used to study this phenomenon. Analytical Ultracentrifugation (AUC) [59a] provides information on protein association strength and stability, by measuring sedimentation velocity and equilibrium (SV and SE, respectively) upon centrifugation. Thiol-Sulfide Exchange Method [62] enables the study of TM helix-helix association in a membrane-mimicking environment. This method promotes TM association by disulphide crosslinking, and allows the detection of dimer interfaces [64]. Since cysteine residues are labelled, and the experiment is conducted in a redox environment, it provides information on TM helices orientation in membrane-mimicking environments. FRET [5h] can be used to report the energetics of a system by following the energy

transfer between a donor and an acceptor molecule, which in turns provide distance information. When TM helices have donor and acceptor molecules, their association distances can be monitored, in membrane mimicking environments such as liposomes, providing information about the conformational changes they induce in each other. Steric Trapping [5e] tests helix interaction strength by competitive binding with monovalent streptavidin (mSA). It can probe different levels of binding strength by using mutated mSA with weakened interaction propensity. Genetic Systems [63] can inform about TM helix association by introducing reporter genes. When a dimer is formed, a DNA-binding domain activates a reporter gene, thus reporting on the dimerization. As an alternative and to complement experimental and lengthy structure determination methods, new, less time-consuming computational approaches to MP structure predictions have been developed over the years, which will be reviewed in the next section.

4. Computational structure prediction of MPs

4.1. Membrane protein structure prediction

Even though various computational methods have been developed for the prediction of soluble protein structures, most of these cannot be directly applied to MPs as the latter are incorporated in a very different environment. All available tools and algorithms had to be adapted, in particular the "solvent" representation has to be changed to create or mimic a hydrophobic layer within a hydrophilic environment. The protocols were developed and tested on well-defined MP databases. To predict MP structure, it can be helpful to consider cytosolic/extracellular partner interactions, since their interacting motifs are easier to study than those responsible for MP-MP interaction. By doing so, part of the cytosolic/extracellular regions of a protein can be determined, making it easier to identify membrane spans. Experimentally determined MP structures in different conformations may also help in achieving more accurate predictions, as some predictions might recreate only one conformation, while important interactions are also taking place in other possible conformations. Furthermore, membrane lipid composition should be considered when attempting to predict the structure of a MP as it affects the conformation and activation state of membrane-embedded proteins [65].

For the determination of tertiary structures, the most common methods are de novo methods and homology modeling [66]. De novo methods can also make use of already determined features such as secondary structure, or TM spans and topology. Incorporating that information can drastically reduce the computational costs, which nonetheless remain too high for these methods to be routinely applied on most cases of interest. Sequence-based homology modeling provides the best results within a reasonable time-frame, but depends entirely on the availability of homologous proteins with resolved 3D structures. The availability (or rather scarcity) of homologues is particularly relevant for MPs since, as was previously discussed, the number of unique MP 3D structures is significantly lower than that of soluble proteins. Some methods have been developed specifically for membrane protein modeling, namely MEMOIR (Membrane protein modeling pipeline), [67] which can model the 3D structure of a protein of known sequence provided there are available homologous MPs with determined 3D structures, and MEDELLER [68], which has provided interesting results thanks to its tailor-made MP structure prediction – a sequential prediction of protein core and loops. MEDELLER will not generate 3D coordinates for regions for which the prediction is uncertain. This has the advantage of rendering the models more accurate but also slightly more incomplete. Structural homology modeling (threading) can overcome the lack of homologues for given sequences, however, as already mentioned, the small number of experimentally available MP structures can lead to insufficient sampling. An example of a pipeline using threading is TMFoldWeb [69], a web implementation of TMFoldRec [70]. Upon topology prediction, systematic sequence to structure

alignment is performed, resulting in the selection of several templates which are ordered according to energy and reliability. Rosetta has also been widely applied to MP prediction [71]. The main improvement over soluble protein prediction was the implementation of a new membrane-specific version of the original Rosetta energy function, which considers the membrane environment as an additional variable next to amino acid identity, inter-residue distances and density [71]. Rosetta has been used to reveal important structural details in voltage sensor MPs, namely the K(v)1.2 and KvAP channels [72], and gain insight into voltage-dependent gating [73]. Recently, RosettaMP was developed as a general framework for membrane protein modeling, featuring modeling tools developed in the past few years [74].

ML methods are becoming highly popular in biological fields [75], and TM structure prediction is no exception. ML is a general-purpose approach defined as the automatic extraction of information from large amounts of data by efficient algorithms, to discover patterns and correlations and build predictive models. ML involves the creation of algorithms that improve their own performance when undertaking a certain task based on their own experience. They should be fully automatic and *off-the-shelf* methods that process the available data and maximize a problem-dependent performance criterion [76]. They aim to be statistically consistent, computationally efficient, and simple to implement and interpret. The choice of a ML algorithm for a specific problem should be made in light of its characteristics, deep familiarity with the theoretical foundations of the field, data source and prediction performance [77].

PsiPred [78] is a broadly utilized platform for secondary structure prediction that utilizes Position Specific Scoring Matrix (PSSMs) as inputs to an Artificial Neural Network (ANN) approach. However, this is hardly specific for MPs. Adding hydrophobicity scales to the prediction of secondary structures, should yield better results [79]. Initially, the utilized scales were focused on ranking single amino acids or small peptides [9]. More recent advances in hydrophobicity scales include the energy of amino acids in fully folded proteins, such as the hydrophobicity scale developed by White and von Heijne [80], which was shown to deliver the best results along with scales such as the Unified Hydrophobicity Scale [9]. Other possible features to take into account are the regions of the protein that actually face the membrane, cytosolic or extracellular sides, and which are the motifs responsible for interactions, whether they are membrane-protein interactions or secondary structure-secondary structure interactions [81].

MP topology prediction by ML techniques has benefited from the recent Big Data explosion. When applied to the study of MP, these techniques progressively increase in complexity and difficulty, beginning with the prediction of secondary structure, followed by that of 3D domains, including super-secondary structures. They are also used to predict the TM protein segments, nowadays often making use of direct residue coevolution features, which are then translated into residue-residue contacts [82,83], or to characterize proteins as MPs from genomic sequences – such as the work of Gromiha and Suwa for outer membrane proteins (OMPs) [84]. Predicting which MP regions are α-helixes has also benefited from ML methods [85]. Martelli et al. [86] developed in 2003 ENSEMBLE, a topological predictor for all-α MPs. Their method can determine how far is an α-helix in the membrane through a combination of two Hidden Markov Models (HMM) and one ANN in what is known as an ensemble approach that combines both methods. Although the individual methods were also tested separately on a dataset of 59 known, well resolved MPs, the final approach is an average of the scores coming from the different methods. With a slight improvement in predictive power, TMSEG [87] was developed in 2016. It first characterizes the protein as soluble or transmembrane, and in the latter case predicts its topology. Based on sequences and PSSM scores, this method utilizes two Random Forests (RFs) and one Neural Network (NN) in a four step process consisting of: i) a per-residue analysis; ii) a per-protein analysis; iii) a refinement method and, finally, iv) a topology prediction step. The first three steps are considered by the authors as

filters that categorize the protein as soluble or TM, while the last is the actual topology predictor. Whereas the previously addressed methods focus on α-helices, *BETAWARE* [88] has a bolder task: Detection of TM β-barrel proteins and topology assignment. It combines an extreme learning machine, a type of NN with a single hidden layer that assigns the weights between input and hidden layers in a single training step, as one would do with a linear model, [89] to decide whether a protein is a TM β-barrel or not. If this is the case, a Grammatical Restrained Hidden Conditional Random Field [90] (GRHCRF) model predicts the topology of the protein. This uses a type of Conditional Random Field [91] (CRF) model. Such models have become popular in biological sequence analysis as methods that can make stepwise predictions of MP structure, just as HMMs. Other tools have also been developed for the prediction of β-barrels and their membrane span, such as the one by Ou et al. [92], which uses sequence information and a Radial Basis Function (RBF) network – an artificial neural network that uses radial basis functions as activating functions for individual neurons [93]. TOPCONS [94], another method for the identification of MP topology works both as a meta-server and as a ML approach. It combines the topology profiles from several other MP prediction platforms and feeds these outputs to a HMM, which creates a final output. Its key aspect, compared to other methods, is its ability to distinguish MPs from signaling peptides. Merging several of the previously mentioned methods, MemPype [95] is a Python pipeline for the prediction of signal peptides, based on homology and other computational methods.

A few methods managed to combine various sources of information to predict TM α-helices and α-helical bundles, as well as β-barrels. OCTOPUS [96] may be one of the most complex ML approaches for TM α-helical spans, as it combines four different ANNs – membrane, interface, loops and globular residues – through a HMM. HMMs consist of a set of sequential states, whose progress is dependent on the confirmation of the current state [97]. TMs were also predicted using Support-Vector-Machine (SVM) - Memsat-SVM [98]. BOCTOPUS [99], developed by the same group as OCTOPUS, allows to predict β-barrels. BOCTOPUS combines local predictions through SVMs and a HMM to combine all local SVM predictions.

Another determining factor when predicting MP structure is the prediction of membrane-facing regions and interaction motifs. LIPS [100] for example takes into account hydrophobicity scales and is able to predict the orientation of helices and amino acids. PRIMSIPLR [101] is another method that operates differently, namely through the training of the algorithm with several helical MPs of known structure. To predict interaction motifs, some approaches for helical MPs have been created, such as MemBrain [102] – a ML trained server that is, so far, possibly one of the best ranking systems of its kind.

Some peculiar MP secondary structures are still not correctly predicted when applying these methods. According to Leman et al. [9], these are usually re-entrant helices (sometimes mentioned as "P-loops"), half-helices (α-helices that do not span the entire membrane), amphipathic helices (α-helices that lie on the surface of the membrane, with both hydrophobic and hydrophilic regions), TM helix kinks and β-barrels whose composition is made up by more than one chain. A good example of software able to deal with more complex proteins is TMkink [103], whose main aim is to determine helix kinks.

4.2. Membrane proteins databases

Genome-wide annotation, relating gene expression with protein expression and activity, is a useful resource that underlies many computational studies. Regarding the original human genome sequencing project, it was estimated that around 20% of the genes coded for membrane proteins [104]. For MPs it becomes relevant to annotate not only the overall protein associated-genome, but also the secondary and tertiary structures formed, so that this information can be further used for application of other bioinformatics methods [105]. THGS (Transmembrane Helices in Genome Sequences) [106] is an example of

structural annotation for membrane proteins, focusing on transmembrane helices. The Membrane Protein Hub, for instance, used sequence annotation to predict human α-helical transmembrane proteome [107]. Regarding β-barrels MPs, OMPdb (Outer Membrane Protein database) [108] focuses on outer-membrane proteins from Gram-negative bacteria, registering information on both structure and function, further organizing the entries by families, regarding evolutionary information.

As previously discussed, both the experimental and computational structural determination of MPs present unique challenges that require specialized approaches. Additionally, the community has a need for databases that specialize in the collection, validation, analysis and publication of 3D MP structures. Some of these databases are of general interest as their purpose is curation and storage of 3D structures. Examples include MPs of known 3D STRUCture (mpstruc) and MPNMR (MPs of known structure determined by NMR). MPs Molecular Dynamics (MemProtMD) [109] is a meta-database which emphasizes analysis and presents the results of MD simulations of some of the proteins in mpstruc. Finally, there are even databases of specific interest such as the G-Protein Coupled Receptor Database (GPCRdb) [110], which stores and analyses data pertinent to GPCRs.

Mpstruc is the largest repository of unique MP structures. As of June 29th 2017 it contains 702 unique entries. It is a manually curated database and its entries serve as the foundation for the identification of MPs in the PDB. Entries are categorized by their biological features, their novelty and experimental conditions under which the structure they represent were determined. More specifically, biological classification takes into account he structural elements of each protein and the way they are inserted in the membrane, in order to categorize the protein into one of the following categories: i) Monotopic MPs; ii) β-Barrel TM proteins and iii) α-Helical TM proteins. As implied by its the name, proteins in categories ii) and iii) differ from those in category i) since ii) and iii) are embedded in the membrane whereas i) has only one domain inserted in the membrane. All proteins are grouped in protein families regardless of the category they belong to. As for the novelty and experimental conditions of an mpstruc entry, they are distributed across three categories: i) unique/primary entries, ii) redundant/secondary entries, and iii) related entries. A unique entry represents a protein that has been identified as a MP by the curators of the database and no single structure of this MP is available. The unique entries can contain duplicates when multiple structures for the same protein from different organisms are available. If, on the other hand, this is not the first structure that is solved for a protein family and organism, the entry will be characterized as secondary relative to the unique structure. These entries always represent proteins from the same organism as an unique entry, and they often showcase the structure of the unique protein under different experimental conditions or with different binding partners. Finally, the related entries are always associated with a unique or secondary entry and represent the structure of the associated entry under different experimental conditions, often originating from the same publication. Fig. 2 illustrates the breakdown of the various types of entries and of the proteins they refer to. Regardless of the entry type, helical proteins are the most popular type. As mentioned, the unique entries set consists of 702 proteins, 516 of which are α-Helical, 139 β-Barrel and 47 Monotopic.

PDBTM [111] is an automatically constructed MP database. Several protein structures were retrieved from the PDB and subjected to the TMDET algorithm [112] for membrane prediction. This information was used to build PDBTM, which comprises to date 3.227 proteins – 2.848 α-proteins and 366 β-proteins (accessed in June 29th of 2017) – and acts as a basic repository of membrane proteins [111]. ExTopoDB [113] is a comprehensive database with various information on several trans-MPs topology. The topology of a protein is the simplified description of the primary structure of a given secondary structure, as well as the latter's relative spatial position and orientations [114]. Another related database is the Orientations of Proteins in Membrane (OPM) database, featuring all unique MPs or in the case of MP families with multiple solved structures, one representative structure per family, some peripheral proteins and membrane-active peptides, with adjustable membrane thickness [115]. The MemProtMD database is an analytical pipeline which embeds MPs in lipid bilayer simulations and stores and publishes the results along with instructions that allow independent researchers to set up their own simulations. The pipeline consists of identifying MPs in the PDB, converting the coordinates into a coarse-grained model (see the Molecular Dynamics of Membrane Proteins subsection) adding membrane lipids and solvent to the system and simulating it for
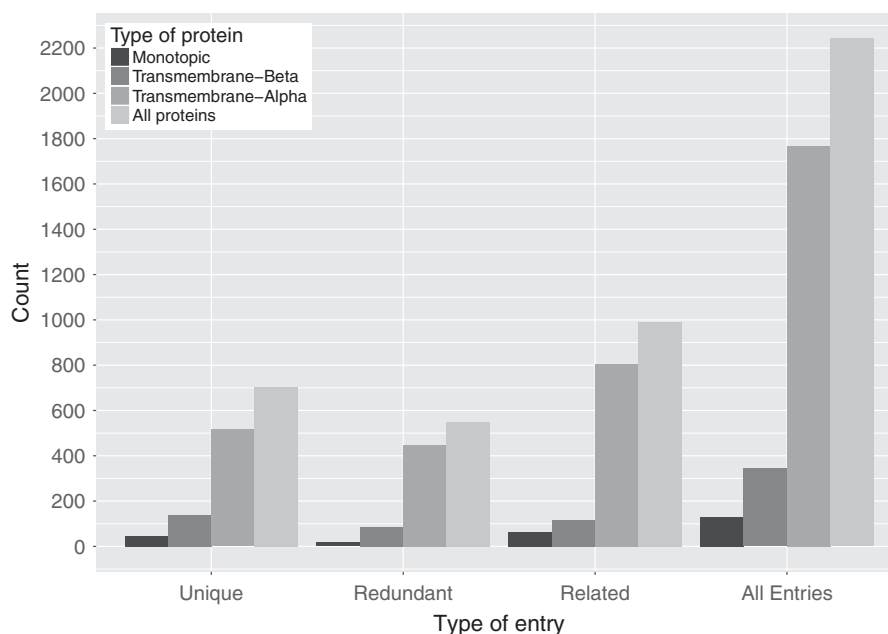

Fig. 2. Number of entries in mpstruc (http://blanco.biomol.uci.edu/mpstruc/) broken down by protein type and grouped by entry type. The α-Helical proteins are, by far, the most populated group (~78%), followed by the β-Barrel ones (~16%) and the Monotopic ones at the bottom (~6%). The most common type of entry are the related ones (~44%), followed by the uniques (~31%) and the secondary (~25%).

100 ns allowing the membrane to self-assemble in a bilayer, and followed by a 900 ns production run in order to study the membrane dynamics for a total simulation time of 1 μs. The systems are then converted back into atomistic detail and are analyzed. All TM proteins found in mpstruc can be found in MemProtMD as well. There are also specialized databases that gather information regarding important biological targets such as GPCRs and Transporters. GPCRdb [110a], GPCR Consortium and GPCR Network have made important contributions to the cataloguing of known data on GPCRs and their understanding. In particular, GPCR Consortium aggregates researchers and data on the matter, in partnerships with both GPCRdb and GCPR Network. GPCRdb provides information on specific GPCRs, their structures, known mutations, homologues, ligands and phylogenetic relationships. It contains 46 unique crystalized entries (statistics of June 29th 2017), varying in resolution between 1.7 and 7.7 Å, covering various receptor types as shown in Fig. 3. Table SI-1 contains all the aforementioned databases with a short description and their website.

GPCRdb also offers other services:

i) GPCRM: Generation of GPCR models using the available template structures;

ii) scPDB: using structure-based approaches, PDB structures are used to identify binding sites on the GPCR suitable for drug-like ligand docking;

iii) GPCR-SSFE: A GPCR – Sequence-Structure-Feature-Extractor – provides template suggestions and homology models of the helical regions of >5000 family A GPCRs;

iv) GOMoDo: Automatic homology modeling and ligand docking of GPCR receptors;

v) GPCR-ModSim: Generation of homology-based 3D models using GPCR sequences and further model equilibration using MD simulations.

Linked to GPCRdb, GPCR-OKB [116](G-Protein Coupled Receptor Oligomerization Knowledge Base) provides detailed information on GPCR oligomerization. GPCR-I-TASSER [117] predicts GPCR structure with the aid of a software program (LOMETS [118]) responsible for identifying a suitable template. It assembles full-length models through a template-based fragment approach.

The Transporter Classification Database (TCDB) is another important database that gathers functional and phylogenetic information on transporters. It features 943 transporter structures from well-defined biological systems (Fig. 4, accessed in June 29th of 2017). TransportDB [119] is another example of a comprehensive transporter database; however, it has not been updated since 2013.
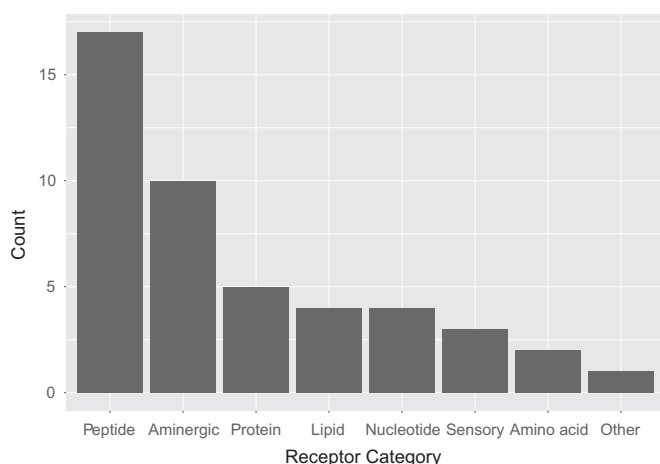

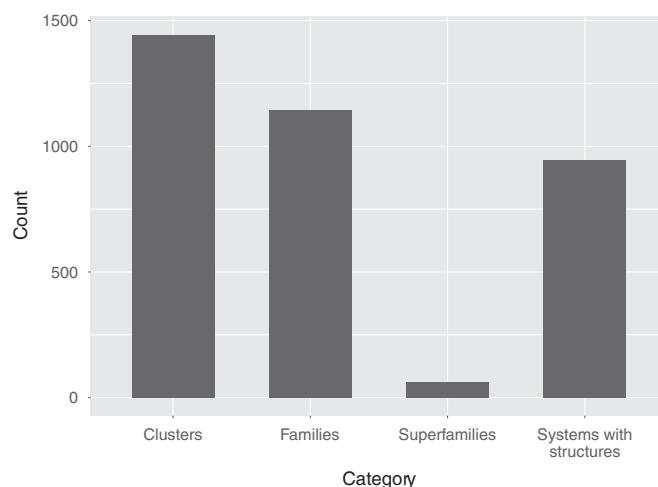**Fig. 3.** GPCRs available at GPCRdb (at June 29th 2017).

Fig. 4. TCDB information, regarding transporters (at February 29th of 2017), in http://www.tcdb.org/.

4.3. Molecular dynamics of membrane proteins

Molecular dynamics (MD) simulations have benefited greatly from advances in recent years and proven a valuable resource for the study of MPs. Various force fields have been developed for membranes such as: CHARMM36 [120] (implemented in the Chemistry and HARvard Macromolecular Mechanics – CHARMM – software) and AMBER Lipid14 (implemented the in Assisted Model Building with Energy Refinement – AMBER – software) [121]. The main difference when compared to other force fields is the number of supported acyl chains, head groups and lipids. For example, Lipid14 features 12 acyl chains, 9 head groups and 8 lipids, which enables the creation of a wide array of molecules. Although constructing these systems might seem challenging, some platforms provide simple solutions, such as the CHARMM-GUI [122], QwikMD [123] and High Throughput Molecular Dynamics (HTMD) [75f]. CHARMM-GUI is an online webserver (http://www.charmm-gui.org/), which takes input from a number of MD software programs such as AMBER [121] or GROMACS [124] and constructs a simulation-ready system with a membrane-embedded protein. QwikMD and HTMD are standalone software programs which offer accessible interfaces for both beginners and experts to perform several MD tasks, such as the insertion of a protein in the membrane, among others. When performing MD simulations of membrane-inserted proteins, protein-lipid interactions are an important factor to consider as they can be greatly affected and should be carefully modeled. For example, hydrophobic mismatch [125] may produce profound modifications in the system: When the bilayer is thicker than the hydrophobic region of a TM protein, hydrophobic residues exposure to water leads to an energy penalty, which causes an increase in the membrane thickness and a slight tilt of the protein. When the opposite occurs, the membrane is compressed to prevent interaction between hydrophilic residues and the membrane.

The membrane can be represented in MD in two ways: i) explicitly (all-atom or coarse-grained (CG)) and ii) implicitly. All-atom models represent the membrane explicitly with as little approximations as possible. One of the first models was gramicidin in a DMPC bilayer back in 1994 [126]. The system comprised 4390 atoms and the simulation was run for 0.5 ns. The access to powerful computational resources and advanced sampling techniques allow now longer MD simulations for membrane protein systems (reaching the microsecond timescale in some cases) [75l,127]. Such long simulations allow observing and characterizing interesting mechanistic phenomena. Just to name a few, Ogata et al. [128] were able to determine that transfer of oxygen,

water and protons across the thylakoid membrane by the photosystem II is actually done through different pathways [129]. Allostery is an important phenomenon which gives rise to complex intramolecular mechanisms and is often used as an information transfer mechanism across the membrane. MD can also be used to study relevant phenomena and aspects of some techniques, such as x-ray crystallography: For example, a 2016 study showed that small detergents at high concentration can bind to important protein sites, something which does not happen with larger detergents [130]. This was made possible by making use of a particular technique, accelerated MD, which allows for better search of the conformational space [131]. A similar advanced sampling method was applied to determine binding site characteristics and the differential interaction of agonists, partial agonists and antagonists in the M3 muscarinic receptor [132]. To reduce the computational expense associated with all-atom models, Markov models can be integrated. Such models assume that only the current state is important for future states. This requires an initial dimensionality reduction (which eliminates redundant data on atom coordinates) and grouping similar kinetic modes [133]. The Markov model itself is then able to project longer timescales from shorter simulations [134]. This combination allowed Razavi et al. to determine the most likely sodium release pathways for the human dopamine transporter (hDAT) triggered by hydration of the Na2 (at sodium binding sites of hDAT) [135]. As advanced sampling techniques in MD have become very relevant for the study of membrane proteins we will review a few here (for a more thorough description refer to Mori et al. [75l]). Replica exchange MD (REMD) is an advanced increased sampling technique in which several replicas of the system are sampled in parallel, each with a different temperature or Hamiltonian. Exchange between different replicas can take place if the Metropolis-Hasting criterion is satisfied [136]. REMD has been applied to study the insertion mechanism of α-helical peptides, suggesting that folding is more likely to happen inside the membrane than on the membrane/water interface as previously thought [137]. Steered MD (SMD), a technique which uses external forces to drive the simulation, was used to determine the gating mechanism of MscL – a mechanosensitive channel of large conductance – by applying external forces of 35–70 pN to residues near the membrane-water interface [138]. Metadynamics is another advanced sampling technique used to calculate several state functions – such as free energy – and it works essentially by adding positive Gaussian potential to already searched spaces. By doing so, it prevents the system from sampling previously sampled conformations, allowing a faster and more efficient description of the full energetic landscape [139]. One of its application to MP systems was the characterization of ion permeation through ion channels [140].

Despite the improvement observed in both harware and software for explicit MD simulations of MPs, the time- and length-scales are still prohibitive for a large number of systems. One approach that has been gathering interest in recent years is the use of CG models. These are able to represent lipids (and also proteins) by approximating ensembles of atoms as single particles. MARTINI [141] is one of the best known CG force fields. It uses a "four-to-one mapping", which considers four heavy atoms and their respective hydrogen atoms as a single particle. By doing so, it greatly reduces the computational cost, allowing for longer MD simulations. However, MARTINI fixes protein secondary structure, which prevents this method from being able to predict protein structure or detect conformational changes. As such, it can preferenciably be used as a tool for the prediction of the oligomerization and interactions of MPs. An interesting case study on the effect of cell membrane's curvature on protein and lipid dynamics for the F_1F_o-ATP synthase revealed that the energetic cost of membrane deformation can be reduced through side-by-side association of multiple dimers [142]. Another example was the study of β-barrel outer MPs turnover in E. coli, which led to the conclusion that an accumulation of proteins occurs in the E. coli poles due to a slowdown of their diffusion through the formation of homologous and heterologous assemblies, mostly mediated by aromatic residues [143]. An interesting study on the formation

of Ras nanoclusters in extensive CG simulations revealed that these nanoclusters influence the membrane curvature and that their clustering depends on palmitoylation and farnesylation [144]. CG simulations were also performed to observe Ras clusters and their effect on the formation of cholesterol-rich domains in the membrane [145]. All-atom and CG models can also be combined as, for example, in the study of the binding site of phosphatidylinositol 4,5-biphosphate (PIP_2) in Kir2.2: PIP_2 was found to have conserved binding sites in receptor tyrosine kinases, which are constituted by basic amino acids responsible for the clustering of PIP_2 in juxtamembrane regions [146].

Next to the explicit models discussed above, the membrane can also be represented implicitly, by some continuum model as a mathematical function, with the remaining of the system – the proteins – represented at an all–atom level. This allows to greatly increase the computational efficiency. When using an implicit solvation model, the membrane is modeled based on its solvent free energy, which depends on the free energies of (i) the electrostatic solute-solute and solute–solvent interactions, (ii) the cost of cavity formation for protein insertion in the membrane, and (iii) the solute-solvent vdW interactions. Simulating membranes implicitly can be done through equations such as the Poisson-Boltzmann (PB) eq. [147]. This equation is able to describe the electrochemical potential of a solution perpendicular to a charged surface (a membrane), which can be hard to implement in MD simulations due to its high computational costs [148]. As such, approximations such as the Generalized Born (GB) method have been considered to reproduce the PB model [149]. GB with a simple SWitching (GBSW) [150] is an implementation of a GB method in the CHARMM software [120, 151]. It considers identical dielectric constants for the membrane and the protein (represented as the solute), and a smoothing function that models membrane-water and solute-water interactions. The membrane is represented as a solvent-inaccessible infinite planar low-dielectric slab. A similar example of a GB model for membranes is the Heterogenous Dielectric Generalized Born (HDGB) method, which considers the membrane as several layers with different dielectric constants [152].

Initially these models represented the protein as cylinders or point particles inside the membrane [153], which is a rather poor representation of a MP. Since then several other aspects have been incorporated, namely considering individual α-helices, the hydrophobic effect, the large free energy penalty associated with peptide desolvation [154], hydrodynamic effects (as reviewed on [155]), or the macroscopic strain energy of the membrane [156]. Different mathematical models have been implemented, including the already mentioned PB model [157], with a wide range of applications, such as studying the interaction of peptides and ions with the membrane [158]. For example, Argudo et al. [153c] have used a model that takes into account surface area, mean curvature, Gaussian curvature, preferred curvature, and bending and Gaussian moduli [159] of the membrane as well as its compression [160] and lipid tilt. The latter considers that not all lipids are perfectly oriented according to the bilayer's normal vector [161]. The validity of implicit models has been assessed by comparing them with atomistic models. One study that looked into the epidermal growth factor receptor dimerization in the membrane concluded that atomistic Monte Carlo simulations yield dimerization rates that could differ by two orders of magnitude compared to simple partial differential equations [162]. Grand Cannonical Monte Carlo (GCMC), a technique yielding promising results when used in MP simulations, allows to simulate systems at constant chemical potential, volume and temperature (μVT). A 2014 study combining GCMC with other MD techniques shed light on the interaction between single-stranded DNA homopolymers and the α-hemolysin pore, concluding that cations act as primary charge carriers through α-hemolysin pore, and that polymer passage through the channel might lead to a flickering gate behaviour [163]. Similar techniques have been used to study ion permeation through an α-hemolysin channel in a comparative study with a continuum model showing that GCMC seemed to give better results than a continuum model

[164]. Other studies have compared MD simulations to continuum models for electropores [165] and CG models to continuum models for lipid bilayer fusion pores [166], both with not so favourable results for the continuum models, suggesting that hybrid approaches (combining both methods) might lead to better results.

4.4. Prediction of interactions between a membrane protein and its soluble partner

Membrane proteins play an essential role in key cellular functions by interacting with several different molecules and particles, ranging from photons to macromolecules such as other proteins. Typically, MPs interact with soluble partners. The modeling of those interactions becomes easier if the involved binding interfaces can be identified. Many approaches have been developed over the years for this purpose. Sequence-based methods are gaining an increasing importance for MPs. They compare sequence related proteins and identify the surface residues and even contacts that are significantly conserved, as conservation is overall correlated with functional importance [167]. Other approaches such as PS-HomPPI and NPS-HomPPI [168] predict interfaces using data on interfacial residues belonging to homologous proteins. ML methods are also used and can predict protein interfaces based on either structure, sequence or both. There are meta-servers as well, which, instead of implementing their own method, provide an interface that gathers structural and/or sequence features from several other servers and software programs and analyzes them to identify protein interfaces. An example is CPORT [169], which gathers information from six different servers and combines this information, provides results better than those ones presented by the individual servers. It is important to mention that most methods have been developed for soluble proteins and might have limitation when it comes to binding site prediction for MPs.

ProMate [170] is an interesting example of a structure-based method, which uses several features such as secondary structure, length of non-secondary structure protein regions and pairwise amino acid residues distribution to calculate an interface propensity value for each residue. Part of the development of ProMate involved the elimination of redundant or highly correlated features, which reduces computation and search space. PPIPP [171] is a good example of a sequence based method by using propensity scores based on the presence of a given residue compared to any other residue at the interface. To solve the lack of partner information, the model was trained by comparing residues in intermolecular protein-protein interface with intra-protein contacts. PPIPP is built on 24 ANN and returns the average score as final score, using PSSMs as one of its main features. PAIRpred [172] is an hybrid approach, using both sequence and structure-based features: the structure-based features consist of relative Surface Accessible Surface Area (SASA), residue depth, half sphere amino acid composition and a protrusion index, while the sequence-based features are based on PSSMs and predicted relative accessible surface area. All these are combined through a SVM to predict protein-protein interactions.

An important note made by Xue et al. in their 2015 review [173] is that partner information is very valuable for protein interface prediction, which is often overlooked. A comparison of the results obtained through PPIPP and PAIRpred – with partner information – with the ones from PSIVER [174] (sequence-based) and SPPIDER [175] (structure-based) proved that partner information greatly improves the predictions made. Evolutionary conservation of residues, and co-evolution [176], is also a growingly utilized feature in protein interface prediction [177]. It is based on the conservation of amino acids at the interfaces, as it relies on scoring residues or residues pairs, depending many times on Multiple Sequence Alignment (MSA) methods [82–83,178].

4.5. Prediction of interactions between membrane proteins

It is well known that membrane proteins can associate. Several methods have been developed to predict their interfaces. In the case of β-barrel MPs, the Transmembrane Strand Interaction Potential (TMSIP) has been recently used to predict oligomer interfaces [179]. By analyzing each strand independently, information can be gathered that hints at the location of an interface – whether it is with other β-barrels or with other structural elements. A higher TMSIP in a β-barrel OMP region indicates a high chance that there lies a protein interface that needs to be stabilized in order to lower the TMSIP for that region [180].

BTMX (β-barrel TransMembrane eXposure) [181] is a server dedicated to the prediction of β-barrel interfaces by predicting the exposure of known TM residues. It uses a simple feature based on the orientation of hydrophobic residues. Table 1 summarizes some of the most commonly used features in the literature for contact prediction of proteins.

4.6. Modeling of membrane protein complexes by docking

The determination of the 3D structure of protein complexes is of the utmost importance for molecular biology and drug discovery. Docking, a computational method that assembles the biologically relevant complex from its known constituents, is the method of choice for this task [197]. It involves "search and scoring", both of which will be addressed in the following. First, during the search phase, a large number of candidate association models must be generated. Any available biological information on the location of the interface can in principle be used to direct this step, which should generate a set of poses as close as possible to the native structure. Search methods can be split into three different approaches [198]: i) rigid docking, where both proteins are considered as rigid structures; ii) semi-flexible docking, where only one of the components (usually the smallest) or limited regions in both components are flexible; and iii) flexible docking, where both proteins are allowed to be flexible. As one would expect, the latter is more demanding when it comes to computational resources, but will probably yield the best results as it will be prepared to deal with possible conformational changes upon complex formation.

Once models (poses) have been generated, the next stage is scoring, which aims at ranking the poses and identifying the native-like models. Various metrics have been used for this purpose such as geometric complementarity, exclusion of solvent from the interface and associated entropy changes (desolvation), electrostatic and van der Waals interactions, and hydrogen bonds. A current limitation of existing scoring functions is that they do not provide accurate identification of native-like solutions. This is particularly true for MPs, as most docking software programs were developed for quaternary structure prediction of primarily water-soluble proteins. Membrane proteins are surrounded by the lipidic environment of the membrane, and consequently commonly used scoring functions are not suited to deal with these systems. Moreover, search algorithms typically do not consider the membrane, which does provide additional restraints on the possible orientations of the components of a complex.

Despite this, some methods have been specifically developed for MP docking. For single-spanning TMHs, TMDOCK [75d] is one of the most recent one, using an all-atom model for TMHs and inserting them into the membrane following the folding of membrane-associated peptides (FMAP) [75m] protocol [199]. By using templates for right- and left-handed TMH, several models are created by parallel translation of the helices, followed by energy minimization of both single TMHs and the TMH dimer. The scoring function estimates the free energy of helix association based on a combination of van der Waals interactions, H-bonds, solvation energy, changes in entropy upon helix association and helix dipole electrostatic interactions. PREDDIMER [200] is one of the oldest TMH docking software and can be described as a three-step process consisting of: (i) TMH structure prediction requiring user input for the protein sequence, pH and relative orientation of the helices in a dimer (parallel/antiparallel), (ii) TMH docking and, (iii) characterization of the TMH dimer with hydrophobic properties and contact regions. CATM [201] is another method which, based on observed recurring patterns in TMH dimer interfaces, predicts homodimeric

Table 1

Features used on several servers and methods, with the respective biological reasoning and the ML algorithm applied to program and/or train the predictor. The target for each feature is mentioned between parentheses.

Feature	Remark	Method	Reference
Amino acid occurrence in Trans Membrane Helices (TMH) segments and their amino acid frequency in proteins with different size windows (predicting TMH).	Using solely the amino acid occurrence leads to a lack of prediction accuracy. The variation of windows in order to find the perfect size and incorporating the amino acid frequency improves it.	Modified genetic algorithm	[182]
Propensity values for individual amino acids according to the Beuming and Weinstein (BW) scale and evolutionary conservation (EC) of amino acids with different window sizes (predicting the burial status of TMH proteins).	BW scales had been already used, however by adding EC to the function, a better scoring function can be made.	Support vector classifier; Fisher's indexes were utilized to assess which of the windows elements were more significant for what particular features (amino acid presence or EC).	[183]
Amino acid physicochemical properties (charge, polarity, aromaticity, size, electronic character). Each amino acid was filed in a category for each property. Compositional index (amino acid composition for TMH) (predicting TMH).	The physicochemical properties and composition index are input to different support vector decomposition machines and these were trained together in a weighted random forest. The hybrid method proved to be more efficient.	Singular Value Decomposition (reduces dimensions with strong correlation and reduced signal to noise ratio).	[184]
Normalized accessible surface area (ASA values for residues from MSMS/reference ASA values for Gly-residue-Gly)(solvent accessibility of TM residues).	ASA from membrane and non-membrane segments are different.	PSI-BLAST profiles were used on the non-redundant NCBI DB. ε-insensitive support regression vector (defining the loss function that ignores errors – values over a given distance from the SVR).	[185]
PSSM for a 9-element window centered on the target residue. 6-letter exchange group representation (20 amino acids are filed into 6 categories – letters) (identification of TMH segments).	With PSSM, patterns are recognized through a 20*n matrix display in a n-element window; amino-acids can be classified into 6 classes (which reduces the dimension, making it easier to compute).	Particle swarm optimization to optimize the weights of the different residues and 6-letters; Fuzzy SVM for machine learning.	[186]
Relative solvent accessibility, secondary structure, relative solvent accessibility, multiple alignment, KD and WW hydropathy profiles (TM domains).	Hydropathy profiles led to the confusion of MPs with globular proteins and were later excluded.	Neural networks. The main changes done were with the number of hidden layer nodes, sliding window, training protocols. In the training set globular proteins and signal peptides were also utilized (reduces confusion with these moieties in the final classifier).	[187]
Windowed PSSM profiles, position of the residue in the TMH (cytosolic side, hydrophobic, extracellular side), orientation of the sidechain (with LIPS [100]), sequence distance between residues and residue coevolution, protein length and number of helices (helix-helix contacts and interacting helices).	These features were considered as out-of-context features (apparently would not affect the predictions). As such, several features for globular and soluble proteins were taken into account (PSSM profiles, sequence distance, coevolution and protein length) and some particular MP features were added. The protein length was considered to be solely the length of the TMHs.	ANN with several Boolean vectors describing all of the features.	[188]
Solvent inaccessible residues (buried polar residues) - entirely conserved within protein families and superfamilies - and hydrogen bonded to an equivalent main chain atom in each family member (helix capping).	Conservation of polar residues and the hydrogen-bond interactions that they form play an important role in maintaining protein structure, by promoting strong restraints on amino acid substitutions during divergent protein families and superfamilies evolution.	The residue propensity to form hydrogen bonds to main chain atoms was calculated as: $$P_{arch(x)} = \frac{\left(\frac{n_{arch(x)}}{N_{(x)}}\right)}{\left(\frac{n_{arch(total)}}{N_{(total)}}\right)}$$ Where P_{arch} is the particular architectural context, $n_{arch}(x)$ is the number of residues forming hydrogen bonds to main chain atoms in a P_{arch}, $N(x)$ is the number of type x residues in the total dataset and N (total) is the total number of residues in the dataset of 131 families.	[189]
Salt Bridges under varying ion (Ca(II)) concentrations with proteins, namely membrane-anchored proteins (SNAP25) – concentration-dependent ion-induced protein oligomerization. Preferred interaction partners of Ca(II) ions.	Ion-selective effects concerning channel permeability, enzyme activity and protein oligomerization have shown to be ubiquitous, concerning ion-protein interactions, however, the underlying molecular ion-binding patterns and the effect of ions on proteins in cellular multicomponent environment have not been resolved.	Radial distribution functions (RDFs) between Ca(II) and the carbon atoms of the carboxylate groups of Asp, Glu and the C-terminus; oxygen atoms of the hydroxyl groups of Ser and Thr and nitrogen atoms of the side chain amino groups of Gln, Arg and Lys. The RDFs yielded increased probabilities for locations ranging from 0.25 (carbonyl oxygens) to 0.5 nm (amino nitrogen).	[190]
Mean value of burial propensity within a 19 residues sliding windows (B-value of the residue) – defined as the average fraction of the buried, solvent-accessible surface area relative to the total solvent-accessible surface area (proposed as hydrophobicity scales).	Topology prediction of Transmembrane Helices (TMHs); most TM proteins are TMH, strongly associated to the membrane by hydrophobic interactions.	THUMBUP – TMH proteins' topology predictor using Hidden-Markov-model based in UMDHMM (University of Maryland HMM) – UMDHMM™HP (UMDHMM for topology prediction of TMH proteins) assigning one of five states to each residue	[191]
Surface Propensity (SP) – defined by the inside/outside propensity of amino acids, Surface Fraction (SF) – reflects the probability of finding a residue on the surface of the TM protein; (also, conservation, hydrophobicity, etc.).	The method was most successful in predicting residue orientation in TMHs by combining conservation and knowledge-based features such as surface propensity.	Jack-knife approach was used to determine the SFs for the SP scale; MSA (Multiple Sequence Alignment) for the prediction of the lipid-facing probability of residues in a protein sequence, assigning a cut-off value for the Probability of the residue being inside.	[192]
One dimensional Equilibrium Structural Features (ESFs) – eight one dimensional structural features: SASA (three features), Helix-helix Contact (HCC – one feature) and Backbone conformational angles (four features).	There is little explanation on why some residues are predicted as belonging to a wrong structural class or with large errors in the absolute values of these features.	Multilayer feed forward neural network, which input is the position specific scoring matrix using Leave-one-out (LOO) method to avoid overfitting.	[193]

Table 1 (continued)

Feature	Remark	Method	Reference
Prediction of mean secondary structure, solvent accessibility and coiled-coil regions from multiple sequence alignments or amino acid sequences.	The use of different multiple sequence alignments of the same protein proves to improve greatly the results.	Neural network-based prediction. Training was done with different multiple sequence alignments for the same proteins for several different proteins.	[194]
Functional impact of amino acid substitution from proteins in the Uniprot or NCBI RefSeq databases and the residue substitution.	Functional impact is based on evolutionary conservation of the amino acid being replaced in close homologues.	Several numerical estimates for amino acid residue substitution. These are calculated through the difference in entropy, which is use as a measure of the impact of a mutation.	[195]
Evolutionary information encoded in multiple sequence alignments.	Traditional sequence-based prediction methods might not be sufficient for structure and function prediction.	Mutual information, chi-square statistic, Pearson Correlation, Joint Entropy.	[83a]
Evolutionary residues couplings.	3D complex structure determination.	Interacting proteins' sequence pairing and comparison.	[196]

interfaces – it considers important amino acid residues motifs, namely Gly-Ala-Ser (GAS$_{right}$) [202]. Its surprisingly simple scoring function mainly consists of hydrogen bonding and van der Waals interactions. Evolutionary-based TMH docking can also be performed by EFDOCK-TM that combines several approaches and features, such as EVFold [203] (evolutionary features), LIPS [100] (selection of interacting surface region for TMHs) and OCTOPUS [96] (determining intramembrane protein segments).

Most methods described in Sections 4.4 and 4.5 are summarized in Table SI-2, which contains a short description of the method as well as the easiest way to access it.

5. Case studies

In this section, we will limit ourselves to GPCRs, transporters and ion channels which constitute the main target of many computational/experimental drug targeting strategies.

5.1. G-protein-coupled receptors

GPCRs belong to one of the largest superfamilies of membrane associated proteins with the most diverse functions [204]. GPCRs share a typical pattern consisting of seven TM helixes (TM1–7) and similar intracellular binding partners. Three High Variability Regions (HVRs) have been identified between TM5 and TM6 and at the N- and C-terminal regions [205] (Fig. 5 A) [206]. Even though GPCRs share high structural similarity, their ligands can range from a photon to a protein [207]. GPCRs can receive distinct stimuli, having roles on metabolic, neuronal, hormonal and immunological functions, as well as in cell growth and cell death [208]. Apart from their ligands (e.g. G-proteins, arrestins and GPCR-interacting proteins (GIPs), membrane-inserted GPCR-binding proteins [209]) the lipid membrane environment also has an active role in modulating GPCR structure and function. For example, interaction with cholesterol significantly changes GPCRs conformational flexibility [210] and modulates their interactions. As such, it was suggested that rather than "binding sites", GPCRs, many times, have "high occupancy sites", when associated to these cholesterol "hot-spots" in the membrane. Constitutive internalization of GPCRs, a crucial cellular function responsible for receptor regulation, is regulated by GPCR interactions and can be clathrin-dependent or clathrin-independent, stressing the large array of interactions and the versatility of GPCRs [211]. Trafficking of GPCRs, which can be agonist dependent or independent, commonly displays an important role on the signaling routes these receptors are involved in [211].

GPCRs play a central role in an enormous variety of cellular mechanisms in human physiology and disease and are the targets of 40% of
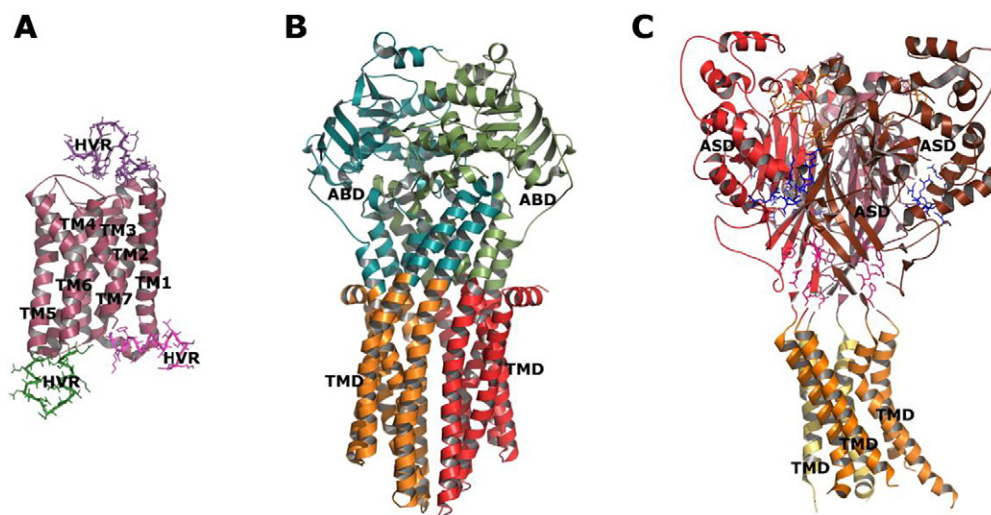

Fig. 5. Examples of membrane protein structures. (A) 2.65 Å resolution crystal structure of bull opsin (PDBID: 4J4Q [206]). The 7 transmembrane α-helical domains (TM1–7) – a key aspect of GPCRs – and the three high variability regions (HVR) are indicated. The latter correspond to the C-terminus region (pink), the extracellular loop between TM5 and TM6 (green) and the N-terminus region (purple) [206]. (B) 3.42 Å resolution crystal structure of the E. coli ATP-Binding Cassette (ABC) Transporter McjD (PDBid: 5EG1 [212]). Its key features are its TM and ATP-binding domains (ABD). The latter are responsible for binding and degrading ATP in order to power the drug-pumping function typical of ABC transporters. McjD is a good example of how the lipidic environment affects the stability of the MP. Through MD simulations, Mehmood et al. [212] were able to determine the synergistic effect of zwitterionic lipids on its stability, besides from McjD negative lipid-dependent function and the impact of both on the structure of McjD. (C) 1.9 Å resolution crystal structure of the chicken acid-sensing ion channel (ASIC) 1 in its closed state (PDBid: 2QTS [213]). The key aspects of this homotrimeric ion channel are its acid sensing domains (ASD) which, upon activation, lead to the opening of the channel, formed by its transmembrane domains (TMD). The ASD where named as such because of their ability to bind both protons and chloride. Each ASD has 3 regions of amino acids (colored in pink, blue and orange) which provide essential interactions for the structure of the ion channel [213].

all commercialized drug targets. As such, they are the subject of major efforts toward understanding their function and signaling selectivity [214]. New insights have been provided by recent GPCR structures in selected conformations, stabilized by a variety of ligands with pharmacologically distinct properties (agonists, inverse agonists, etc.), by nanobodies mimicking signal transducers [215], and in some cases by full heterotrimeric G-proteins (GTP-binding protein) [216,217]. Still, the molecular mechanisms connecting GPCR structure to these states and how these states are related to GPCR functional mechanisms remain undetermined.

Computational methods can be useful in understanding structure-activity relationships [218], conformational changes upon ligand binding [219], and complex mechanisms such as allostery [220]. Such methods have also been used to study of GPCR dimerization and oligomerization [221], explaining more complex GPCR signaling pathways [222], and to clarify molecular mechanisms of signaling selectivity by exploring the dynamic properties of the systems [75i,223]. Using evolutionary conservation, a GPCR-independent mechanism of trimeric G protein activation [224] was elucidated, proving the significance of common regions across these proteins and illustrating how co-evolution can elucidate complex GPCR-related mechanisms.

GPCRs comprise a large number of different receptors. Dopamine receptors (DR), for instance, have been studied under the scope of determining structural relevant elements, such as intracellular and extracellular loops (ICLs and ECLs, respectively, which have been deemed critical for their interaction with many other effectors) as well as agonist and antagonist binding [225]. When considering drug design against GPCRs, studying the differences between the active and inactive state [226] is important, as is the case for Dopamine receptors 2 and 4 (D2R and D4R, respectively) [227]. Computational studies have also been able to assess how post translational modifications (PTMs), such as phosphorylation, influence GPCR-arrestin interaction [228]. The interactions GPCRs with G-proteins is another relevant field of study since their complexes can generate several biological effects. Many of the published studies have concentrated on the rhodopsin-like family (class A) [229]. The effect of dimerization and allosteric communication on GPCR activation has also been studied computationally [230], particularly for D2R. In particular Helix 8 (HX8), a perimembrane substructure, has been identified as a key player in D2R-PDZ domain interaction [231]. Another GPCR, Serotonin-2A, a serotonin receptor, has also been studied through computational methods: A hybrid approach using experimental data (NMR and mass spectrometry, for example) and computations (MD) revealed the importance of the ICL2 substructure when considering conformational changes upon ligand binding [232]. Metabotropic Glutamate Receptors (mGluR) are yet another thoroughly studied GPCR family, since they are involved in a large array of neurologic disorders. For example, it was found that orthosteric modulation by ligands is apparently not as relevant as allosteric modulation. This led to the design of allosteric drug (both negative and positive) against mGluR [233].

5.2. Transporters

Transporter proteins are one of the most interesting and diverse groups of TM proteins. The TCDB database [234] contains about 10,000 transporter protein sequences distributed across > 1110 families. Such a high number of families has given rise to algorithms for the prediction of transporter targets using RBF networks [235], which simplifies the assignment of families to individual transporters. The ATP-binding cassette (ABC) transporters superfamily is one of the largest and most important transporter superfamilies [236], with 7 different gene families [237]. 48 human ABC transporters have been identified, with several different targets and cellular locations [238]. Their involvement in diseases such as Alzheimer's, Tangier's, Harlequin Ichthyosis and Stargardt's [239] makes them highly relevant and, as such, common drug targets [240].

ABC transporters are structurally organized in at least 4 domains: 2 nucleotide-binding domains (NBDs), also known as ATP-binding cassettes, responsible for the binding and hydrolysis of ATP, and two TMs, responsible for substrate transport and typically consisting of 6 helices, across 2 different chains [241], as shown in Fig. 5 B for the E. coli ABC transporter McjD [212]. Even though ABC transporters are expressed in both prokaryotes and eukaryotes, few structures are available in the PDB [242]. Pinto et al. [241] have reviewed how structure relates with drug interaction in ABC transporters and how computational methods, namely web platforms, can be used to determine drug inhibition and transport mechanisms. The major drawback seems to be the absence of a generalized method applicable to all ABC transporters since each method is custom made for a single receptor or for a small subset of receptors. Interesting strategies to further understand ABC transporter-drug interactions could be the identification of recurring features through different methods and their combination to build models to predict these interactions, or the study of transporters according to their targets, as was done in a recent study by Gromiha et al. [243], leading to the identification of mutations responsible for activity and drug response alterations.

Glutamate transporters [244] are an interesting case when discussing transporters, since various computational methods were used to understand their structure and mechanism of action, such as homology modeling and MD. The major setback is the lack of homologous structures, since the only available structure was of the archaeal glutamate transporter Glt_{Ph}, which was resolved in 2007 [245]. Functionally, it is highly similar to its prokaryote and eukaryote homologues [246]. Glt_{Ph} is a trimer, each monomer being composed by eight primary α-helices (TM1–8) and two helical hairpins (HP1 and HP2). TM3, TM6–8 and both hairpins in each monomer are responsible for the co-transport of 3 Na^+ and 1 aspartate. Most MD simulations have been conducted to describe this transport mechanism [247]. Homology modeling studies were able to show that, despite the low sequence identity between Glt_{Ph} and Excitatory Amino Acid Transporters (EAATs), the mammalian counterparts of Glt_{Ph} and LeuT, their binding pockets are 60% homologous, with important amino acids being present in both Glt_{Ph} and EAATs, which should explain their different mechanisms [247] (EAATs co-transport 1 glutamate, 3 Na^+, $1H^+$ to the inside of the cell, while exporting 1 K^+ [248]). MD simulations of EAAT3, based on a homology model of the Glt_{Ph} homologue, have highlighted the necessity of protonating E374 to stabilize the binding of glutamate [249], revealed the location of the potassium binding site and shed light on the actual opening and closing mechanism.

Glt_{Ph} belongs to a rather large group of membrane transporters called sodium symporters. As already mentioned, their main driving force for transport is a sodium ion (and sometimes chloride) gradient, whose dissipation enables the transport of other substances, such as leucine and aspartate, besides glutamate. LeVine et al. [129] recently reviewed the information from different techniques, including MD, and compared different symporters, namely Glt_{Ph} and LeuT. They showed that these transporters share common features in their allosteric regulation: Regardless of sequence and 3D structure, they share common functional motifs that allow them to bind specifically both ions and substrate for their transport. Such findings lead to a better understanding of a protein class that also encloses sMATs (MATs will be further discussed in the following paragraph) and EAATs.

Another transporter protein, MonoAmine Transporters (MATs), is responsible for the reuptake of monoamine neurotransmitters (serotonin, dopamine and norepinephrine) in presynaptic neurons [250]. MATs are named accordingly to their substrate, hSERT (human Serotonin Transporter), hDAT (human Dopamine Transporter) and hNET (human Norepinephrine Transporter). The transport is energetically driven by the co-transport of sodium and chloride ions [251]. Unfortunately, no high-resolution structure is available for MAT. However, in 2015, Koldso et al. [252] developed models for each of the human MATs using Drosophila melanogaster DAT (dDAT) and an engineered

LeuT (LeuBAT) as templates. Their binding to substrate and to different psychostimulants, antidepressants and Mazindol, an anorectic, was assessed, revealing important parts of the mechanism of all human MATs.

Several studies throughout the years have revealed recurring structural motifs on different transporter proteins. Shi [253] gathered all recurring folds and secondary structures motifs presented by secondary transport proteins. These were considered the Major Facilitator Superfamily (MFS) fold, the LeuT fold and the Na^+/H^+ antiporter (NhaA) fold. They mostly consist of different rearrangements of α-helices into structurally identical monomers. Upon dimerization, these monomers forming the secondary transport protein. Another key feature of transporters is how monomers orient in an anti-parallel fashion upon dimerization.

5.3. Ion channels

Ion channels enable the crossing of hydrophilic molecules (ions) through a hydrophobic environment (lipid bilayer) [254]. These MPs are key at regulating the concentration of ions and are central to several biological functions, such as the generation and maintenance of electrochemical gradients [255]. Ion channels connecting intracellular and extracellular environments are generally narrow and very sensitive, while ion channels connecting intracellular environments are larger and allow for a steadier flux of ions. It was shown that these channels are ion selective and have "open" and "closed" states, allowing for the maintenance of electrochemical gradients. To better understand their role in neuronal communication, it is necessary to cross-reference ion channels with membrane carriers and pumps [256].

Ion pumps are essential for the maintenance of intracellular ion concentrations, being able to generate gradients at the expense of energy. Ion channels, on the other hand, feature sensitivity and high ion conductivity, allowing them to rapidly disrupt gradients generated by pumps by allowing ions to move according to their electrochemical gradient. This makes them decisive, for example, in the generation of electrical impulses, which can propagate and shape a well-functioning nervous system. In some cases, ions can also act as secondary messengers (e.g. zinc, calcium magnesium divalent cations), which is relevant in innate and adaptive immunity, where ion channels, pumps and carriers play a role in this process [257]. The membrane electrical potential is largely influenced by ion concentration and fast leakage, making these ion channels highly relevant in therapy, particularly in neurodegenerative diseases [258].

The voltage-gated ion channel superfamily, including the K^+-, Na^+- and Ca^{2+}-channels, with four homologous functional units consisting of six TM domains are key examples due to their important therapeutic and functional role. The fourth domain has been shown to work as the voltage sensor, while the linker between the fifth and sixth domains forms the pore structure for the passage of ions. The ligand operated receptor channel superfamily, associated to neurotransmitter receptors such as GABA$_A$ receptor, glutamate receptor and nicotinic acetylcholine receptor are MPs composed of five homologous subunits, each containing a large hydrophilic amino-terminal domain which binds ligands and/or competitive blockers, followed by four TM segments [259]. Computational algorithms have been developed for ion channel classification. VGIchan [260], makes use of amino acid sequences and ML-based tools to classify channels into sodium, potassium, calcium or chloride channels, while the Voltage-Gated $K+$ Channel Database (VKGDB) [261], analyses and compiles data regarding channels using phylogenetic analysis and HMMs. Due to the relevance and increasing information available regarding ligand-gated ion channels, the Ligand-Gated Ion Channel Database (LGICdb) was created [262], containing 554 entries (accessed in June 29th of 2017). Channelpedia is another database with information on most ion channels [263].

A few studies on structural prediction on ion channels were published recently. Ion channels usually undergo conformational changes to perform their designated biological functions. A structural model of the "down state" of a potassium channel voltage sensing domain (VSD) was predicted through homology modeling from a supposedly up-state – the gating state corresponding to the activated receptor [264]. The up and down states of VSD are important to understand conformational changes happening upon activation as they represent the states for positive and negative voltages, respectively. Acid-Sensing Ion Channels (ASICs) are voltage-insensitive cation channels sensitive to extracellular photons whose activity can be altered by several agents [265]. These channels are believed to be composed by 3 chains, each containing one large, bulky acid-sensing domain (ASD) and one TM domain (TMD). The ASDs of the Acid-Sensing Ion Channel 1 (ASIC1), besides binding both protons and chloride ions, are also responsible for the key interactions that maintain the homotrimeric structure of ASIC1, across three different regions [213] (Fig. 5C). Since ion channels typically visit a large ensemble of possible conformations, the combination of different computational techniques can lead to a better understanding of their structure-function relationship.

Homology modeling and evolutionary couplings were used in the study of Cyclic nucleotide-gated ion channels, involved in visual and olfactory sensory transduction [266]. The results support a modular model of allosteric gating, according to which protein domains can move independently, but are coupled to each other. Despite the lack of a proven global approach to the prediction of ion channel structure, they remain targets for drug design with computational methods such as homology modeling, ligand-based methods and structure-based methods being used on a case-by-case basis [267].

An important research focus for ion channel function are lipid-protein interactions, which have been shown to be an important factor affecting the channel structure and function [22]. To name a few examples, nonanular lipid binding to the pore domain of a mutant KcsA-Kv1.3 channel – a prokaryotic potassium channel and a human potassium channel, respectively – was shown to increase its activity [268] and, through an hybrid experimental-computational approach, Kir2.1 was shown to bind cholesterol in two nonannular hydrophobic regions that affect the hinge motion responsible for channel gating [269].

6. Conclusions

In this review, we have presented an overview of both theoretical and experimental methods used for the structural characterization of membrane proteins and their interactions. We have shown that the combination of computational tools and MP-associated data offers approaches that are complementary to the more expensive and time-consuming experimental studies. Even though computational methods for MP study depend on available data, the rising trend for experimental methods to perform better at characterizing MPs draws an exciting future for novel integrative approaches to study these highly relevant proteins. The many developments in computational methods for predicting MP topology, structure and interactions, together with the ever-increasing amount of experimental data, computational power and new user-friendly interfaces to access these methods, should boost research in this field and enable researchers with little training in computational biology to further advance the field of MP structure.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.07.008.

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