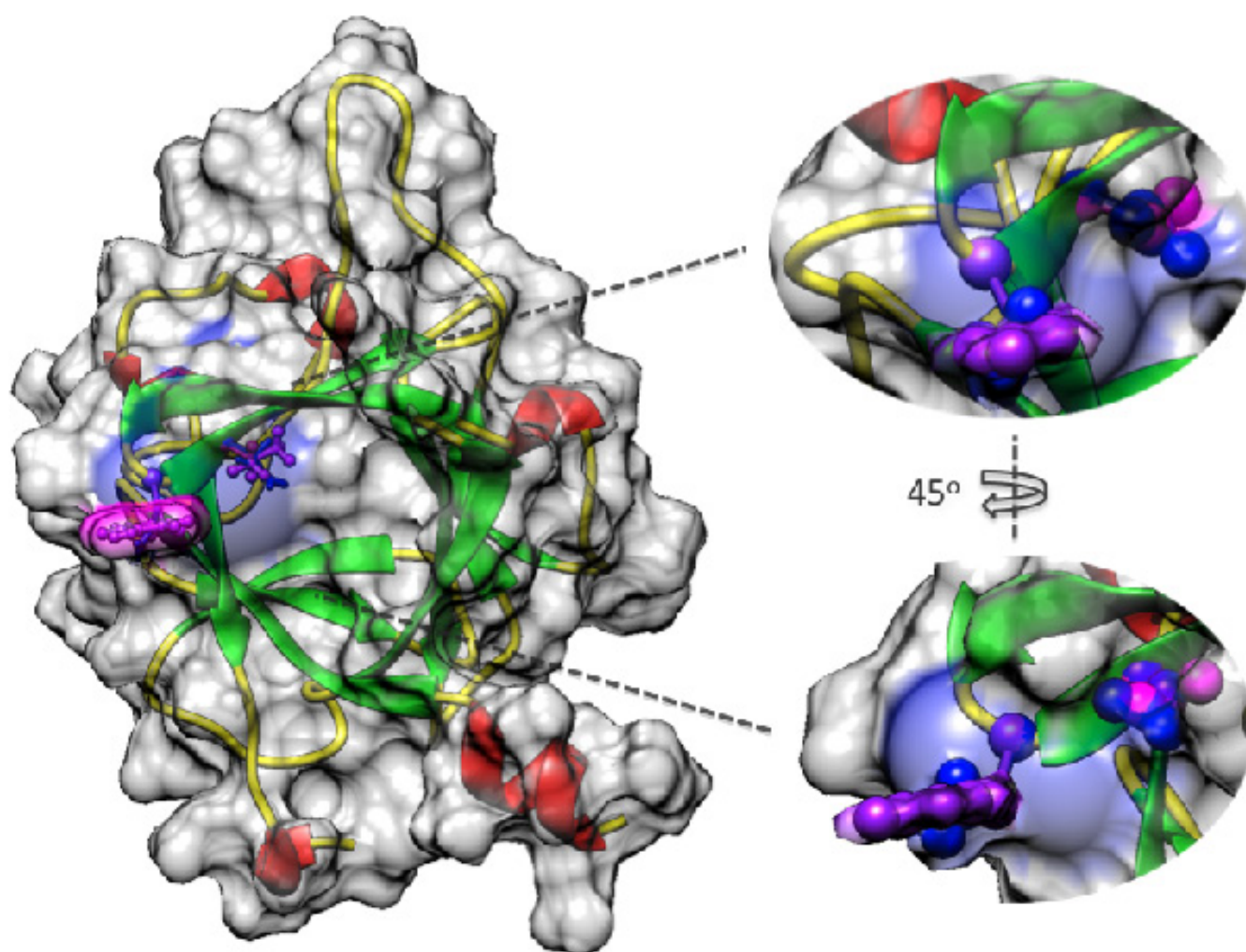


## Protein-Protein Interactions as New Targets for Ion Channel Drug Discovery



**Superposition of FGF14 wild type with FGF14 Y153N/V155N.** The secondary structures are colored as follow: alpha-helices in red, beta-strand in green and beta-turn in yellow. Tyrosine (Y) 153 is colored in purple, Valine (V) 155 in magenta, Asparagine (N) N153 and N155 are in blue. Cover illustration by Dr. Svetla Stoilova-McPhie.

Review Article

# Protein-Protein Interactions as New Targets for Ion Channel Drug Discovery

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## Introduction

Proteins are fundamental players in cell cycle, cell proliferation, growth, differentiation, signal transduction and programmed cell death, but the understanding of how these processes occur remains an outstanding scientific question [1]. A fundamental concept that has emerged in the post-genomic era is that proteins do not operate in isolation, but are rather part of multi-macromolecular complexes [2] that dictate protein function and influence the cell environment. The identification of these interactions and the comprehension of how this intercatome operates in the cell will definitely further our understanding of human diseases and open new horizons in therapeutics providing new targets for drug development.

Ion channels, the large transmembrane proteins that control the flux of ions in and out the cell, are emerging as a critical group under the regulation of PPI. These interactions control trafficking and biophysical properties of both voltage-gated and ligand-gated ion channels with implications for human diseases, especially in the CNS. In this review we will discuss prominent features of PPI and available methods to study these interactions in the light of pharmacotherapeutic development targeting ion channels.

## Properties of PPI

Although recognized as key elements of all cellular processes, PPI have emerged as potential drug targets only recently [3]. Historically,

## Abstract

Protein-protein interactions (PPI) are key molecular elements that provide the basis of signaling in virtually all cellular processes. The precision and specificity of these molecular interactions have ignited a strong interest in pursuing PPI surfaces as new targets for drug discovery, especially against ion channels in the central nervous system (CNS) where selectivity and specificity are vital for developing drugs with limited side effects. Ion channels are large transmembrane domain proteins assembled with multiple regulatory proteins binding to the intracellular portion of channels. These macromolecular complexes are difficult to isolate, purify and reconstitute, posing a significant barrier in targeting these PPI for drug discovery purposes. Here, we will provide a short overview of salient features of PPI and discuss successful studies focusing on protein-channel interactions that could inspire new drug discovery campaigns targeting ion channel complexes.

PPI have been known for their flat, featureless, and large interacting surfaces that made them unattractive for drug discovery purposes. Over the past decade, though, the identification of “hot-spots” has revolutionized the concept of PPI surfaces. Though the average size of a PPI surface is estimated to range between 1150 Å and 4660 Å [4], it was discovered that interactions between proteins are governed by only few amino acid residues, named “hot spots” that contribute much to the free energy of interaction. “Hot-spots” are usually surrounded by hydrophobic regions and engaged in hydrogen bonds through their side chains. Studies have shown that conversion of these “hot-spots”, usually tryptophan (Trp), tyrosine (Tyr) and arginine (Arg), into neutral alanine (Ala) [5] significantly decreases PPI, indicating that intervention on single residues are sufficient to induce drastic structural changes at interacting protein surfaces. With these milestone studies, the idea of using “hot-spots” as druggable targets has become appealing in the drug discovery field [3,6].

Despite the enthusiasm raised by these discoveries, challenges in studying and targeting PPI for drug discovery purposes still remain. Difficulties in studying PPI arise from limited accessibility and instability of certain “hot-spots”. For example, in convoluted PPI surfaces “hot-spots” are often buried within small and deep cavities and as such are hard to probe [7,8]. In other cases, interactions are transient and not visible with classical high-resolution structural methods [9].

Another critical element in considering PPI for drug discovery purposes is the choice of compounds to target “hot-spots”. Traditionally, PPI have been targeted by peptides mimicking interacting domains. Peptides can provide a combination of potent binding and exquisite selectivity. In practice, though, peptides make often poor drugs. Peptides have restricted membrane permeability [10], are rapidly degraded, and are structurally unstable in solution. In addition, their active conformations are not easily predictable *in silico* or defined experimentally. As such, peptides have poor pharmacokinetic properties with low absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles [11,12] and require chemical efforts to be converted into pro-drugs.

Usually, the conversion of a peptide into a drug-like molecule is achieved by creating non-peptidic scaffolds that mimic peptidic motifs by converting side chains into diverse chemical groups, by generating cyclic peptides, or by substituting part of the molecule with  $\beta$ -turn mimetics. These modifications are typically designed to impose structural constraints on the peptide mimicking its active conformation and to improve the peptide ADMET profile. This lengthy, time-demanding approach requires a substantial amount of trial-and-error validation. An emerging cutting-edge and more rapid approach in medical chemistry is to target protein-binding interfaces using small molecules either alone or chemically conjugated with peptide-based fragments [13-18]. Small molecules are membrane-permeable, promiscuous, and highly active in functional PPI. With the advent of new chemistry and chemoinformatic efforts, large libraries of small molecules have become available to the academic and pharmaceutical communities, providing a new base for drug discovery against PPI [19]. In summary, the milestone identification of critical "hot-spots" as the key elements of PPI, the knowledge of druggability of these "hot-spots", and the availability of large and diverse chemical libraries of small molecules have opened a new era in pharmacology with PPI playing a central role in the drug discovery process [20,21].

### Targeting PPI within ion channel complexes

In the CNS, ion channels play fundamental roles mediating synaptic transmission and neuronal excitability and also participate in governing the ability of neuronal circuits to adapt to environmental stimuli [22]. Ultimately, the fine-tuning modulation of ion channels encodes the molecular mechanisms underlying complex cognitive and motor functions. Not surprisingly, ion channels are linked to a variety of human disorders [23] and as such are appealing targets for therapeutic development. For decades, though, pharmacological efforts targeting ion channels have been directed against toxin-binding sites, voltage-sensor domains, or orthosteric ligand-binding sites, which are highly conserved across the ion channel family [24] and pose potential problems with selectivity and toxicity of the resulting drug. Emerging directions in pharmacology include the search for alternative, less-conserved drug targets within macromolecular complexes of proteins that bind to and control the function of ion channels *in vivo*. Because PPI interfaces are highly specific and flexible and are governed by few key residues, they could serve as ideal scaffolds for drug design—especially against ion channels in the CNS where selectivity and specificity are vital for developing drugs with limited side effects. Given that this approach is nascent there is still a significant lack of adequate platforms to screen and study protein-channel interactions, hampering drug discovery initiatives in this area of pharmacology.

Ion channels are large transmembrane proteins that are hard to purify, reconstitute, and study in isolated systems. Traditional methods using X-ray crystallography, nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), or fluorescence polarization (FP) require high protein yield, which is difficult to achieve for an ion channel alone or even more challenging if the channel is combined with binding partners. Other approaches employed for studying ion channels include low resolution in-cell methods using heterologous cell lines transiently or stably expressing ion channels which could be assayed through manual and/or automated patch-clamp electrophysiology [25], fluorescence-based methods [26] or ion flux assays [27]. These approaches have certainly proven useful in many

drug discovery campaigns targeting ion channels [28], but have not yet been commonly employed to screen PPI within channel complexes. For this purpose, additional molecular procedures would be required to transiently and/or stably co-express the ion channel binding partner and reconstitute the complex in live cells. Furthermore, in the context of a protein-channel complex, functional assays require a full understanding of the mechanism of channel regulation by a given interactor. This information might not always be available in early-stage studies (i.e. as soon as an interaction is identified through high-throughput screenings). Thus, a more desirable approach would be to define the PPI interface of the protein-channel complex prior to any functional assay or other more complex evaluations (i.e. *in vivo* recordings and behavioral studies). Approaches restricting the analysis to the ion channel fragment and its interacting partner could serve as an optimal starting platform to begin the evaluation of PPI interfaces at protein-channel complexes, bypassing the reconstitution of the full complex and rapidly gaining structural information on the PPI interface. This approach might be especially advantageous in the hit identification phase of drug discovery campaigns in large chemical screenings or to gain mechanistic information on the role of a given regulatory protein. Once the basic properties and key residues of interacting surfaces within protein-channel complexes are identified, *in silico* analysis [29] and bioinformatics engines [30-32] might be used to refine the selection of "hot-spots" and identify druggable pockets. In the next paragraphs we will discuss a few *in vitro* and in-cell studies applied to either gain structural information on protein-channel complexes or to identify small molecules/peptides targeting these complexes.

### Examples of reconstitution of protein-channel complexes

Successful examples of structural studies on protein-channel interacting complexes include the recent X-ray crystal structure resolution of calmodulin (CaM) and the C-tail of the voltage-gated Na<sup>+</sup> channel (Nav) isoform 1.6 complex via the channel's IQ motif [33]. In this study the complex was reconstituted by engineering a chimeric construct expressing the Nav<sub>v</sub>1.6 IQ motif linked to the C-terminus of CaM through a glycine flexible linker [34]. Another example is the reconstitution of the fibroblast growth factor 13 (FGF13) and the Nav1.5 C-tail complex, which was obtained by expression of the two interacting partners through separate vectors [35].

In addition to high-resolution crystal structures, which can be problematic even in case of channel fragments, in-cell reconstitution of protein-channel complexes have emerged as a powerful approach when high yield expression of the protein complex partners is not efficient or simply to provide a more physiological microenvironment for the protein-channel complex reconstitution. Protein-fragment complementation assays (PCA) respond to these needs [36,37].

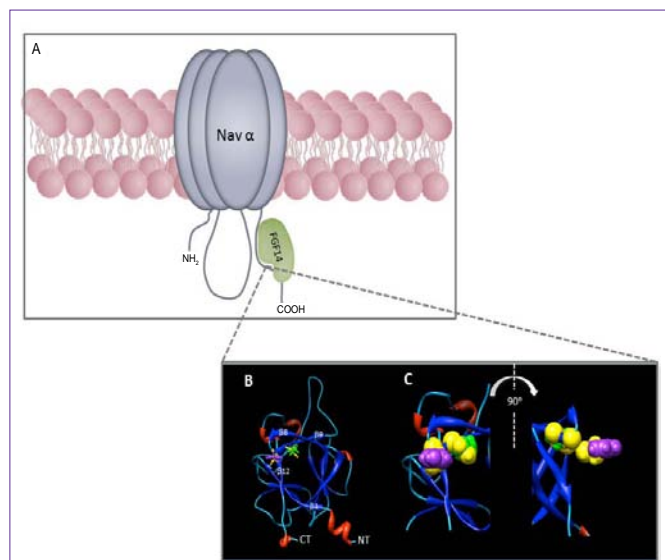
A new approach combining the split-luciferase complementation assay (LCA) [38-41], a luminescence-based PCA, and *in silico* analysis was recently utilized to identify "hot-spots" at the fibroblast growth factor 14 (FGF14) and voltage-gated Na<sup>+</sup> channel (Nav) complex [29] (Figure 1A) and to screen for intracellular pathways governing this interaction [42]. In this LCA, two complementary N-terminus and C-terminus fragments of firefly luciferase, inactive on their own [41], were respectively fused to a chimera of the CD4 transmembrane segment and the C-tail of Nav1.6 or FGF14 [29], a functionally relevant regulator of neuronal Nav channels [43-47]. Upon addition of the appropriate substrate, in-cell reconstitution

of the FGF14-Nav1.6-C-tail complex is detected as a luminescence signal representing the strength of interaction between the two binding partners [29]. When combined with *in silico* analysis, this method resulted in the identification of two “hot-spots”, Y153 and V155 (Figure 1B,C), at the FGF14-Nav1.6-C-tail interaction site [29]. The same strategy has been applied to perform a high-throughput screening (HTS) of a chemical library of kinase inhibitors which led to the discovery of a new signaling pathway controlling the FGF14-Nav1.6-C-tail complex through the enzyme glycogen synthase kinase 3 (GSK3) [42].

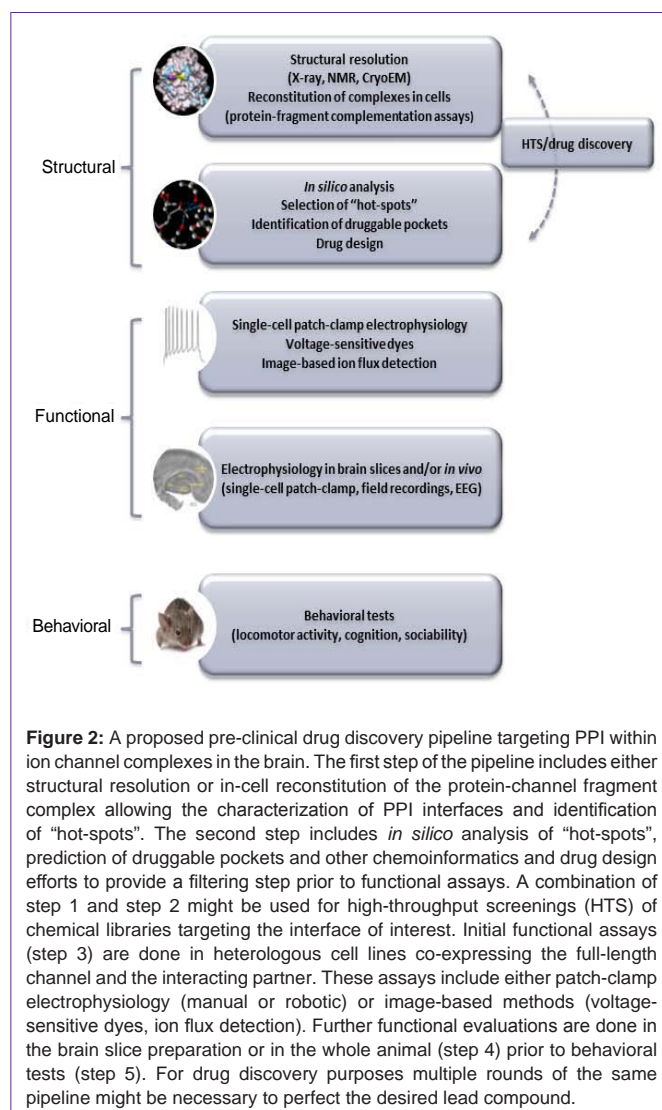
### PPI-based drug discovery campaigns targeting ion channels

Examples of drug screening campaigns targeting protein-channel interactions are still rare. Nevertheless, two examples are worth mentioning. The first successful screening campaigns against protein-channel complexes was employed to identify small molecules inhibiting the interaction between the voltage-gated  $K^+$  (Kv) channel, Kv1.1, and its accessory  $\beta$ -subunit, Kv $\beta$ 1[48]. Hits were identified with an in-cell yeast-two hybrid screening restricted to the channel fragment and its interacting partner and validated through  $Ca^{2+}$  flux assays and patch-clamp electrophysiology. The campaign led to the identification of chemically diverse compounds, some of which with promising anti-seizure activity *in vivo*[48].

An alternative approach to identify active compounds targeting a protein-channel complex is to focus directly on the interacting partner of a particular ion channel in search for ligands that could potentially



**Figure 1:** Schematic representation of the FGF14:Nav channel complex. A. The Nav channel is a transmembrane voltage-gated ion channel protein. FGF14 binds directly to the proximal region of the C-terminal tail of the Nav channel. Y153 and V155 are “hot-spots” at the FGF14:Nav channel complex interface. B. Ribbon representation of the secondary structures of overlapped FGF14 wild type and FGF14<sup>Y153N/V155N</sup> mutant. The  $\alpha$ -helices in red, the  $\beta$ -strand in blue and the random structures in light blue are shown as ribbons. The C-terminal and N-terminal tails are denoted as CT and NT, respectively. The side chains corresponding to the Y153 (purple) and V155 (green) in the FGF14 wild type, and the side chains corresponding to the N153 and N155 (yellow) in the FGF14<sup>Y153N/V155N</sup> double mutant are superimposed on the FGF14 wild type structure and are shown as ball and sticks. C. Magnified orthogonal views of Y153 (purple), V155 (green), N153 and N155 (yellow) side chains from B are shown as spheres.



**Figure 2:** A proposed pre-clinical drug discovery pipeline targeting PPI within ion channel complexes in the brain. The first step of the pipeline includes either structural resolution or in-cell reconstitution of the protein-channel fragment complex allowing the characterization of PPI interfaces and identification of “hot-spots”. The second step includes *in silico* analysis of “hot-spots”, prediction of druggable pockets and other cheminformatics and drug design efforts to provide a filtering step prior to functional assays. A combination of step 1 and step 2 might be used for high-throughput screenings (HTS) of chemical libraries targeting the interface of interest. Initial functional assays (step 3) are done in heterologous cell lines co-expressing the full-length channel and the interacting partner. These assays include either patch-clamp electrophysiology (manual or robotic) or image-based methods (voltage-sensitive dyes, ion flux detection). Further functional evaluations are done in the brain slice preparation or in the whole animal (step 4) prior to behavioral tests (step 5). For drug discovery purposes multiple rounds of the same pipeline might be necessary to perfect the desired lead compound.

act as competitive inhibitors at the protein-channel interaction site. This approach, though, requires a detailed understanding of the interaction site and relies exclusively on one component of the complex. The discovery of FSC231, a small molecule inhibitor of the complex formed by the glutamate receptor GluA2 and the protein interacting with C kinase 1 (PICK1) [49] falls in this category. This small molecule was identified using a fluorescence-polarization assay targeting the PSD-95/Discs-large/ZO-1 homology (PDZ) domain 1 of PICK1, which was previously known to mediate the interaction of PICK1 with GluA2.

### Conclusion

The large network of PPI that composes ion channel macromolecular complexes provides a rich source of vastly overlooked targets for drug discovery [50,51]. Yet, effective methods for screening these macromolecular complexes in search of new compounds targeting protein-channel interfaces [52,53] are still missing links in this newly emerging field of pharmacology. Figure 2 illustrates a simplified pipeline to study PPI at the level of ion channels that we propose as a starting point for characterizing interfaces and targeting

them in drug discovery campaigns. We envision that a combination of efforts including structural information on the protein-channel complex, in-cell screenings, *in silico* analysis, functional assays, and behavioral tests could provide an integrated platform for exploiting protein-channel complexes [54-56]. Overall, we envision that one of the most critical steps in the pipeline is to be able to rapidly gain information.

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