CHAPTER 9

SUPRAMOLECULAR SIGNALLING COMPLEXES IN THE NERVOUS SYSTEM

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Abstract: It is now apparent that multiprotein signalling complexes or "signalling machines" are responsible for orchestrating many complex signalling pathways in the cell. The synapse is a sub-cellular specialisation which transmits and converts patterns of electrical activity into cellular memory. This processing of electrical information is mediated by the protein components of the synapse. The organisation of synaptic proteins has been investigated over the last number of years using proteomic methods and with the application of bioinformatics; a landscape of modular protein complexes at the synapse is emerging. Many share a common organisation centred on a receptor/channel, a protein scaffold, (in which the signalling molecules are localised) and membrane to cytoskeleton interactions. The use of PDZ-domain based protein scaffolds is a particularly common feature in the construction of neuronal protein complexes and the differential presence of these proteins in complexes can have functional consequences. Here we overview current proteomic methodologies for the analysis of multiprotein complexes. In addition, we describe the characterisation of a number of multiprotein complexes associated with ion channels (NMDAR, P2X7 and Kir2) and GPCRs (5-HT2A/5-HT2C, D2 and mGluR5) and discuss common their common components and organisation.

1. INTRODUCTION

The field of proteomics aims to characterise proteins at many levels of complexity in a biological system. This ranges from identification of the complement of protein expressed in that system to how they are modified by posttranslational events. Comprehensive proteome analysis also seeks to elucidate protein sub-cellular distribution and protein-protein interactions, which integrate proteins into functional molecular complexes that ultimately perform cellular functions. Protein-protein interactions provide the basis for the macromolecular organisation of cells and the architecture of these interactions, for example connectivity of proteins in a network, reflect the assembly of proteins into complexes.

The composition of multiprotein complexes or organelles is variable between cell types, tissues, regions of tissues and between species. In addition, the content of these complexes is likely to be highly dynamic and temporal and thus such complexes can exist in many different forms in a cell at a single time point. Classical plasma membrane associated complexes generally contain receptor or channel protein(s) with an associated matrix of scaffolding proteins which may be linked to the cytoskeleton and other subcellular organelles. Within this scaffold there exists signalling proteins which mediate the transmission of incoming signals from the membrane to the interior, e.g. signaling to the nucleus to regulate gene expression. The types of protein interactions in protein complexes range from stable structural protein domain interactions such as PDZ domain-based interactions to highly transient interactions mediated by phosphorylation or other posttranslational events.

The capture of multiprotein complexes in sufficient quantity and purity whilst maintaining their integrity has been and still remains quite a challenge. There are many approaches to study multiprotein complexes and here we overview methods for the isolation of tagged or native complexes from brain and how the components

of these complexes can be characterised. The application of these approaches will be reviewed to highlight the diversity of proteomic applications in neuroscience.

2. METHODS FOR ISOLATION OF PROTEIN COMPLEXES

2.1. Biochemical Isolation

The approach employed to biochemically isolate a protein complex depends heavily of the abundance of the complex in the starting material. Abundant complexes can be isolated by a physical fractionation scheme, which exploits physical characteristics such as the density or ionic charge of the complex (Dynlacht et al. 1991). However, many proteins and the multiprotein complexes that they form are of low abundance and such physical fractionation schemes are not suitable. In these cases, generation of highly enriched samples containing the target molecule can be achieved by employing various affinity chromatography steps. These can range from ligand affinity (drugs, substrates or co-factors) to the more widely used approach of immunoprecipitation, involving specific antibodies to the molecule of interest.

2.1.1. Immunoprecipitation/Immunoaffinity Chromatography

The use of antibodies for the precipitation or isolation of proteins and protein complexes has been the most common approach for many years. Antibodies raised to whole recombinant proteins or short peptides can be generated that exhibit high affinity binding and specificity to the target protein. In immunoprecipitation, the antibody is incubated with the solubilised protein extract and then the antibody-target protein complex with associated proteins, is precipitated using beads or resin conjugated with Protein A or Protein G. Immunoaffinity chromatography involves coupling the antibody to a resin for example, via amine groups to N-hydroxy-succimide (NHS)-activated Sepharose. The antibody-coupled resin is incubated with the solubilised protein extract and the protein complex is captured directly (Figure 1A). Extensive washing of the resin in both of these antibody-based isolation methods is necessary to reduce non-specific binding. Elution of the complex can be achieved by a variety of methods differing in their efficiency and specificity. In a standard experiment, the washed resin with attached protein complex is boiled in SDS-PAGE loading buffer that contains a high concentration of SDS and a reducing agent to interrupt the antibody-antigen interaction. An aliquot of the supernatant is run on a 1D SDS-PAGE gel and protein-containing bands, as visualised by colloidal coomassie staining, can be excised for downstream analysis by mass spectrometry or for western blotting. A major drawback in using antibody-based approaches for proteomic analysis is the introduction of the IgG itself, which interferes with the analysis by overshadowing proteins of interest that co-migrate during the gel-separation. This can be greatly reduced by using alternative elution strategies such as competitive elution with the immunising peptide. This approach is much cleaner with dramatically less non-specific binding proteins, however complete elution of target protein from the

resin is difficult to achieve. An alternative elution method involves raising or lowering the pH, thereby disturbing the antibody-antigen interaction. This method is still cleaner than boiling elution but some prominent non-specific protein interactions are still evident.

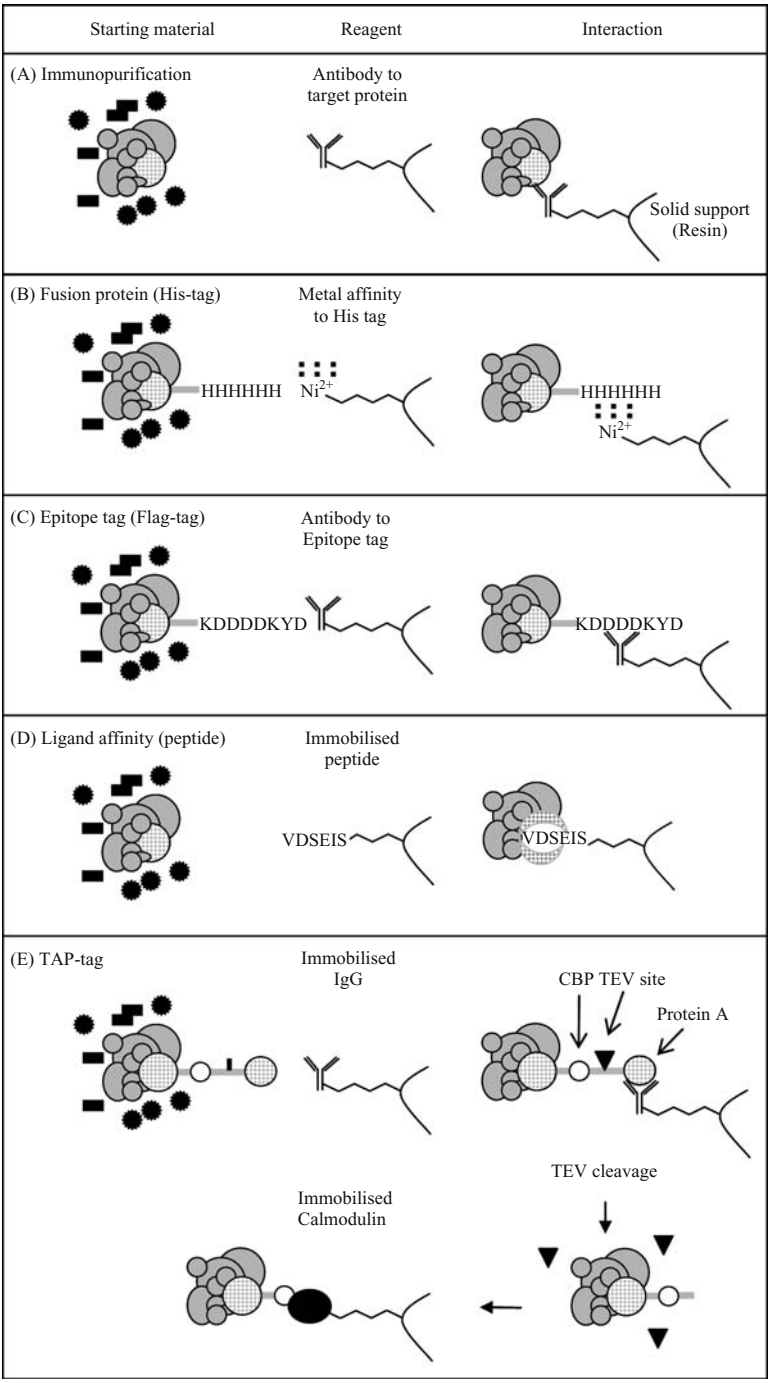
2.1.2. Fusion-protein pull-down

The two most commonly used fusion proteins for protein complex purifications are GST (Glutathione-S-transferase) and His (>6 Histidine residues) tagged. In this approach, the tagged protein is expressed in *Escherichia coli* as a recombinant fusion protein and subsequently immobilized on a resin. The GST-tagged protein is purified using a Glutathione sepharose resin whereas the His-tagged protein is purified by metal-affinity chromatography using for example, a nickel-sepharose resin (Figure 1B). These tagged proteins are incubated with a solubilised protein extract and interacting proteins are co-purified. The main advantages of such fusion-proteins are that it is useful for detecting weak interactions and for low abundance proteins. However, the drawback for such an approach is that the complex must form *in vitro*; post-translational modification of the protein may be necessary for protein complex formation and competition with the pre-assembled complex may occur.

2.1.3. Epitope-tagging

This method combines the use of antibodies and the expression of fusion proteins to deliver an approach which can be applied in a very generic way. The protein of interest is expressed in a cell-line with an epitope-tag which is recognised by an antibody (Figure 1C). Many different cDNA's can be fused to the same tag allowing purification of many different complexes in parallel using the same strategy. Many

Figure 1. Methods for isolation of multiprotein complexes. These methods can be broadly divided into two categories; one which is independent of the starting material such as immunopurification and ligand affinity purification and those which are based on the introduction of a tag into the target protein in the biological sample, for example Fusion protein, Epitope tag and TAP-tag methods. Immunopurification of protein complexes is achieved by the production of antibodies with high affinity to the target protein and isolation of the complex by incubation of the immobilised antibody with a protein extract (Panel A). Peptide affinity purification exploits the affinity of the target protein to a short immobilised peptide for example PDZ domain-containing protein PSD-95 binding to the PDZ motif contained in the last six amino acids (SIESDV) of NR2B (Panel D). The other methods all require engineering of a tag into the gene of the target protein and expression of this tagged protein in a cell line or model organism. The tag can be a short peptide sequence which can be isolated using an antibody specific to that sequence (Flag-tag, Panel C) or a peptide sequence which can be captured by metal-affinity chromatography (His-tag, Panel B). All of these methods suffer from some degree of non-specific binding which can be problematic. A tandem affinity approach reduces the amount of contaminating non-specific binding by using sequential purifications based on for example Protein A/IgG interactions and CBP (Calmodulin binding protein)/Calmodulin interactions (Panel E). The complex is eluted from the first purification by cleavage at a TEV protease site between the tags and from the second purification using a chelating buffer to disturb the calmodulin interaction which is calcium-dependent.


Starting material
Reagent
Interaction
(A) Immunopurification
Antibody to target protein
Solid support (Resin)
(B) Fusion protein (His-tag)
Metal affinity to His tag
HHHHHH
Ni2+
HHHHHH
Ni2+
(C) Epitope tag (Flag-tag)
Antibody to Epitope tag
KDDDDKYD
KDDDDKYD
(D) Ligand affinity (peptide)
Immobilised peptide
VDSEIS
VDSEIS
(E) TAP-tag
Immobilised IgG
CBP TEV site
Protein A
TEV cleavage
Immobilised Calmodulin

such tags exist (e.g. Flag, Myc) and high affinity antibodies to these tags are commercially available. This approach has the advantage that large numbers of complexes can be purified in a consistent way; however problems concerning levels of expression of the tagged-protein and interference of the tag with protein interactions are recognised caveats.

2.1.4. Ligand affinity purification

An alternative to antibody-based or tagging-based methods to isolate protein complexes is to utilise the affinity of a ligand to capture the target protein and associated proteins. Membrane receptors and channels and many classes of enzymes are quite amenable to this kind of approach as many already have well characterised ligand binding pockets. Analogs of their natural ligands have been developed and are used as drugs to modulate the activity and therefore can be used as bait for affinity purification. Single proteins and (Furuichi et al. 2000) or protein complexes (Husi and Grant, 2001) can be isolated by immobilisation of the ligand on a suitable matrix, incubation with a protein extract, and after extensive washing the captured protein(s) can be eluted. The success of such an approach depends of the availability of functional groups or "handles" with which the drug can be immobilised and also upon the specificity of the drug in question.

There are many discrete protein domains which bind to defined linear peptide sequences in their interacting proteins. Often these kinds of interactions are central to the structure of multiprotein complexes and can be exploited to capture these complexes biochemically (Husi and Grant 2001), (Collins et al. 2006), (Becamel et al. 2004). The peptide sequence which forms the binding site for the interacting protein can be synthesised and immobilised readily using the N-terminal amino group (Figure 1D). As these types of interactions are mediated by such short peptide sequences, a synthesised and immobilised version of the sequence is adequate to capture the *in vivo* interaction. As the peptide has to compete with an interaction in a pre-assembled complex in the protein extract, a longer incubation time is usually required for satisfactory capture. Elution can be performed by boiling of the resin, pH elution or by competitive elution with the free synthesised peptide. As for peptide elution of antibody-antigen interactions, peptide elution in this case is also specific and therefore a cleaner elution is achieved. However, long incubation times with the peptide are necessary to increase the efficiency of elution.

2.1.5. Tandem affinity purification

Purification of protein complexes in a high throughput manner requires a method which is generally applicable to any protein and must be reproducible, specific and efficient. Tandem affinity purification (TAP) was developed in the late 1990s and has proved to be very useful for large-scale analysis of protein complexes (Gavin et al. 2002). In this approach, two tags engineered into the protein are used for sequential purification of the complex. The TAP tagged protein is usually stably expressed in a cell line along with the endogenous protein or can be targeted into

the genome of an organism by homologous recombination to replace the endogenous gene. The protein of interest is engineered in frame with an N-terminal or a C-terminal tag; usually both are used for the purification to check for disruption of protein interactions caused by the tag. The tag module is composed of two different affinity tags separated by a tobacco etch virus protease (TEV) cleavage site (Figure 1E). The first purification exploits the protein A tag which is captured efficiently on IgG Sepharose. Then the tag is cleaved at the protease cleave site, thereby releasing the rest of the tag module and protein complex. Next the second tag, which can be a calmodulin binding peptide (CBP) (Gavin et al. 2002), a 6 Histidine residue repeat (Rubio et al. 2005), hemagglutinin (HA) peptide or a myc repeat (Rubio et al. 2005) is captured by calmodulin sepharose, metal chelate sepharose, by antibodies to HA and finally by antibodies to myc, respectively (see Cheeseman and Desai 2005 for review). The final elution of the protein complex is achieved by using EGTA (a calcium chelator) in the case of the CBP or by imidazole in the case of the His-tag. These two elutions are efficient and are free from contaminating IgG. The main advantages of using such two-step purification are that less stringent washing conditions can be used to allow capture of less stable complexes and that non-specific protein binding is low. In addition, because the same purification strategy can be applied to many complexes, the resultant data is directly comparable, a point which is quite important in reciprocal or reverse tagging for protein complex network expansion.

2.2. Identification of Components

2.2.1. Western blotting

Identification of isolated proteins can be readily achieved using western blotting or mass spectrometry. Western blotting (also known as immunoblotting) utilises specific antibodies to detect a protein that has been transferred from a gel onto a membrane. A major advantage of western blotting is its sensitivity and ability to detect amounts of protein beyond the range of current sequencing-based or mass spectrometry methodologies. In fact, the range of protein abundance which was detected by western blotting in yeast was from 50 to more than 10^6 molecules per cell (Ghaemmaghami et al. 2003) which is at least an order of magnitude more sensitive than current LC-MS/MS analyses of complex samples (de Godoy et al. 2005).

It is obvious that this approach is biased by the assumption that a given molecule might be present, and cannot be used to identify unknown or unsuspected proteins. However, when there is a large amount of evidence concerning the putative involvement of molecules that influence many target proteins and such proteins pose ideal targets for western blotting approaches. Additionally, changes in protein levels, as a result of modulation or modification of a receptor's environment, can easily be visualised using specific antibodies against the proteins under investigation. A disadvantage of this method is its labour intensive nature and thus difficulty in performing on a large scale.

2.2.2. Proteomic mass spectrometry

Mass spectrometry (MS) permits the identification of proteins by the determination of the exact mass of peptides, and the fragmentation of these peptides to determine the amino acid sequence. Protein samples, such as purified organelles or protein complexes are usually separated by their relative molecular weight on SDS-PAGE gels. The protein bands are visualized by dyes such as Coomassie brilliant blue and then are excised from the gel. The protein-containing gel slices are washed and incubated with proteolytic enzymes such as trypsin and the resultant peptides extracted from the gel-slices.

A mass spectrometer consists of an ion source, a mass analyzer that measures the mass to charge ratio (m/z) of peptides and a detector that counts the numbers of ions at each m/z value. Electrospray ionization (ESI) mass spectrometers use a heated capillary needle through which the peptide solution elutes and are subsequently ionized and introduced into the mass spectrometer. This type of ion source is readily compatible with liquid chromatographic (LC) separations resulting in an LC-MS/MS setup in which thousands of peptides maybe identified in a single experiment. In a typical LC-MS/MS experiment a peptide mixture is separated by analytical reversed-phase (RP) chromatography in a HPLC (High pressure liquid chromatography) system and is introduced into the mass spectrometer via the ESI source (Figure 2). Multiply

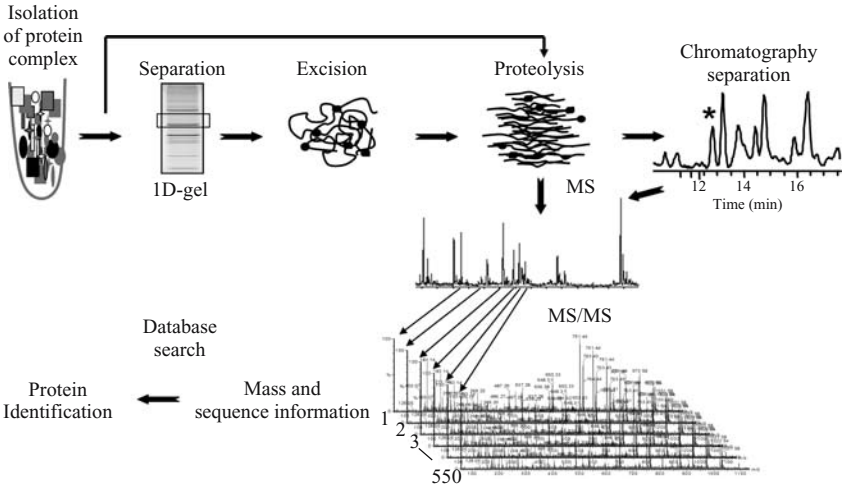


Figure 2. Workflow of an LC-MS/MS experiment. A mixture of peptides from a protein sample digest is separated by reversed-phase chromatography on a nano-flow HPLC. The peptides elute from the RP column and are ionized by an electrospray source. In the first stage of mass spectrometry, m/z values and charge states for each precursor ion are determined and the most abundant precursor ions are selected for analysis in the second stage. The ions are then fragmented with by collision-induced dissociation (CID) a gas to produce fragment ions which are detected. Using the mass (from MS-1) and sequence information (from MS-2) protein sequence databases are searched to provide peptide identifications and protein matches.

Table 1. Multiprotein complexes in the nervous system. Summary of the numbers of identified components and methods used to characterise three ion channel and 4 GPCR multiprotein complexes

Complex	No. # of proteins	Methods	Reference
Ion Channel			
NMDA receptor	186	Peptide-affinity chromatography/ Immunoprecipitation	(Husi et al. 2000)/ (Collins et al. 2006)
P2X7 channel	11	Immunoprecipitation	(Kim et al. 2001)
Kir2.x channel	11	GST-tagging	(Leonoudakis et al. 2004)
GPCR			
5-HT2A receptor	7	GST pull-down/Peptide-affinity chromatography	(Becamel et al. 2002)
5-HT2C receptor	17	Peptide-affinity chromatography	(Becamel et al. 2002)
D2 receptor	39	Various/many single protein studies	(Kabbani et al. 2005)
mGluR5 receptor	76	Immunoprecipitation	(Farr et al. 2004)

protonated peptides give rise to the first mass spectrum (MS1) and then the computer generates a prioritised list of the most intense precursor ions detected that are subsequently subjected to fragmentation by energetic collision with gas (collision induced dissociation, (CID)) in the collision cell. This MS/MS spectrum contains data relating to the amino acid composition of the peptides and the pattern of fragmentation that occurred. This process of tandem mass spectrometry provides m/z values of peptides and fragmentation data for each peptide. This data is then used by sequence database searching programs such as MASCOT (Perkins et al. 1999) or SEQUEST (Yates et al. 1995) and provides probability-based peptide matches and therefore protein identifications.

3. MULTIPROTEIN COMPLEXES IN THE NERVOUS SYSTEM

Many of the methods described in the previous section have been applied to the study of multiprotein complexes in the nervous system. The first proteomic study of a neurotransmitter receptor complex in 2000 (Husi et al. 2000) has been followed by similar studies of important classes of brain receptors and channels (Table 1). As shown in Table 1, complexes associated with ion channels such as members of the ionotropic class of glutamate receptors and GPCRs such as the metabotropic class of glutamate receptors have been described. Here we review a number of such complexes and discuss how their components relate to nervous system biology.

3.1. Ion Channel Complexes

3.1.1. NMDA receptor-adhesion protein signalling complex

The first proteomic analysis of a multiprotein complex relevant to the brain involved the purification and identification of the molecular constituents of the NMDA

receptor-adhesion protein signalling complex (NRC). Using an antibody based approach targeting the NR1 subunit of the NMDA receptor, the NRC was shown to comprise 77 proteins organized into receptor, adaptor, signalling, cytoskeletal and novel proteins, of which 30 are implicated from binding studies and another 19 participate in NMDAR signalling (Husi et al. 2000).

NMDAR and metabotropic glutamate receptor subtypes are linked to cadherins and L1 cell-adhesion molecules in complexes lacking AMPA receptors (Husi et al. 2000). These neurotransmitter-adhesion receptor complexes are bound to kinases, phosphatases, GTPase-activating proteins and Ras with effectors including MAPK pathway components (Husi et al. 2000). A striking feature of the composition is that there appears to be "modules" or sets of signalling proteins that are known to comprise key components of signal transduction pathways that can be distinctly regulated. For example, all of the molecules necessary to induce the phosphorylation of mitogen-activated protein kinase (MAPK) following NMDAR stimulation are present in the NRC (including CaMKII, SynGAP, Ras, MEK and ERK). These modules may allow the NMDAR and mGluR to integrate signals within the complex and then couple to down stream cellular effector mechanisms, such as trafficking of AMPA receptors and cytoskeletal changes that mediate structural and physiological plasticity. Furthermore, at least 18 NRC constituents are regulated by synaptic activity indicating that the composition of the complex is dynamic. In the hippocampus, these activity-dependent genes are known to undergo specific temporal changes following the induction of plasticity.

3.1.2. NMDAR/MAGUK-associated signalling complexes

This NRC dataset was further expanded using a combination of peptide affinity chromatography and mass spectrometry based identification of new components of the complex. Purification of MASCs (MAGUK (membrane-associated putative guanylate kinase)-associated signalling complexes) was achieved by peptide affinity chromatography using a hexapeptide corresponding to the C-terminus of the NR2B subunit that binds MAGUK proteins (including PSD-95). Purified complexes were separated by SDS-PAGE gel electrophoresis, bands excised, digested, and analysed by mass spectrometry. Western blotting of peptide-purified complexes for candidate proteins was also performed. These complexes are similar in composition to NMDA receptor complexes and MAGUK complexes isolated with antibodies (Husi and Grant 2001; Husi et al. 2000). This approach extended the NRC dataset to comprise 186 proteins which constitute MASC complexes (Collins et al. 2006). Out of the previously identified 100 proteins identified in the NRC by analysis of anti-NR1 immunopurifications, 84 of these were also found by the peptide approach. In addition, 86 new proteins were identified by the peptide approach, many of which were in the molecular weight range obscured by the contaminating antibody chains in the immunopurification strategy. The composition and organisation of MASC has been investigated in a number of ways from the use of protein interaction data to construct molecular networks (Pocklington et al. 2006) to the analysis of domains and other protein sequence-based qualities of the components of this complex. The presence of Interpro (Mulder et al.

2005) domains in MASC proteins was investigated using a hierarchical clustering (Figure 3). Proteins in this MASC dataset that contained Interpro domains that were present more than once, and were present in more than one protein were clustered and the resultant clusters were annotated according to their protein classes. MASC protein domains cluster into 14 discrete sets of proteins (Figure 3) reflecting many of the structural and functional aspects known about this set of proteins. This complex is centred on interactions between glutamate receptors and PDZ-containing scaffolders (Table 2). This provides a framework in which a host of kinases, phosphatases and signalling molecules transduce information to gene expression regulatory pathways and to cytoskeletal and transport networks. The MASC dataset represents a comprehensive account of NMDAR-MAGUK complexes identified by large-scale western blotting and mass spectrometry based analyses of both antibody and peptide affinity-based purifications. In order to gain an overall view of the postsynaptic proteome and to validate the components of MASC complexes, a systematic analysis of purified postsynaptic densities (in which these complexes are embedded) was performed. PSD proteins were separated on an SDS-PAGE gel and gel slices were analysed by LC-MS/MS (Collins et al. 2006). 7402 peptides were identified

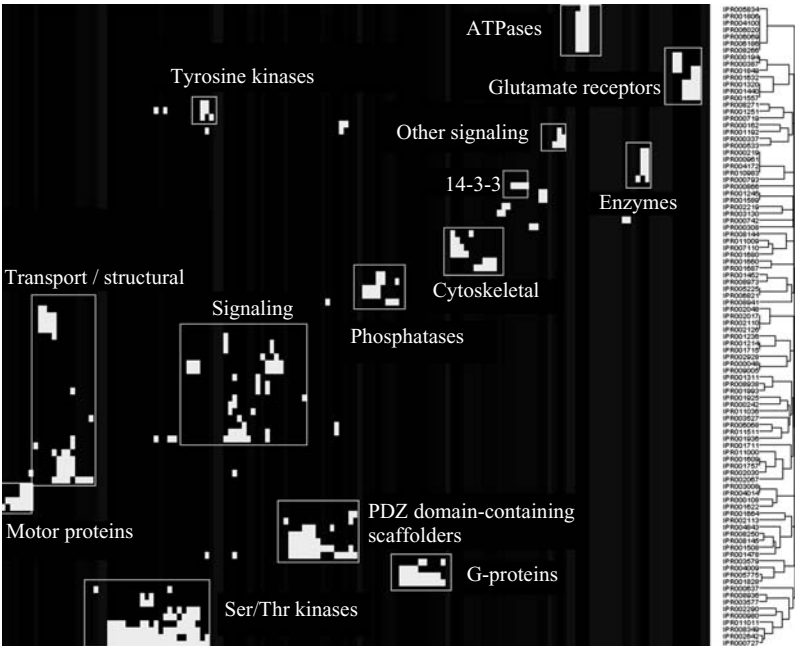


Figure 3. Hierarchical domain clustering of MASC proteins. Interpro domains in MASC proteins occurring more than once in the MASC dataset were clustered. Discrete clusters (grey boxes) were annotated with the protein class of which the majority proteins belonged to. Clusters are formed from co-occurrence of two or more domains characteristic of a protein class

Table 2. Distribution of PDZ domain-containing proteins in neuronal multiprotein complexes. As MAGUK proteins and other PDZ domain-containing proteins provide important scaffolding functions in protein complexes their distribution was analysed. The most widely used PDZ domain-containing proteins are DLGH3 and SAP97. The differential use of these proteins in complexes containing subtypes of 5-HT receptor highlights the heterogeneity and variability in complexes even in closely related receptors

UniProt	Name	MASC	mGluR5	Kir2.2(B)	Kir2.2(H)	P2X7	5HT-2a	5HT2c	D2	# times
O88910	DLGH3	**X**		**X**		**X**	**X**	**X**		5
Q62402	SAP97	**X**		**X**	**X**		**X**	**X**		5
Q62108	PSD-95	**X**		**X**			**X**	**X**		4
O70589	CASK			**X**	**X**			**X**		3
P70175	SAP102	**X**		**X**				**X**		3
Q9Z251	VELI1	**X**		**X**	**X**					3
O88952	VELI3			**X**	**X**		**X**			3
Q63622	CHAPSYN-110	**X**		**X**						2
Q9WV34	DLGH2	**X**		**X**						2
Q9Z216	HOMER1	**X**	**X**							2
Q9WU13	SHANK1	**X**	**X**							2
Q02410	MINT-1			**X**				**X**		2
Q9WVQ1	MAGI2						**X**	**X**		2
Q925T6	GRIP		**X**							1
P39447	ZO-1	**X**								1
Q61234	SYNTROPHIN				**X**					1
Q63ZW7	CIPP						**X**			1
O35274	PPP1R9B								**X**	1
Q9Z0G0	RGS19IP1								**X**	1

corresponding to 620 non-non-redundant protein identifications, with an average of 16.7 proteins identified per gel slice (Collins et al. 2006). This dataset was merged with six other published proteomic studies of the PSD (Walikonis et al. 2000; Satoh et al. 2002; Jordan et al. 2004; Li et al. 2004; Peng et al. 2004; Yoshimura et al. 2004) and 119 individual papers reporting PSD localised proteins. This combined Total PSD dataset contains 1124 proteins and the distribution of protein identifications in the Total PSD dataset show that the majority of proteins were detected only once (58%) and that 198 (18%) proteins were detected twice. In order to define a set of higher confidence proteins, those that were identified two or more times (466 proteins) were grouped and termed the "Consensus PSD" (cPSD) (Figure 4).

It is important to note that the majority of the components of MASC complexes have been validated as PSD proteins in this way and the major overlap between these complexes occur in the cPSD (Figure 4), supporting the notion that the cPSD is an important subset of the Total PSD dataset. This is consistent with a postsynaptic organization where the MASC signalling complex is connected to multiple cell biological effector mechanisms organized into their respective complexes. In addition to MASC and AMPA complexes, components of other complexes such as cell adhesion, growth factor, cytoskeletal, transport and ribosomal complexes were found in the PSP.

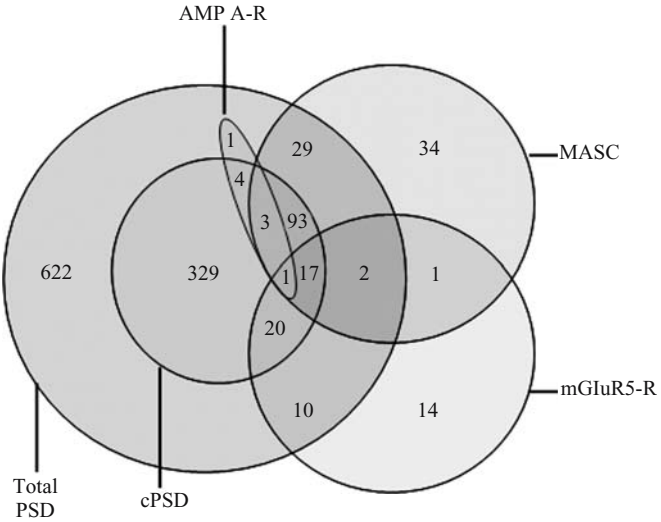


Figure 4. Multiprotein complexes in the postsynaptic density. Venn diagram illustrating the overlap of three glutamate receptor complexes with the Total and cPSD datasets. It can be seen that the majority of overlap between components of these complexes and the PSD occurs in the high confidence cPSD dataset. Proteins detected in these multiprotein complexes, which were not found in any of the PSD datasets, are generally of low abundance that are enriched in immuno-purifications of complexes compared to whole PSD analyses.

Functional annotation of each component protein in a multiprotein complex from the literature can be very useful for determining shared functions within a complex and ultimately functions and pathways specific to the protein complex in question. Using such an approach the importance of the MASC complex was highlighted by the fact that mutations or polymorphisms in 47 MASC genes are associated with 183 human disorders, of which 54 are nervous system disorders (Grant et al. 2005). In addition, 43 MASC proteins have been reported to be important in synaptic plasticity and 40 have been associated with rodent behaviour (Grant et al. 2005). Combining such phenotypic data with protein interaction data allows investigation of the distribution of functions within a network. A protein interaction network with 650 protein–protein interactions for 281 PSP proteins is shown in Figure 5 (**see colour insert**). The individual proteins in this network are represented by filled circles (nodes) and the interactions are represented by connecting lines (edges). An in-house database of mouse targeted mutations, in which a change in LTP or LTD was observed for a given gene knockout was used (see databases on synaptic plasticity and behaviour of mouse knockouts on www.genes2cogntion.org). PSP genes for which such mutant phenotypic data was available are coloured in green in Figure 5. 19 MASC genes, 29 cPSD and 8 Total PSD (not in MASC or cPSD) display changes in LTP/LTD when mutated. The size of nodes displayed in this network is proportional to the number of protein interactors or connectivity of that node. It can be seen that many of the highly connected nodes are found in the MASC complex and that nodes with a phenotype

in LTD/LTP are the mostly highly connected and many are in the MASC complex. This is in agreement with the notion that synaptic plasticity is relatively robust and that perturbation with more highly connected proteins in a network is more likely to produce a phenotype than less connected proteins (Jeong et al. 2001; Pocklington et al. 2006).

Clearly, this kind of phenotypic evidence is very useful and especially when combined with diverse data sources such as gene expression and protein interaction information. Using such information one can assess the heterogeneity of the complex by for example, looking at co-expression of the protein components in brain regions or in developmental time courses in culture (Valor et al. 2007). Furthermore, functional subsets of the complex can be highlighted by using protein interaction network tools, for example such analysis of the MASC protein interaction network resulted in the identification of modularity in the complex where sub-complexes or modules were predicted to perform distinct functions that is there was distributed functionality within the complex as a whole (Pocklington et al. 2006).

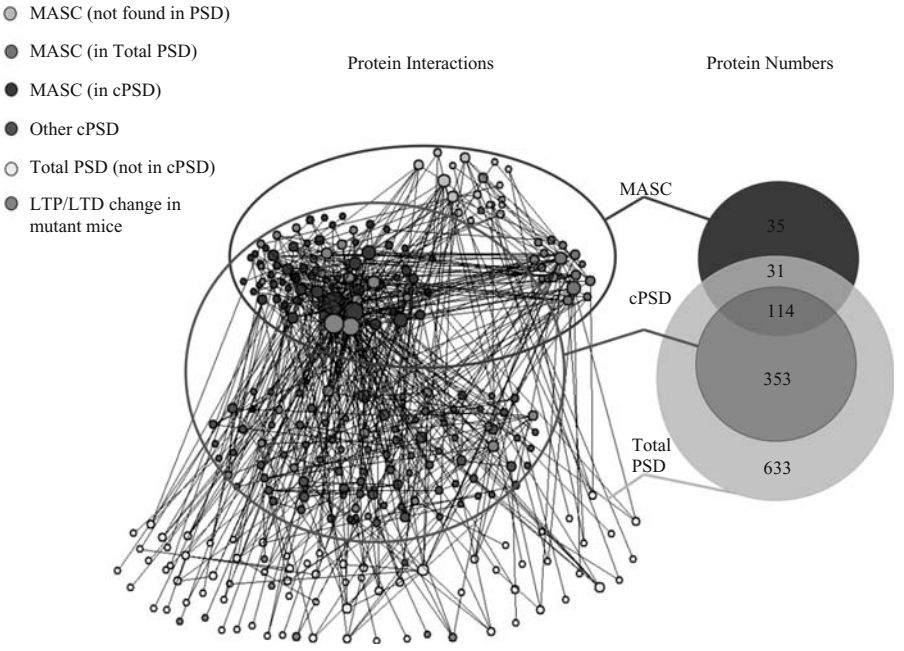


Figure 5. Network illustration of protein interactions and knockout mouse phenotype in PSP proteins. 650 protein–protein interactions for 281 PSP proteins are shown. These PSP interactions are divided into MASC (Red circle), cPSD (Blue circle) and nodes outside the circles are other components of the Total PSD. Also, a Venn diagram of the total numbers of proteins in the MASC, cPSD and Total PSD is shown. Proteins in this network for which an electrophysiological phenotype (change in LTP/LTD) has been reported in the literature are indicated by green nodes. The size of a node is proportional to its connectivity (number of interactors) and it can be seen that the largest nodes with an electrophysiological phenotype are in MASC.

3.1.3. P2X7 receptor complex

P2X receptors are ATP-gated ion channels which are exclusively found in vertebrates. There are seven subtypes of P2X receptors which may be found as heteromers or homomers composed of between three and six of these subunits (Nicke et al. 1998). The $P2X_7$ subtype form functional homomeric channels and have functions in distinct neuronal, astroglial, and microglial cells as well as in cells in non nervous tissues. The exact functions of $P2X_7$ receptors in the brain are diverse but most involve sensing of intracellular ATP levels. When such ATP levels are low, there is evidence to suggest that it can cause $P2X_7$ receptors to directly affect synaptic neurotransmission, when high ATP levels occur, widespread activation of $P2X_7$ receptors stimulate many pathological cascades and finally under persistent activation these receptors, cell death signals are generated (see Sperlagh et al. 2006 for review).

A small complex of proteins associated with the $P2X_7$ receptor has been characterised in HEK293 cells (Kim et al. 2001). The complex was immunoprecipitated with an anti-$P2X_7$ antibody from the membrane fraction of HEK293 cells stably expressing the rat form of the $P2X_7$ receptor. The components were separated by SDS-PAGE electrophoresis and prominent protein containing (as assessed by Coomassie blue staining) bands were excised, digested and the resultant peptides extracted and analysed by MALDI-TOF mass spectrometry. Eleven proteins were identified, including structural/cytoskeletal proteins such as alpha-actinin and MAGuK P55 subfamily member 3 and signalling proteins which included PI4K and RPTPbeta. The presence of a member of the MAGUK family of scaffolding proteins is reminiscent of the NMDA receptor complex indicating perhaps a common mode of protein complex organisation. The association of the complex with the cytoskeleton is expected as activation of the receptor in HEK293 cells results in cytoskeletal rearrangements which result in membrane blebbing (Virginio et al. 1999).

The presence of the tyrosine phosphatase RPTPbeta prompted investigation of tyrosine phosphorylation of the receptor and it was found that the $P2X_7$ receptor was functionally modulated by tyrosine phosphorylation and the exact site of phosphorylation was determined by mutational analysis (Kim et al. 2001). PI4K is involved in the generation of the second messenger precursor PIP_2 and its presence in the complex would confer the ability of the receptor to signal downstream through inositol triphosphate and diacylglycerol. Although this complex was characterised in a non neuronal cell type, the components identified so far hint to the signalling capacity of the receptor. A similar analysis of the complex from neuronal tissue and comparison with complexes associated with the other P2X sub-types is warranted and would be very useful in defining the potential functional similarities as well as differences in P2X signalling complexes.

3.1.4. Kir2 channel complex

Potassium channels are key regulators of cell excitability in the brain and also in other electrically active tissues such as the heart. They control cell membrane potential and

aspects of action potential waveform frequency and shape. Potassium channels consist of pore-forming alpha subunits of which, there are at least 70 different types and are often associated with auxiliary subunits. The activity, trafficking and expression of potassium channels are regulated by interactions with such auxiliary proteins and identification of associated proteins may aid elucidation of channel function.

One subclass of potassium channels, the strong inward rectifier potassium channel (Kir2.x), has been characterised by proteomic analysis (Leonoudakis et al. 2004). Three of these Kir2.x channels contain PDZ interaction motif's at their N-terminus that provides a binding site to scaffolding proteins which contain PDZ domains. A C-terminal GST-Kir2.2 fusion protein was used to affinity-purify interacting proteins from brain and heart tissue. Using mass spectrometry, immunoblotting and N-terminal microsequencing 11 proteins were found to be in a complex with Kir2.2 in brain tissue. Eight members of the MAGUK family were found in the complex, seven of which are also in the MASC complex interacting with the C-terminal PDZ binding site of the NR2 subunit (Table 1). The composition of Kir2 channel complexes in cardiac tissue was also investigated and in addition to many members of the MAGUK family, components of the dystrophin-associated protein complex were identified. These additional components presumably reflect additional Kir2 channel functions in cardiac cells where cytoskeletal and extracellular links are particularly important and alterations in dystrophin can lead to muscular dystrophy (Albrecht and Froehner, 2002). The most likely function of these groups of proteins associated with Kir2.2 channels is to regulate the targeting and trafficking of the channel to discrete subcellular locations as well as scaffolding and stabilisation of the channel in the plasma membrane.

3.2. G-protein Coupled Receptor Complexes

The G-protein coupled receptor (GPCR) family is the largest receptor superfamily in mammalian genomes (Kabbani et al. 2005) and its members are involved in diverse functions from neurotransmission to chemotaxis and many other important physiological processes. This family of receptors is the target for over 50% of all medicines and characterisation of associated proteins in GPCR complexes may aid the discovery of novel modes of pharmaceutical modulation of GPCR-associated functions.

3.2.1. 5-HT-2A/C receptor complexes

All 5-HT receptors (except for 5-HT3, which is a ligand-gated channel) are members of the GPCR family and many psychoactive drugs have been discovered to target the 5-HT-2 sub-types. The 5-HT_{2A} and 5-HT_{2C} subtypes are quite similar in terms of amino acid sequence and pharmacological profile and both contain a canonical type 1 PDZ ligand ((S/T)Xϕ, where ϕ is a hydrophobic residue, SCV and SSV, respectively). In order to investigate whether these differences in the PDZ binding motif in the C-terminal of these two receptors recruited different sets of proteins, a differential proteomic approach was employed. (Becamel et al. 2002, 2004). Synthetic peptides corresponding to the last 14 amino acids of each receptor were coupled to a resin and

peptide-affinity chromatography was performed using brain extract. The co-purifying proteins were identified by a combination of two-dimensional electrophoresis and MALDI mass spectrometry and by western blotting. In total, 7 proteins were found to be associated with the 5-HT_{2A} subtype and 17 proteins with the 5-HT_{2C} subtype of the receptor. Both of these sets of proteins were rich in PDZ domain-containing proteins with 9 such proteins being present in one or other of these complexes (Table 2). The presence as well as the quantity of these PDZ domain-containing proteins varied between these two purifications. This differential recruitment of scaffolding proteins due to a single amino acid change in the consensus PDZ motif is striking and illustrates the specificity inherent in PDZ domain interactions (Nourry et al. 2003).

The 5-HT_{2C} receptor preferentially interacted with PSD-95, SAP102 and MPP-3 when compared with the 5-HT_{2A} receptor. However, the most striking difference between these complexes is the presence of the tripartite Veli3-CASK-Mint scaffolding sub-complex in the 5-HT_{2C} but not the 5-HT_{2A} complex. Electron microscopy revealed that the 5-HT_{2C} receptor is highly concentrated at pre- and post-synaptic thickenings, consistent with the notion that the 5-HT_{2C} receptor is associated with protein networks that are important for its synaptic localisation and its coupling to the signalling machinery (Becamel et al. 2002, 2004). The 5-HT_{2A}receptor on the other hand, which does not interact with this scaffolding complex, is localised to mainly the cytosol of dendritic shafts. This differential localisation of these 2 sub-types of 5-HT_2 receptor correlate well with the differential binding of PDZ domain-containing scaffolding proteins determined by the single amino acid difference in the -1 position of their C-termini.

3.2.2. D2 Dopamine receptor complex

The dopamine receptor is another relatively well characterised and pharmacologically relevant GPCR, which appears to exist in a discrete receptor signalling complex (Kabbani et al. 2005). Dopamine receptors are involved in many functions, from motor control to memory formation and they mediate these functions through regulation of ion channels, sodium pumps and ion exchangers (Kabbani et al. 2005). There are five subtypes of dopamine receptor with differing affinities for dopamine, specificity for coupling to G-proteins and post-translational processing. A systematic analysis of the components of dopamine receptor complexes has not been carried out to date but collection of data from individual published studies reveals that there are at least 39 proteins which directly interact or transiently associate with the D2 type of dopamine receptor (Kabbani et al., 2005). These interacting proteins range from the usual GPCR-associated G-proteins to scaffolding/trafficking proteins and some signalling proteins. Two PDZ domain-containing proteins are associated with the D2 receptor (Table 2). GIPC (GAIP (RGS19) interacting protein C-terminus) binds to the carboxyl-terminal tail of the D2 receptor, thereby recruiting RGS19 which regulates downstream G-protein signalling. The PP1 phosphatase regulatory subunit PPP1R9B (Neurabin II) interacts directly with the third intracellular loop of the D2R by a mechanism which is independent of its PDZ domain and actin-binding domains (Smith et al. 1999). This interaction may mediate linkage of the receptor to the cytoskeleton via

actin and also bring the phosphatase PP1 to the vicinity of the receptor to regulate its state of phosphorylation.

However, the most striking feature about D2 receptors is their direct interaction with many receptors and ion channels. It is generally accepted that GPCRs exist as dimmers or as part of larger oligomeric complexes formed by other types of receptors. Signalling complexes composed of receptors for different neurotransmitters might result in more efficient processing of incoming signals than if they were physically uncoupled. D2 receptors can associate with SST5 Somatostatin receptors (Rocheville et al. 2000), CB1 Cannabinoid receptors (Kearn et al. 2005) and A_{2A} Adenosine receptors (Ciruela et al. 2004). D2 receptors also interact with many ion channels including NMDA (Fiorentini and Missale, 2004), AMPA (Fukata et al. 2005) and GABA receptors (Seamans and Yang, 2004). It is highly unlikely that the dopamine receptor exists in a supramolecular complex with all of these channels and receptors but rather in many different specialised smaller complexes. Elucidation of the components of such a spectrum of complexes would be very interesting and might allow resolution of the diverse functions of the dopamine receptor and dopamine receptor/other receptor or channel complex combinations, at a molecular level.

3.2.3. Metabotropic glutamate receptor 5 complex

Metabotropic glutamate receptors, which are GPCRs and thus couple to G-proteins, modulate synaptic transmission and cell excitability. There are three sub-groups of receptor based on their G-protein coupling preference. mGluR5 is a member of group I mGluRs which couple to the Gq signalling pathway and prior to any proteomic investigation it was well known to be involved in many signalling pathways and to interact with other receptors. The mGluR5 receptor complex was isolated using an immunoprecipitation approach combined with MS-based analysis of a one-dimensional gel separation of the eluted protein and also by direct tryptic digestion of the antibody-conjugated mGluR5 complex. Seventy-six proteins were identified in mGluR5 complexes with varying degrees of confidence based on the presence of each protein in multiple experiments and not in control experiments (Farr et al. 2004). This analysis confirmed the association of the mGluR5 receptor with an intracellular calcium cascade with identification of the IP3 (Inositol 1,4,5-triphosphate) receptor, Homer and Phospholipase C-beta-1. Activation of mGluR5 results in the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP2) to diacylglycerol (DAG), which activates PKC, and inositol triphosphate (IP3), which in turn activates the IP3 receptor to release intracellular calcium (Kawabata et al. 1998).

Interestingly, the IP3 receptor binds to a signalling protein called IRBIT (membrane associated IP3 (inositol triphosphate receptor) binding protein)), in an IP3 sensitive manner (Ando et al. 2003). A phosphoproteomic study of the synapse discovered phosphorylation sites in mGluR5, PLC-β and IRBIT (membrane associated IP3 (inositol triphosphate receptor) binding protein)) indicating potentially novel mechanisms of signalling through the mGluR5 receptor(Collins et al. 2005). The N-terminal region of IRBIT responsible for the interaction with the IP3 receptor was found to contain three

novel phosphorylation sites, and phosphorylation in this region had been postulated to regulate this interaction (Ando et al. 2003).

mGluR5, G-proteins and PLC-β form a multiprotein complex with the IP3 receptor and IRBIT via the scaffolding properties of Homer (Tu et al. 1998). This complex seems to facilitate a signalling pathway from mGluR5 to modulation of intracellular calcium, which is known to regulate many intercellular signalling activities. Homer and Shank are prominent PDZ domain-containing proteins at the synapse (Table 2) and they seem to provide a scaffold (possibly by homo-multimerisation) between the plasma membrane bound, mGluR5 and endoplasmic membrane bound IP3 receptor (Sala et al. 2005).

mGluR5 has an important role in many NMDAR associated synaptic functions. Co-activation of mGluR5 and NMDA receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons (Kotecha et al. 2003) and this process requires IP3R-mediated release of intracellular calcium and activation of PKC for maintenance of potentiation. There is biochemical evidence to suggest that these receptors are physically associated; mGluR5 is a component of the NMDA receptor complex (Husi et al. 2000), and indeed NR2A (an NMDA receptor subunit) is present in the mGluR5 receptor complex (Farr et al. 2004). In fact, 21 proteins are shared by both the NMDAR and the mGluR5 receptor complexes (Figure 4) raising the possibility that these two receptors may co-exist in a signalling complex.

4. EMERGING THEMES IN PROTEOMIC STUDIES OF BRAIN COMPLEXES

What do these multiprotein complexes have in common? The most striking attribute of neuronal multiprotein complexes is their content of scaffolding proteins. The family of proteins which contain PDZ domains are likely to be crucial to the structure and function of many of these complexes. Nineteen PDZ domain containing proteins are found in the eight brain complexes listed in Table 2. Some of these proteins are very common with proteins such as SAP97 and DLGH3 being present in five complexes while others such as GRIP and CIPP are much more specific. Although it is interesting to compare these complexes to look for commonalities that may explain shared structure or function it is likely that the molecular differences may be more informative about the specific functions of these complexes. The association of discrete sets of proteins such as G-proteins, specific kinases and their adaptors (e.g. PKA/AKAP complexes) or other signalling modules are good indicators of what a multiprotein complex actually does. As more data concerning the components of brain complexes accumulates, extraction of what is common and what is specific to each complex will be possible. Systematic characterisation of large numbers of complexes especially within a subclass of complexes such as neurotransmitter receptor complexes, would allow identification of complex specific proteins. Such an approach might accelerate functional validation of complexes by highlighting the most likely candidates for inferring multiprotein complex function.

It is interesting to note that the size of brain protein complexes varies from less than 10 proteins to nearly 200. It is unlikely that the 186 proteins contained in NRC/MASC complexes exist as a single complex but rather this dataset is an average of a number of smaller variant complexes. Protein interaction network analyses suggest that complexes do not exist as discrete units but are linked together and share common modules. The idea of modules or sub-complexes which are used in a generic way in complexes is intriguing, indicating yet another level of molecular architecture. In addition, the physical association of complexes which are functionally linked, such as that described for the NMDAR and mGluR5 points to the fact that signalling pathways between these receptors may be physically embedded in the components of the respective complexes and not remote from each other.

5. CONCLUSIONS

In the last 6 years the concept that multiprotein complexes are employed extensively in cells has been driven by the application of many proteomic technologies, and in particular, mass spectrometry. Systematic mapping of protein complexes in yeast has revealed its molecular architecture in unprecedented detail. Characterisation of protein complexes in the nervous system has been achieved for a relatively small number of channels and receptors using mainly labour intensive approaches. The future of mapping and characterisation of protein complexes lies in the use of generic strategies such as epitope tagging or TAP tagging. So far these are the only technologies that are realistically scaleable for whole proteome analysis of protein complexes. Together with recent advances in high-throughput and sensitive mass spectrometry platforms, characterisation of protein complexes on a proteomic levels is becoming more feasible. Systematic charting of the landscape of protein complexes in higher organisms would certainly be a valuable exercise and will most certainly mark the start of a new era in cellular and molecular biology.

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