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Review

Ion channels and their molecular environments – Glimpses and insights from functional proteomics

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ABSTRACT

There is emerging evidence from functional analyses and molecular research that the role of ion channels in cell physiology is not only determined by the pore-forming subunits but also depends on their molecular environment. Accordingly, the local and temporal specificity of channel-mediated signal transduction is thought to result from association of these integral membrane proteins with distinct sets of partner proteins or from their assembly into stable macromolecular complexes. As yet, however, the molecular environments of most ion channels have escaped direct investigation, mostly because of technical limitations that precluded their comprehensive molecular analysis. Recent advances in proteomic technologies promoted an experimental workflow that combines affinity purification of readily solubilized protein complexes with quantitative high-resolution mass spectrometry and that offers access to channel-associated protein environments. We will discuss advantages and limitations of this proteomic approach, as well as the results obtained from its application to several types of ion channels including Cav channels, Kv channels, HCN channels, AMPA-type glutamate receptors and $GABA_B$ receptors. The respective results indicate that the approach provides unbiased and comprehensive information on (i) the subunit composition of channel cores including identification of auxiliary subunits, on (ii) the assembly of channel cores into 'signaling entities' and on (iii) integration of channels into extended protein networks. Thus, quantitative proteomics opens a new window for the investigation of ion channels and their function in the context of various types of cell.

Contents

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1. Introduction

The major tasks of ion channels in the central nervous system (CNS) – as in most other excitable tissues – are perception, processing and propagation of electrical signals as the fundamentals for cell–cell communication. For these purposes the channel-mediated signaling must exhibit exquisitely high precision in both time and (subcellular) space and display activity-dependent dynamics as required for promoting storage and retrieval of bits of information. Molecular and functional investigations offer more and more evidence that these characteristics cannot be exclusively met by the pore-forming subunits of ion channels operating as stand-alone tools. Rather, channel-mediated signaling is thought to result from multiple protein–protein interactions, transient or stable in nature, occurring in the 'molecular environments' of the ion channel proteins.

Conceptually, these molecular environments are formed by proteins that are either directly or indirectly associated with the pore-forming α subunits and that are able to modulate channel properties and processing, affect downstream signaling pathways or shape spatio-temporal concentration gradients of ions and other diffusible messengers. In the literature, molecular environments are often referred to as micro- or nano-domains, depending on their structural dimensions and are thought to represent a general principle for how membrane protein-based signaling is organized to guarantee specificity and adequate rate of signal transduction. As a result, ion channels embedded in such entities may exhibit variable properties and functions depending on the state of excitation and level of activity, subcellular localization, developmental stage of the cell and expression of distinct sets of partner proteins.

Despite their fundamental importance, the present knowledge on molecular environments of ion channels is rather limited, mostly because of technical problems including poor solubilization, small amounts of source material and lack of sensitive protein analysis techniques that have precluded their experimental access for a long time. Recent developments in both biochemistry and mass spectrometry, however, enabled the first comprehensive investigations of channel-associated environments and unbiased identification of the respective protein constituents. In this review we will first introduce the novel approach of 'functional proteomics' and then discuss the results obtained with their application to various ion channels in the CNS.

2. Approach of 'functional proteomics': strategy and experimental workflow

A broad range of approaches has been used for identification of ion-channel associated proteins, including recombinant high-throughput assays such as the conventional yeast-two-hybrid or the split-ubiquitin platforms [1,2], genetic screens [3,4] and linkage studies [5,6], biochemical cross-linking experiments [7,8] and affinity-based co-purification [9,10]. The latter has recently been coupled with unbiased protein identification by mass spectrometry [2,11–13], a combination referred to as *AP-MS* [14] or *functional proteomics*. Although each method offers distinct advantages and is capable of identifying individual protein-protein interactions, *functional proteomics* is the only approach providing a comprehensive view on protein assemblies formed in native systems at all levels of complexity. When combined with adequate controls and quantitative evaluation procedures, it also delivers highly reliable and detailed results, key advantages in the light of rather costly and laborious functional follow-up work.

2.1. Protein complexes and networks

Apart from the availability of powerful tools and technologies, the success of proteomic approaches is based on the kinetic stability of biochemical protein–protein interactions during their isolation from cells and tissues. These stable interactions give rise to higher-order protein **assemblies** that, together with membrane-based compartmentalization, form the structural and organizational framework of any living system [15]. With regard to ion channels, assemblies with distinct biochemical properties and different levels of complexity have been detected and isolated from physiological systems (Fig. 1). Thus, ion channel **complexes**[1] are formed by pore-forming α ubunits, auxiliary (β) subunits and other stably associated proteins with a defined (saturable) stoichiometry. They may contain up to eight or more proteins and typically range from 200 to 1000 kDa in size [11,16–18]. Larger functional units made up from stable assemblies of distinct and functionally independent protein complexes, like channel–channel complexes [16], enzyme–channel complexes [19] or receptor-channel complexes [20] may be termed **supercomplexes** (as proposed by Schägger and Pfeiffer [21]). Finally, ion channels may be associated with a multitude of other proteins and protein complexes into extended **networks**,[1] as found for postsynaptic NMDA receptors [22] and presynaptic Cav2 channels [23]. Although all these protein assemblies can essentially be resolved by functional proteomics, they present with distinct technical challenges: For example, supercomplexes tend to be biochemically unstable and are, therefore, hard to isolate [16,21]; protein networks are difficult to resolve on a molecular level due to their heterogeneous composition, extended surface for non-specific binding, and poorly defined boundaries.

2.2. Functional proteomics: overview

The introduction of liquid nano-HPLC coupled high-resolution mass spectrometry (nano-LC–MS/MS [24]) promoted the revival of the rather classical AP approach [14,25]. The ability to identify and quantify hundreds of proteins in affinity-purified protein samples has opened a new dimension of studying protein interaction complexity, but also uncovered a broad spectrum of experimental artifacts. This has stimulated the development of more elaborate workflows with multiple APs, stringent quality controls and advanced evaluation tools. A schematic overview of proteomic techniques used to characterize protein complexes and higher-order assemblies is depicted in Fig. 2. The displayed methods and steps have been optimized to address the major technical issues, as discussed in more detail in the following sections:

(1) Extraction of the target proteins from native source material under conditions preserving their association with other proteins, relative abundance levels and modification status.

[1] Note: It should be noted that the terms "complex" and "network" are frequently used to describe entities that are not biochemically tangible, like proteins interacting functionally or temporarily, or virtual protein interaction clusters.

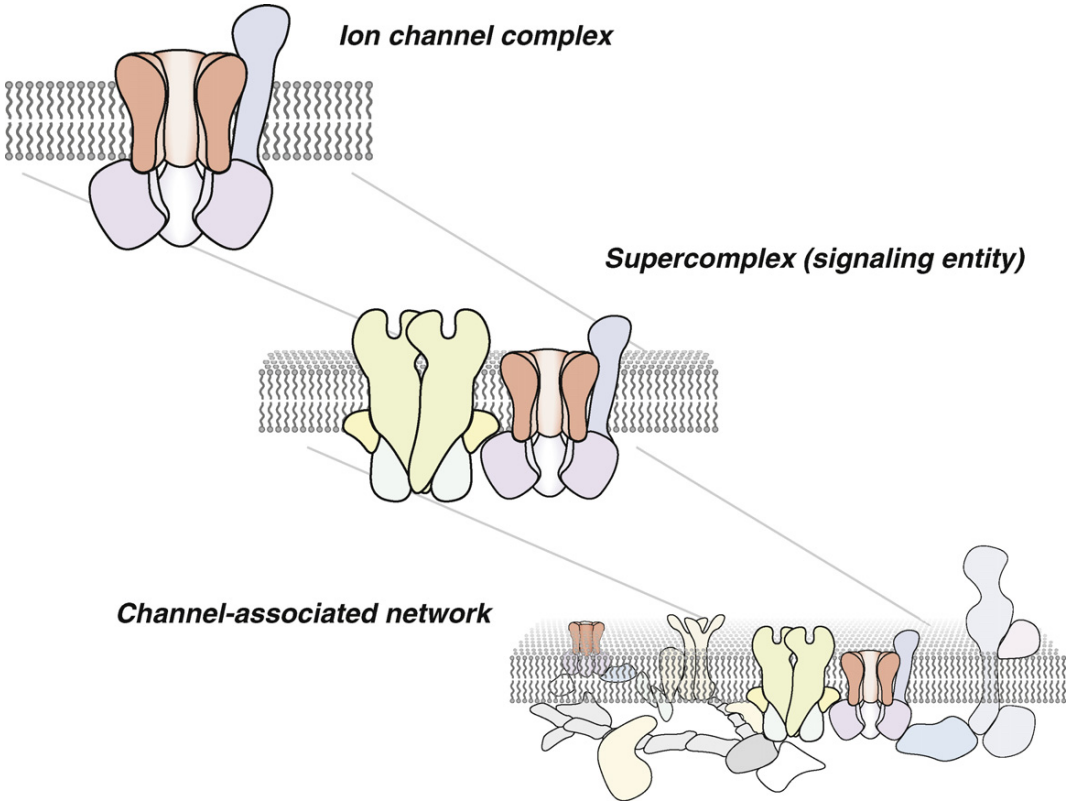

Fig. 1. Organization of ion channel-associated proteins as complexes (top), supercomplexes representing signaling entities (middle), and membrane-associated protein networks (bottom). The core complex is coloured in red; note that these types of assembly may co-exist and dynamically interconvert in native cells.

(2) Efficient, specific and representative purification of solubilized target protein assemblies.
(3) Unbiased identification and quantification of proteins with high sensitivity and over a broad range of abundances.
(4) Processing and biochemical interpretation of the complex AP and control datasets obtained from MS analysis.

The displayed proteomic workflow is not yet widely distributed, and until now only a few ion channels have been analyzed using a comparable approach ([11,12,16,23,26–29]; see also Table 1).

2.3. Preparation of protein assemblies from native tissue

The first step in isolating membrane proteins is the preparation of source material, membrane vesicles from cells and tissue. Different protocols have been described, either for preparation of total membranes or aiming at enrichment of specific subcellular compartments. Apart from feasibility considerations, there are several factors to be considered:

- Only about 5% of the total cellular protein is associated with the plasma membrane.
- Cytosol and some organelles like mitochondria or ribosomes contain highly abundant proteins that tend to enhance the background of APs and thereby reduce the sensitivity of the approach [14,25].
- Any pre-fractionation step introduces biases that may strongly affect the outcome of the approach (e.g. effective co-purification of α2δ subunits with the voltage-gated calcium channels through pre-enrichment by lectins [30]).
- In contrast to a widespread assumption that cell physiological relevance is confined to proteins directly associated with ion channels at the plasma membrane, proteins involved in proper maturation, trafficking or degradation of channel proteins and/or complexes might be highly relevant and should, therefore, not be excluded from any study.
- Finally, it appears noteworthy that even the most elaborate membrane preparation is of limited purity (<90%) and may be rather disadvantageous given the substantial loss of material usually encountered by multi-step isolations and the concomitant negative effects on the structural integrity of proteins.

Consequently, 'plasma-membrane enriched fractions' that are partially depleted of mitochondria, nuclei and cytoplasm, but contain all vesicular structures related to the plasma membrane appear a reasonable compromise between purity, completeness and integrity [31,32].

Solubilization of the membrane protein assemblies is the second experimental step (Fig. 2), mandatory and critical due to the required use of detergents. Many different detergents have been described for this application, most of them neutral, anionic or mixtures thereof, but systematic investigations are lacking. Solubilization buffers have a large impact on the proteomic approach, as not only the target ion channel but also putative (or often unknown) partner proteins must be effectively solubilized. With this respect, a few points must be considered:

- Detergents inevitably affect protein–protein interactions, particularly those occurring within the lipid bilayer that depend on binding to specific lipids or that are stabilized mainly by hydrophobic forces.
- Furthermore, detergents are known to at least partially denature proteins, thereby adding to the protein background in subsequent purification steps.

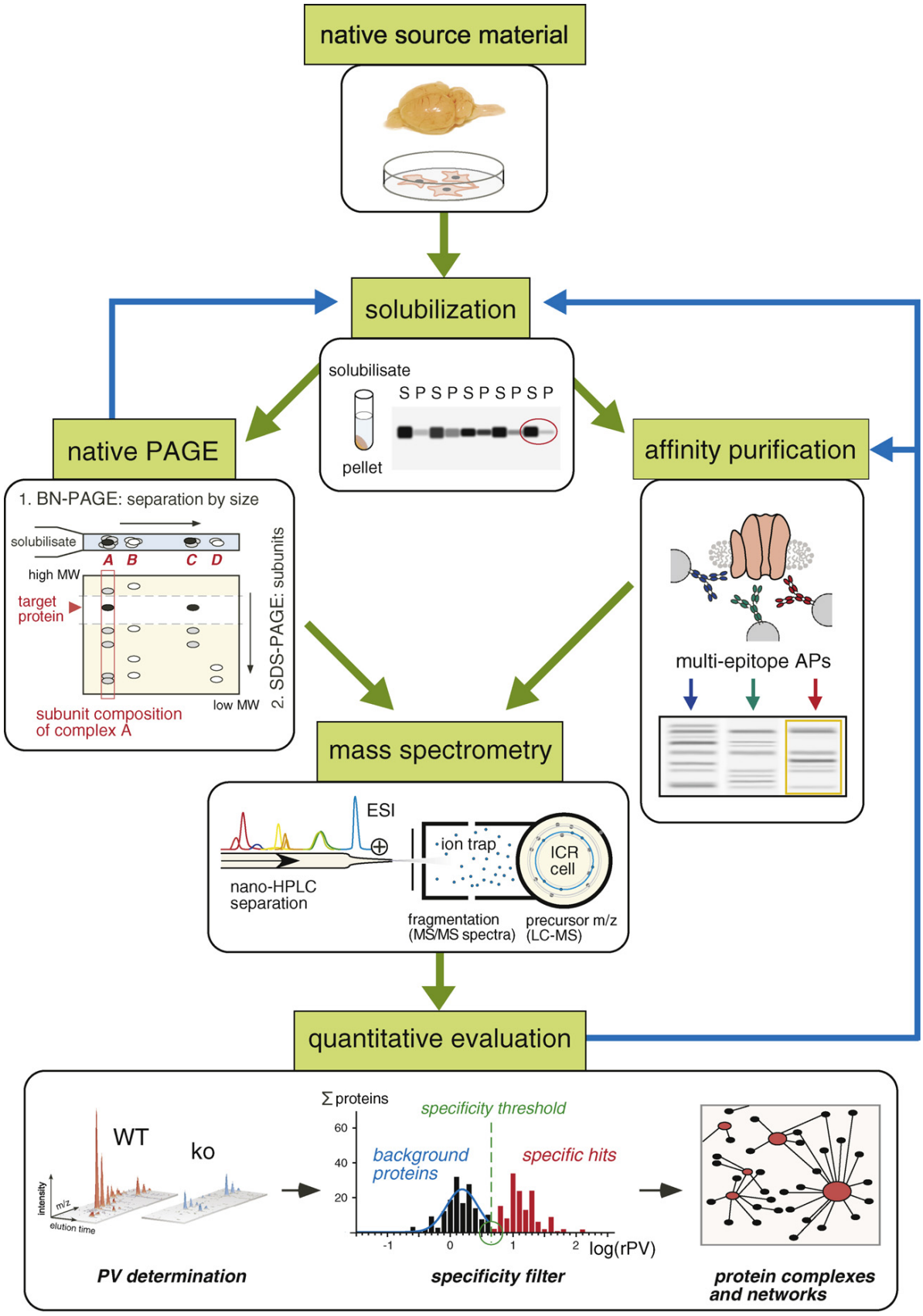

Fig. 2. Workflow of the 'functional proteomics' approach (see text for details). Arrows indicate how individual methods and steps are combined (green) and results are fed back (blue) to obtain optimal results.

Table 1
Overview of published proteomic approaches targeting ion channels in native tissues.

Channel target	Source tissue	Reference	Ligand	Epit./Baits	Var.	Contr.	Solubilization	MS setup	Specificity	Biochemical verification
BK	Rat brain	[16]	ab	2	1	up	Verified	HR-LCMS	SpecC	rIP, pd
BK	Mouse brain	[39]	ab	1	1	up	n.d.	Maldi	SpecC	n.a.
BK*	Mouse cochlea	Kathiresan et al., Mol Cell Proteomics 2009; 8: 1972–87	ab	1	1	up, blank	Verified	LCMS	ProtSt	rIP
BK	Rat brain	Kim et al., Proteomics 2007; 7: 2591–602	fp	1	1	up	n.d.	LCMS	ProtSt	IP, pd
BK	Mouse brain	Ma et al., FEBS Lett 2007; 581: 1000–8	fp	1	1	up	n.a.	Maldi	ProtSt	pd
BK	LNCaP cell line	[13]	ab	1	1	up	n.d.	HR-LCMS	SpecC	rIP, pd
CatSpeM	Mouse testis	[2]	tag	1	2	ko	n.d.	LCMS	ProtSt	pd
CatSpeM	Mouse testis	Wang et al., Biol Reprod 2009; 81: 539–44	tag	1	2	ko	n.d.	LCMS	ProtSt	rIP, pd
Cav2.1-3	Rat/mouse brain	[23]	ab	14	3	up, ko	Verified	HR-LCMS	PV	n.a.
Cav2.2*	Rat brain	Khanna et al., J Biochem Mol Biol 2007; 40: 302–14	ab	1	1	up	Verified	LCMS	SpecC	rIP
CLC2	Calu-3 cells	Thelin et al., J Clin Invest 2007; 117: 364–74	fp	1	1	up	n.d.	Maldi	ProtSt	rIP, pd
CFTR	Mouse brain	Dhani et al., J Biol Chem 2003; 278: 16262–70	fp	1	1	up	n.d.	Maldi	ProtSt	IP, pd
CLC4	Mouse brain	Suginta et al., Biochem J.2001; 359: 55–64	fp	1	1	up	n.d.	LCMS	ProtSt	IP, pd
Connexin 43	Mouse brain	Butkevich et al., Curr Biol 2004; 14: 650–8	fp	1	1	up	n.d.	Maldi	ProtSt	IP
KCNQ2, Kv4.2	Rat brain	Klemmer et al., J Proteomics 2009; 72: 82–90	ab	1	1	up	n.d.	LCMS	SpecC	n.a.
HCN2	Rat brain	[29]	ab	1	2	up	Verified	HR-LCMS	SpecC	rIP, pd, BNP
Kir2.2	Brain, heart	Leonoudakis et al., J Biol Chem 2004; 279: 22331–46	fp	1	1	up	n.d.	LCMS	ProtSt	pd
Kir3.3	Rat brain	Lunn et al., Nat Neurosci 2007; 10: 1249–59	fp	1	1	up	n.d.	LCMS	ProtSt	pd
Kv1.1	Rat brain	[103]	ab	1	2	up	Verified	LCMS	ProtSt	rIP, pd
Kv1.2	Rat brain	Ogawa et al., J Neurosci 2010; 30: 1038–48	ab	1	1	up	.d.	HR-LCMS	SpecC	rIP
Kv2.1	Rat forebrain	Chung and Li, FEBS J 2005; 272: 3743–55	ab	3	1	up	Verified	Maldi	ProtSt	n.a.
Kv4.2	Mouse brain	[26]	ab	1	3	ko, up	Verified	HR-LCMS	PV, SpecC	n.a.
SK2, SK3	Rat brain	[19]	fp	2	1	up, blank	n.d.	LCMS	ProtSt	IP, pd
TREK1	Mouse brain	Sandoz et al., EMBO J 2006; 25: 5864–72	ab	n.a.	1	up, ko	n.d.	LCMS	n.a.	pd
TRPV1	Spinal cord	Goswami et al., J Neurochem 2004; 91: 1092–103	fp	2	1	up	n.d.	Maldi	ProtSt	pd
TRPV6	Human placenta	Stumpf et al., J Biol Chem 2008; 283: 18086–98	ab	1	1	up, blank	n.d.	Maldi.LCMS	ProtSt	IP
ASIC1a, 2a	Rat brain	Chai et al., J Biol Chem 2007; 282: 22668–77	fp	3,2	1	up	n.d.	LCMS	SpecC	pd
GluA1	Mouse brain	[28]	ab	1	1	ko	n.d.	Maldi	IsoL	rIP, pd
GluA1,2	Rat/mouse brain	[11]	ab	3	3	up	Verified	HR-LCMS	PV, SpecC	rIP, pd, BNP
GluA4	Rat cerebellum	Santos et al., J Proteome Res 2010; 9: 1670–82	ab	1	1	up	n.d.	LCMS	SpecC	n.a.
GluA6	Mouse brain	Coussen et al., Neuron 2005; 47: 555–66	ab	3	1	up	n.d.	Maldi	SpecC	pd
GluA6, 7	Rat cerebellum	Zhang et al., Neuron 2009; 61: 385–96	ab	2	1	up	n.d.	LCMS	ProtSt	rIP
GlyRb	Rat CNS, liver	Paarmann et al., J Biol Chem 2006; 281: 34918–25	fp, tox	1, 1	l	up	n.d.	Maldi	SpecC	n.a.
nAChR b2	Mouse brain	Gottschalk et al., EMBO J 2005; 24: 2566–78	tag	2	1	up, ko	n.d.	LCMS	ProtSt,SpecC	n.a.
nAChR	*C. elegans*	Kabbani et al., Proc Natl Acad Sci USA 2007; 104: 20570–5	ab, fp	1	1	up, ko	n.d.	Maldi	ProtSt	n.a.
nAChR7	Mouse brain	Paulo et al., J Proteome Res 2009; 8: 1849–58	tox	1	1	ko	n.d.	HR-LCMS	SpecC	n.a.
NR2A, 2B	Rat hippocampus	Al-Hallaq et al., J Neurosci 2007; 27: 8334–43	ab	1, 1	1	up	n.d.	LCMS	SpecC	rIP
NR1.2B	Rat brain, heart	Seeber et al., J Biol Chem 2004; 279: 21062–8	ab	1	1	up	n.d.	Maldi	ProtSt	rIP

Columns list the primary target, the source tissue(s), the Pubmed reference, the ligand used for affinity purification (ab, antibody; tag, tagged target transgene; tox, specific channel toxin or drug; fp, fusion protein bait), the number of target ligands/epitopes used, the number of variations of experimental conditions, the type of purification controls (blank, empty affinity matrix; up, unspecific antibody or bait; ko, target knockout material), validation of solubilization conditions (verified, efficiency and/or integrity of target complexes assayed; n.a., no detergents used; n.d., effects of solubilization not addressed), the used mass spectrometric method (maldi, matrix-assisted laser desorption ionization mass spectrometry; LCMS, liquid chromatography coupled to low resolution mass spectrometry (quadrupole or ion trap); HR-LCMS, liquid chromatography coupled to high resolution mass spectrometry (Orbitrap or ICR detector)), quantification method(s) to determine specificity (ProtS, differential protein spots or bands visualized by protein staining; SpeC, spectral count-based protein quantification; IsoL, isotopic labeling; PV, peak volume-based protein quantification), and the biochemical verification methods used (pd, pulldown assays in vitro or from heterologous cells; (r)IP, (reverse) affinity-(co)purification from native tissue; BNP, co-migration on native gels); asterisks indicate studies which failed to retrieve the primary target protein. Note that only very few studies meet the high quality standards described in Section 2.

- Finally, a number of detergents exhibit individual unfavorable effects. Thus Triton X100 and related polyethoxy alkyl/aryl derivatives have a tendency to induce phase separations in lipid bilayers, thereby leading to 'insoluble' vesicular structures strongly enriched in certain membrane proteins and incorrectly referred to as 'lipid rafts' [33]. Digitonin, a natural saponin and mild detergent, exhibits strong batch-to-batch variation in quality and is well known for forming irreversible precipitates with cholesterol [34] absorbing hydrophobic proteins.

To achieve reasonable balance between experimental requirements and conservation of protein structures, and to minimize detergent-related artifacts, the effects of solubilization must be carefully monitored. Solubilization efficiency may be tested in a straight-forward manner by Western blotting as illustrated in Fig. 2 (inset 'solubilization'). Effects on complex integrity can be thoroughly monitored by native gel electrophoresis [16,21]; incorporation of a target protein into higher molecular weight complexes under different solubilization conditions can be quantitatively analyzed (Fig. 2; inset 'native PAGE'). The structural integrity of solubilized ion channel complexes can be further tested in ligand binding assays [35,36], reconstitution experiments [37,38], or by probing known interaction partners in test APs as positive controls.

It is noteworthy that despite the severe effects of insufficient or disruptive solubilization, these issues are often neglected in proteomic studies.

2.4. Affinity purification of target protein assemblies

APs are predominantly performed with target-specific antibodies, more rarely with fusion proteins or low-molecular weight ligands. Although these tools theoretically allow for rapid and strong enrichment of target proteins, they may have critical adverse effects. A major concern is specificity, since many antibodies also bind to proteins independent from the target. Such unpredictable cross-reactivities can be as high in affinity as that for the primary target and sometimes even dominate the AP (for example, the APC-107 anti-BKα antibody (Alomone) strongly cross reacts with dynamin 1 (unpublished observation, but compare [39]). Thus, in addition to general background resulting from surfaces of the affinity matrix and incubation tubes, antibodies may add a multitude of non-specific proteins through their direct or indirect cross reactivities. A recent study, in which several antibodies were tested in APs from wildtype and compared to knockout controls suggested that around 50% of co-purified proteins may in fact result from antibody off-target effects [23]. Conversely, antibodies may also cause false-negative results. Due to their molecular size and sterical shielding of target epitopes by associated proteins, antibodies may selectively bind to certain subsets of target assemblies, potentially missing major populations of interaction partners. Even worse, antibodies with very high affinity for a particular epitope conformation may actually disrupt target protein assemblies [40]. Not surprisingly, both of these effects may be more frequently observed with monoclonal antibodies [41].

Thus, the AP step represents a major source of error and must be strictly controlled (Fig. 2, inset 'affinity purification'). General background should be minimized by adjustment of AP conditions, the remaining level is usually controlled by comparison with APs using non-specific pre-immunization IgGs. It should be noted, however, that loading of antibodies with protein assemblies also increases the surface available for non-specific binding. This effect has to be taken into account when defining specificity thresholds (see below) to avoid misinterpretation of pseudo-enriched proteins. Any off-target effects can be effectively eliminated by using control APs with membranes isolated from target knockout tissues [12,29]. In case genetic knockouts are not available, control material could also be generated by biochemical depletion of the target with a second antibody ('biochemical knock out'). Selection biases and structural interference with target complex integrity are more difficult to address. A simple but important quality control is verification of AP efficiency, which can be monitored by Western blot analysis of samples taken before and after AP. Antibodies that fail to deplete their target protein from the solubilisate are likely not co-purifying the complete spectrum of interaction partners. In addition, the use of multiple antibodies targeting different epitopes combined with consistency filtering (see Section 2.5) has been shown to successfully eliminate artifacts introduced by individual antibodies [23].

2.5. Qualitative and quantitative mass spectrometry

Mass spectrometry has emerged as the most powerful technology for unbiased identification and quantification of proteins. Current LC–MS/MS instruments of choice are capable of reliably identifying hundreds of proteins with a sensitivity of $\leq$1 femtomol in a single run [42,43]. This high performance is achieved by the optimized and inline combination of nano-HPLC separation, electrospray ionization, signal-dependent fragmentation in a linear ion trap and precise m/z detection, as depicted in Fig. 2 (inset 'mass spectrometry'). In the most widely used setup, protein samples are first digested with trypsin – either in solution or as lanes from silver stained gels – to obtain defined peptides of suitable length (ideally between 6 and 25 amino acids). These peptide mixtures are then resolved by reverse phase nano-HPLC, either directly or subsequent to loading on a pre-column. Eluting peptides are then electrospray-ionized (ESI) and collected in the linear ion trap, from which they are either forwarded to the mass analyzer for high resolution intensity over m/z detection (precursor ion spectrum, LC–MS), or selectively fragmented by collision with gas molecules (CID) for recording of fragment ion spectra (MS/MS). The combined information of accurate peptide pre-cursor mass and fragment ion patterns is then extracted and matched with information from protein sequence databases using established software tools. This workflow, including many bioinformatic tools for detailed protein characterization, is meanwhile well established and standardized [44].

In contrast, quantitative evaluation of MS data is comparably less evolved, lacks generally accepted quality standards, and is scarcely integrated into AP-MS workflows (see Table 1). The most widely used protein quantification methods are based on either metabolic or chemical labeling of proteins or peptides with stable isotopes [45]. Samples to be compared are measured simultaneously thus eliminating variability introduced by sample processing and run-to-run differences and facilitating data processing. However, application of isotopic labeling to AP-MS approaches has substantial limitations: Metabolic labeling is not readily applicable to all native source materials, and chemical labeling reduces sensitivity and, like any chemical modification, increases MS background due to side reactions. Furthermore, both methods have restrictions concerning the number of datasets that can be directly compared: isotopic multiplexing allows up to 8 samples being measured together meaning that larger numbers of samples or data from separate experiments cannot be compared without additional measurements. In addition, isotope purity of labels in practice limits the dynamic range of quantification to about two orders of magnitude [46].

Because of their high sensitivity, potentially broader dynamic range and flexibility, label-free approaches are becoming increasingly popular in functional proteomics. Label-free MS quantification is based either on counting the number of acquired MS/MS spectra (e.g. emPAI score [47] or rPQ/PQ_{norm} score [16]), or pep-

tide ion intensities [48] or peak volumes (PV, calculated as the sum of m/z signal intensities integrated over elution time assigned to a peptide [49]). Spectral counting is not very accurate and often fails, particularly for small proteins and large differences in protein abundance [46,50]. In contrast, PVs are well correlated with protein abundance over a large dynamic range and largely independent from the type of protein, sample composition or instrument setting [51]. Therefore, PV-based quantification is the method of choice for the evaluation of AP samples.

Two sets of quantitative information can be obtained from peptide PVs both useful for the interpretation of AP-MS data: (i) ***Abundance ratio*** (or ***relative quantification***) of individual proteins in AP(s) versus control(s), and (ii) ***molar abundance*** of any protein within a given sample (often incorrectly referred to as 'absolute quantification'). The abundance ratio (rPV) of a protein is usually calculated as mean or median of its peptides' PV ratios in sample versus reference. It may resolve more than 1000-fold differences in protein abundance with reasonable accuracy [46,51], the latter being significantly enhanced by the use of appropriate (fusion) protein standards [52]. rPVs are used to judge specific enrichment of any co-purified protein in a target-dependent manner defining it as a bona fide interaction partner [12,23], to compare relative efficiency and selectivity of different antibodies in APs based on individual target proteins, or to determine subtype preferences of interaction partners in heteromultimeric complexes [12,23]. The molar abundance of a protein can be roughly assessed by its three highest peptide PVs (Top3 [53]) or by the sum of all peptide PVs normalized to the number of amino acids accessible to MS-analysis ($\text{abundance}_{\text{norm}}$ [12,23]). It is predominantly used to discriminate abundant and tightly associated protein partners from rare or dynamically interacting partners [11,12,29], and to identify clusters of interacting proteins by linear correlation of their abundance profiles over multiple AP datasets [23].

Finally, PV-based quantification has also been used to determine stoichiometries of protein complexes [12,54,55]. This requires these complexes to be purified without antibody-induced bias, concatenated protein standards and multiple MS-measurements.

2.6. Evaluation and controls

The proteomic approach in Fig. 2 comprises several steps, controls and complementary experiments read-out either by Western blot analysis or mass spectrometry. The resulting data may be used first to uncover shortcomings of individual steps or protocols and to check the quality of tools. Several considerations and macroscopic parameters may serve as guidelines:

- As a rule of thumb, the yield of target protein recovered by the approach should exceed 50% of the protein input from the source tissue in order to obtain representative results. This implies that both consecutive steps solubilization (Fig. 2, inset 'solubilization') and AP should reach an efficiency of at least 75%. In the case of protein networks the use of highly stringent conditions in addition to a more physiological reference can be advantageous to check for the robustness of protein–protein interactions [23].
- The quality of the antibodies used for APs should be critically investigated for specificity and selectivity. Only antibodies displaying high specific enrichment of the primary target versus IgG and knockout controls (i.e. rPV values >100) and high subtype selectivity as confirmed by target knockout controls should be included in the evaluation. Reliable AP datasets are characterized by rather low amounts of background (i.e. few background proteins with $\text{abundance}_{\text{norm}}$ values exceeding those of the target protein complex subunits), depletion of the target from the solubilisate, and specific co-purification of validated interaction partners (positive controls). Ideally, 2–3 high quality antibodies are available to perform consistency filtering during final evaluation as outlined below.
- The relative coverage of the primary target' s amino acid sequence derived from MS-retrieved peptides should be >50% [12,23,29]. This is mandatory for the identification of splice variations and post-translational modifications [23,41], but also for identification of lower abundant and small interaction partners [11].

In a second step, the MS data of verified AP datasets must be evaluated. Standards for reliable protein identification (m/z tolerance, minimum MS/MS scores and number of identified peptides, false-positive discovery rates (FDR)) have been well established [44]. Likewise, quality filters for protein quantification should be applied, including appropriate LC-retention time and m/z tolerance windows for alignment of different MS datasets as well as total PV intensity thresholds and the availability of at least two quantifiable, specific peptide PVs per protein [11,12,23]. In addition, specificity thresholds for rPV values of proteins may be determined based on the actual AP/control datasets. For this purpose, histogram plots displaying the distribution of all protein log(rPV) values have been developed [23]. Accordingly, APs with high signal-to-noise show a clear separation of the specifically (co)-purified proteins from the population of background proteins, usually at rPVs higher than 5–25 [23]. In addition to enrichment, the significance of co-purified proteins can be corroborated by comparing their consistency throughout APs with different antibodies or AP conditions. For example, with three or more antibodies available, errors caused by individual antibodies can be eliminated by selecting proteins specifically retrieved with a majority of antibodies ('consistency criterion' [23]). The success of these filtering steps can be checked by the obtained list of interaction candidates: organelle contaminations (mitochondrial, nuclear, ribosomal proteins) as well as common background proteins [14] should be largely absent.

As a word of caution, there remain several issues that may not be easily resolved or even aggravated by the proteomic approach:

- The approach as shown in Fig. 2 with its multiple controls operates on the cost of completeness. Thus, rare or highly dynamic interaction partners such as modifying enzymes will likely become eliminated, as well as partners with promiscuous binding properties (for example see [23]).
- Target proteins that are stably integrated into detergent-resistant assemblies or partners/interactions that are sensitive to detergents can hardly be analyzed.
- Small proteins (<20 kDa) with unfavorable sequences, i.e. with lack of MS-detectable tryptic peptides, will escape MS-based identification and quantification.
- Protein abundances determined with the approach do not necessarily reflect quantitative relations in the native system, as antibodies and biochemical effects may cause biases, disruption or rearrangements of protein assemblies during the workflow.
- The dynamic range and sensitivity of MS-analysis and the evaluation procedures define a technical limit for the "interactome". Thus, the most elaborate study so far identified somewhat more than 200 proteins that are specifically connected to a target (directly and indirectly), going down to about 1% of the abundance level of the target protein [23].

3. Application of the proteomic approach

Until now, around 40 studies have been published describing identification of new interaction partners of ion channels using proteomic methods (Table 1), but only few of them meet the outlined quality standards, such as MS quantification, stringent specificity controls or a comprehensive AP-MS strategy. In this section, we will discuss a few examples where specific physiological questions

related to ion channels have been successfully answered by functional proteomics. Following the definitions set forth in Section 2.1, they have been categorized in elucidation of channel cores, identification of complex ion channel signaling entities and characterization of ion-channel-associated protein networks (Fig. 1).

3.1. Subunit composition of channel cores

Ion channels are composed of principal and auxiliary subunits; the latter influence gating, trafficking and/or subcellular localization of the channels and are identified by diverse techniques from genetic screens to large-scale isolation and partial protein sequencing. More recently, unbiased proteomic studies retrieved a number of novel channel subunits, most of them previously unrelated to ion channels or even lacking any assignment of cell physiological function [2,11–13,28].

3.1.1. HCN channels

Hyperpolarization-activated cation channels (HCN) are expressed as homo- and heterotetramers of four principal subunits (HCN1–4) in excitable tissues like neurons and cardiac muscle cells where they contribute to setting the resting membrane potential and trigger rhythmic electrical activity (reviewed in Refs. [56,57]). Hallmarks of these channels are their activation in the negative voltage range (< -50 mV) and their modulation by cyclic nucleotides, mainly cAMP. Binding of cAMP to the nucleotide binding domain in the α subunit shifts activation to more positive potentials and thereby increases the number of active HCN channels in the physiological voltage range [58]. This mechanism is implicated in several functions such as β-adrenergic regulation of heart beat rate [59,60] or transition between sleep and wake states of the brain [61], with cAMP sensitivity showing considerable variability in vivo (reviewed in Ref. [62]).

Since no auxiliary subunits had been described for this channel family, a functional proteomic screen was performed targeting neuronal HCN2 channels [29]. Among the proteins robustly co-purified under different solubilization conditions PEX5/Trip8b was identified, a protein previously found as a HCN channel interactor in a yeast-two-hybrid screen [63]. Importantly, mass spectrometric quantification based on peptide PVs calibrated with HCN-PEX5/Trip8b fusion protein standard and strong enrichment of HCN channels in anti-PEX5/Trip8b (reverse) APs revealed that HCN subunits and PEX5/Trip8b co-purified at comparable molar amounts and without apparent selectivity for particular HCN subtypes. Co-migration analysis on native PAGE gels indeed suggested that the majority of neuronal HCN channels may be associated with PEX5/Trip8b. These prominent features of PEX/Trip8b stimulated more detailed functional analysis revealing that association with this subunit abolished cAMP-modulation of HCN channels in heterologous cells as well as in cultured neurons [29,64]. Cell-type specific assembly with PEX5/Trip8b (and splice variants thereof) may therefore account for differences in cAMP sensitivity observed between different neurons and cardiac muscle cells that do not express Trip8b.

3.1.2. AMPA-type glutamate receptors

AMPA-type glutamate receptors (AMPARs) are ligand-gated ion channels that are responsible for most of the fast excitatory neurotransmission in the CNS. They are formed by tetrameric assemblies of α subunits (GluA1–4, reviewed in Refs. [65–67]) whose large extracellular domains are structurally arranged as crossing pairs of dimers [68]. Characterization of the *stargazer* mutant led to identification of a family of small transmembrane proteins termed TARPs (for transmembrane AMPAR regulating proteins) as auxiliary subunits [69,70] that are structurally related to the γ1 subunit of voltage-activated Ca^{2+} (Cav) channels found in skeletal muscle [71]. Interaction with these proteins has been shown to enhance membrane trafficking of AMPARs, to slow their deactivation and desensitization behavior, and to alter their pharmacological properties [72].

AMPARs solubilized from brain display remarkable homogeneity in molecular size (700–900 kDa) on native PAGE separations indicating that these receptor channels may co-assemble with only a limited number of partner proteins. The latter, however, cannot be recruited from the TARP family of proteins alone as gel shift assays using TARP-specific antibodies demonstrated that the TARPs are only associated with a subpopulation of native AMPARs [11]. Subsequent functional proteomic analyses indeed identified proteins with previously unknown function as novel auxiliary AMPAR subunits. In a first study cornichons 2 and 3 were discovered as highly abundant components of native AMPAR complexes [11]. These small transmembrane proteins belong to a family of proteins homologous to the *cornichon* gene product originally identified in Drosophila where it serves as a cargo transporter for certain secreted growth factors [73,74]. In-depth quantitative MS-analysis including comparison with fusion protein standards and native gel shift assays confirmed that cornichons 2 and 3 are specifically associated with the major portion of solubilized AMPARs in the mammalian brain. In heterologous co-expression experiments, cornichons markedly slowed deactivation and desensitization kinetics of AMPARs, and enhanced their trafficking to the plasma membrane similar to what has been observed with the TARP proteins [72]. In an independent proteomic screen, von Engelhardt and coworkers identified CKAMP44 as another protein specifically co-purifying with native AMPARs. This transmembrane protein is expressed at low levels in brain, but localizes specifically to synapses. When associated with AMPARs, it selectively enhanced their desensitization and slowed recovery from the desensitized state, thereby affecting short term plasticity of excitatory synapses [28].

3.2. Assembly of signaling entities

Functional measurements in native systems have revealed numerous examples for highly specific and rapid signaling processes at membranes which suggested that the involved ion channels and modulating proteins such as G-protein coupled receptors (GPCRs) or effector channels may assemble into larger functional units [75]. Such assemblies or defined spatial arrangements may be realized through specific direct or indirect protein–protein interactions.

3.2.1. BK_{Ca}–Cav channel–channel complexes

Large conductance, calcium- and voltage-activated potassium channels (BK_{Ca}) are tetrameric assemblies of α subunits (BKα) whose hallmark feature is the name-giving dual activation by membrane depolarization and increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This characteristic activation of BK_{Ca} channels forms the basis of their general physiological function, that is translating a mostly local increase in $[Ca^{2+}]_i$ into rapid membrane hyperpolarization (reviewed in Refs. [76–78]). The channels' sensitivity to Ca^{2+} as well as the kinetics of channel activation are fine-tuned by auxiliary β subunits (BKβ1–4, reviewed by [76]).

BK_{Ca} channels in central neurons are primarily fueled by Ca^{2+} ions provided through Cav channels [79–82]. To be robustly activated under physiological conditions, i.e. at membrane potentials around 0 mV, $[Ca^{2+}]_i$ must be $\geq$10 μM [16]. As such levels of $[Ca^{2+}]_i$ are restricted to the immediate vicinity of active Ca^{2+} sources [83,84] this implies stable and close co-localization of BK_{Ca} and Cav channels. The biochemical basis of the coupling between BK_{Ca} channels and specific calcium sources has recently been resolved by application of functional proteomics [16]. Careful adjustment of solubilization conditions in combination with selection of

suitable antibodies for APs allowed isolation of high-molecular weight BK_{Ca}-associated supercomplexes. These contained a set of specifically associated Cav channels as identified by quantitative high-resolution mass spectrometry. Accordingly, Cav1.2 (L-type channels), Cav2.1 (P/Q-type channels) and Cav2.2 (N-type channels) together with their auxiliary beta subunits (Cav β 1–3), but not the highly homologous Cav2.3 (R-type channels), were found to tightly associate with BK_{Ca} channel complexes consisting of BKα and BKβ2 or BKβ4 subunits. Reverse purification of these Cav channel subtypes, control experiments using BKα knockout brains and co-purifications from heterologous expression systems confirmed the formation of specific BK_{Ca}–Cav channel–channel supercomplexes, most likely through direct interaction of their α subunits. Functional coupling of both types of channel was finally confirmed by electrophysiological experiments using different calcium chelators on BK_{Ca}–Cav supercomplexes reconstituted by heterologous co-expression of their respective subunits [16]. Thus, functional proteomics not only resolved a long-standing mystery in physiology but also delivered an explanation for the distinct biophysical properties of Cav–BK_{Ca} signaling observed in different types of neurons (reviewed in Ref. [76]) that actually result from the differences in kinetics and voltage-dependence of the associated Cav channels [85].

3.2.2. $GABA_B$–KCTD complexes

$GABA_B$ receptors belong to the class III of GPCRs and are activated by γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian brain. Two different subunits, $GABA_{B1}$ (coming in two splice variations $GABA_{B1a}$ and $GABA_{B1b}$) and $GABA_{B2}$ have to co-assemble to form functional $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors (reviewed in Ref. [86]). These receptors are expressed in virtually all neurons in the brain and influence synaptic transmission and signal propagation by regulating the activity of Cav and inward-rectifier K^+ (Kir) channels via the βγ dimer of the activated G-protein [86–89]. This functional coupling is restricted to closely co-localized $GABA_B$ receptors and Kir3/Cav channels [90,91], suggesting existence of signaling complexes or interactions with additional linker proteins.

Thus, a functional proteomic approach was applied to native $GABA_B$ receptors that used validated solubilization buffers, multiple APs (each controlled by the respective target knockout) and quantitative mass spectrometry [12]. In addition to (expected) G-protein subunits, four previously uncharacterized members of the 'potassium channel tetramerization domain-containing' (KCTD8, 12, 12b and 16) family of proteins were found to specifically co-purify with the receptor. Their consistent and highly abundant co-purification with $GABA_{B1}$ and $GABA_{B2}$ protein, efficient co-purification of $GABA_B$ subunits in reverse APs with anti-KCTD antibodies and co-migration of the KCTD proteins with $GABA_B$ heteromers in native gel separations qualified them as constitutive and specific subunits of native $GABA_B$ receptors. Furthermore, biochemical analysis combined with advanced mass-spectrometric quantification of reconstituted complexes formed from $GABA_{B2}$ and KCTD12 domains revealed the quaternary structure of this assembly: one $GABA_{B2}$ subunit associates with one tetramer of KCTDs. Accordingly, the minimum size of native $GABA_B$ receptors observed in native PAGE gels is consistent with a stoichiometry of dimers of heterodimeric $GABA_{B(1,2)}$ assembled with two KCTD tetramers. Electrophysiological measurements of $GABA_B$ receptor-mediated Kir3 channel activation or Cav2 channel inhibition demonstrated that receptor-associated KCTD proteins influence the G-protein signaling of the $GABA_B$ receptor by increasing agonist sensitivity, accelerating the onset of the receptor response and determining kinetics and extent of desensitization in a subtype-specific manner [12]. Association of the $GABA_B$ core subunits with the four KCTD proteins as auxiliary subunits expands the functional repertoire of native $GABA_B$ receptors and may help elucidate the mechanism(s) responsible for selective coupling of $GABA_B$ receptors to effector ion channels.

3.2.3. Kv1 channel complexes

Kv1 channels, the first potassium-selective ion channels to be cloned and thoroughly characterized biochemically, primarily assemble as heterotetramers of Kv1 α subunits (Kv1.1–7) and auxiliary β subunits (Kvβ1–3) (reviewed in Ref. [92,93]). Upon membrane depolarization most Kv1 channels elicit non-inactivating K^+ currents, unless Kv1.4 or Kvβ1 subunits are incorporated into the channels. The latter present with particular N-terminal domains that endow the channels with rapid inactivation thus giving rise to the well-known A-type currents [94,95]. Accordingly, depending on their subunit composition, Kv1 channels repolarize and shape the action potential in CNS neurons and thereby influence firing pattern and modulate neurotransmitter release [96–99].

An initial proteomic screen based on large-scale APs of Kv1 channels from rat brain and mass spectrometric analysis of specific protein bands identified a number of known interaction partners (including various Kv1α, Kvβ, several MAGUKs, neurexin, Caspr2) as well as the functionally uncharacterized protein LGI1 (leucine-rich glioma inactivated gene 1 [100–102]) and the transmembrane catalytically inactive metalloproteinases ADAM22 and 23 [103]. Functional characterization of reconstituted presynaptic Kv1 channels (Kv1.1/Kv1.4/Kvβ1 + LGI1) showed that LGI1 selectively disrupted rapid Kvβ1-mediated channel inactivation. This effect was not observed with LGI1 mutants found in patients suffering from autosomal dominant lateral temporal lobe epilepsy, providing an explanation for development and inheritance mode of this LGI1-caused disease [103,104]. Subsequent proteomic studies found that LGI1 forms stable complexes with ADAM22 and 23 [105,106] and confirmed that a subpopulation of LGI1 associates with Kv1 channels [104,107–109]. Although it is likely that LGI1-ADAM22/23 complexes have additional functions independent from modulation of Kv1 channels, it is noteworthy that both complexes were also co-purified as part of Cav2-associated (mainly presynaptic) protein networks (see Section 3.3).

3.3. Channel-associated protein networks: Cav2 channels

Distinct from the aforementioned examples, the proteomic approach in its extended form (Fig. 2) can be used to achieve comprehensive insight into composition and organization of protein networks (Fig. 1).

This may be best illustrated with the subfamily 2 of Cav channels (Cav2.1–Cav2.3 [110]) that are key players in CNS synapses where they initiate a multitude of processes including neurotransmitter release, regulation of excitability, excitation-transcription coupling, synaptic plasticity or axonal growth [111–114]. Most of these processes require free Ca^{2+} ions in micromolar concentrations and are, therefore, expected to reside in the immediate vicinity of the Cav channels, often termed nano-environments (reviewed in Refs. [83,84]). In line with such assumption, APs with individual antibodies targeting the principal α1 and auxiliary β subunits of the Cav2 channels co-purified quite a number of proteins at similarly high yields as the Cav channels [23] and thus imposed all the severe challenges on the proteomic approach described in Section 2.

Consequently, proteomic analysis of the Cav2 nano-environments required the complete set of controls discussed above, including solubilization conditions of different stringency, multiple antibodies to distinct epitopes on the Cav2 target proteins, target knock out material and IgG controls, as well as specificity thresholds and consistency of candidate interactors among the individual APs (Fig. 2, inset 'quantitative evaluation'). As a result,

evaluation of the 64 APs analyzed by 129 nano-LC MS/MS runs (that identified an average of 240 proteins per AP with an average 1970 PVs assigned per AP) provided a wealth set of information on the composition and operation of the molecular nano-environments of the Cav2 channels in the whole mammalian brain. In particular, the following issues and novel insights were set forth by the proteomic approach [23]:

Composition of the channel core: Cav2 channels were found to be made up from α1 and β subunits (Cavβ1–4) that were both co-purified at equimolar ratios independent of the antibody or the solubilization condition. In contrast, two other types of supposed auxiliary subunits [115] were either retrieved at low amounts or failed verification by the proteomic approach. Accordingly, the α2δ proteins, recovered at 0.1–1% of the amounts obtained for α1 and β subunits, must be regarded as either less-stably associated subunits or interactors of a subpopulation of Cav2 channels readily solubilized by digitonin [116,117]. The Cav γ proteins γ2–8 are no subunits of Cav2 channels, but were rather identified as auxiliary subunits of the AMPA-type glutamate receptors [11].

Identification and characterization of the nano-environment constituents: In addition to the core-subunits, proteomic analysis identified ~200 proteins that were all co-purified with the Cav2 channels both specifically and consistently as indicated by abundance ratios with knock out controls and the use of 14 different *anti-Cav* antibodies (see Section 2.6). Quantitative analysis of the protein amounts using PV-based $abundance_{norm}$ values (see Section 2.5) showed that these robustly purified proteins covered an abundance range of about three orders of magnitude with respect to the channel core as a reference. Moreover, the identified proteins exhibited distinct preference for the three Cav2 channel subtypes: one third was uniquely found with one subtype, while two thirds were either shared between two subtypes or were co-assembled with all, Cav2.1–Cav2.3. The largest overlap in 'common protein pools' was seen with Cav2.1 and Cav2.2, reflecting the shared role of both P/Q- and N-type channels in the presynaptic release of transmitters [118].

The number of proteins identified by this comprehensive approach as constituents of the Cav2 nano-environments exceeded by far the number of interactors previously described as partners of Cav2 channels. Notwithstanding, all of these established interactors are represented in the Cav2 proteome at appreciable amounts [119–123]. Quite a number of the other constituents have been implicated in Ca^{2+}-dependent signaling, but have not been structurally linked to the Cav2 channels or their nano-environments. It should be further added, that besides individual interactors the approach also captured en-bloc a number of previously identified 'signaling entities' (see Section 3.2) including the BK_{Ca}–Cav complexes [16], the $GABA_B$ receptor together with the KCTD tetramers [12] or the Kv1-associated protein assemblies [103,124,125].

Organization of the Cav2 nano-environments as networks: The quantitative data on protein amounts obtained from the series of *anti-Cav* APs under different solubilization conditions enabled correlation analyses that can be used to detect direct and more complex protein-protein interactions within the identified pool of nano-environment constituents [126]. In fact, such analyses revealed quite a number of connections between individual constituents of the Cav2 nano-environments and identified a series of sub-clusters therein. Together with database entries on protein interactions (mostly based on one-to-one interactions of proteins or protein domains) this correlation analysis provided the first insights into the organization of Cav2 nano-environments as extended and modular protein networks.

Operation of Cav2 nano-environments: In addition to the more structural/biochemical insights into the Cav2 networks discussed above, the results of the proteomic approach also provided a wealth set of data on their operation. Thus, the primary (biochemical) function of the identified constituents, their established role in cell physiology and their localization within less than a few 10 nm from the Cav2 channels promote a picture where the Cav2 networks function as 'integrative units' or 'signaling platforms' [23,111]. These units are activated by membrane depolarization as an external input which, via opening of the Cav channels, leads to an increase in $[Ca^{2+}]_i$. This Ca^{2+} signal is subsequently integrated by positive and/or negative feedback loops that involve a number of nano-environment constituents (including GPCRs, enzymes, channels and transporters). The resulting $[Ca^{2+}]_i$ finally determines the output of the unit by controlling the activity of 'Ca^{2+}-dependent effector systems' (including BK_{Ca} channels, protein kinase C, CaM-kinase II, nitric oxide synthetase or the machineries of vesicle processing and fusion).

Thus, the proteome of the Cav2 nano-environments reflects the cellular processes that can be initiated by Cav2 channel activity and defines the molecular framework for organization and operation of local Ca^{2+}-signaling by these channels. In addition, it offers a roadmap for detailed investigations of the local Ca^{2+}-signaling in the CNS.

4. Conclusions

The concept of ion channels being embedded into molecular environments has been discussed for quite a while and many efforts have been made to get access to the underlying proteins and protein–protein interactions. High-quality functional proteomics as discussed here now appears able to finally fill this gap and provides comprehensive information on these environments – unbiased and free of any hypotheses. In particular, the proteomic approach is able to unravel the subunit composition of channels and their periphery beyond the borders of known, hypothesized and/or functionally assigned proteins, to discriminate proteins as true complex subunits from more dynamic or indirect interaction partners, and to give insight into the functional properties of the resulting channel-mediated signaling. All these information certainly go far beyond a pure 'list-like' annotation of proteins retrieved by individual APs, but rather set new impulses for further investigations of ion channel function in the areas of molecular as well as cellular research.

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