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Evolution of Synapse Complexity and Diversity

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postsynaptic density, proteome, genome, protosynapse

Abstract

Proteomic studies of the composition of mammalian synapses have revealed a high degree of complexity. The postsynaptic and presynaptic terminals are molecular systems with highly organized protein networks producing emergent physiological and behavioral properties. The major classes of synapse proteins and their respective functions in intercellular communication and adaptive responses evolved in prokaryotes and eukaryotes prior to the origins of neurons in metazoa. In eukaryotes, the organization of individual proteins into multiprotein complexes comprising scaffold proteins, receptors, and signaling enzymes formed the precursor to the core adaptive machinery of the metazoan postsynaptic terminal. Multiplicative increases in the complexity of this protosynapse machinery secondary to genome duplications drove synaptic, neuronal, and behavioral novelty in vertebrates. Natural selection has constrained diversification in mammalian postsynaptic mechanisms and the repertoire of adaptive and innate behaviors. The evolution and organization of synapse proteomes underlie the origins and complexity of nervous systems and behavior.

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Contents

INTRODUCTION

The hallmark of the brain of humans and that of most other species is its high degree of complexity and diversity of neuronal morphology. Comparisons of the neuroanatomy of organisms in different phyla point to the origin of functional neuronal circuits ~600 Mya in the gelatinous Ctenophora and Cnidaria (reviewed in Lichtneckert & Reichert 2009). Despite the apparent anatomical simplicity of these organisms, their neurons possessed chemical (symmetrical and asymmetrical) and electrical synapses, as well as chemical and peptidergic neurotransmitters. Considering that morphological synapses are absent in Porifera (sponges) or earlier multicellular organisms, it is difficult to envision a set of evolutionary pressures that would have selected for the evolution of all the specialized molecular components that construct a synapse in a single step. As with the evolution of the vertebrate eye (Dawkins 1986, 1994; Nilsson & Pelger 1994), it is far more likely that the many molecular components of synapses had already existed in those earlier organisms that lacked neurons and that these components were reorganized into the visibly distinct structure we know as the neuronal synapse.

In this review we focus on the molecular evolution of synapse proteins and their organization. Akin to the neuroanatomical staining methods that exposed the striking cellular complexity of the nervous system discovered by anatomists in the nineteenth century, neuroproteomic methods in the twenty-first century uncovered a far higher degree of molecular complexity in the protein composition of synapses than had been expected from earlier electrophysiological, biochemical, or genetic studies. It is from these proteomic data that investigators can systematically explore the molecular evolution of all the individual proteins as well as their organization into networks and supramolecular structures. We first introduce the composition of synapse proteomes and genome evolution and then review the evolution of synapses.

SYNAPSE PROTEOMICS AS A BASIS FOR STUDYING SYNAPSE EVOLUTION

Because one can readily measure gross neuroanatomy, cell number, and neuronal shape, there is an extensive literature on the evolution of the nervous system based on these measurements (reviewed in Kaas 2009). By contrast, there is a paucity of studies on synapse evolution, presumably because microscopic and electrophysiological measurements are difficult to obtain and compare between species. This has been a major shortcoming in evolutionary neuroscience since synapses were identified, more than one century ago, as the basis for neuronal connections and information transfer within neuronal circuits.

In the late 1980s and 1990s, the application of complementary DNA (cDNA) cloning

methods identified the genes for a number of ion channel subunits and a relatively small number of other synaptic proteins. At this point genome sequence data were sparse, and thus the scope of enquiry into the range of species and ultimately the ancestry of the genes could not be readily determined. Moreover, these were mostly single gene studies and did not explain the evolution of the synapse itself: The synapse is a macromolecular subcellular structure that is assembled from the protein products of many genes, and it is necessary to examine the evolution of its composite sets of proteins.

The year 2000 was a significant turning point for two reasons. First, proteomic methods were applied to the study of synapses, which revealed unexpectedly large numbers of proteins (Husi et al. 2000). Proteomics has discovered more synapse proteins than has any other approach and has provided the necessary starting information for molecular studies of synapse evolution. Second, the draft sequence of the human genome was released, and the ensuing explosion of genome sequencing from many organisms provided essential data for systematic studies of the phylogeny of individual genes, sets of genes, and whole organisms. The combination of synapse proteomics and genomics has been at the center of the first systematic studies of synapse evolution.

MOLECULAR COMPLEXITY IN THE SYNAPSE PROTEOME

Catalogs of proteins found within mammalian synapses have been derived from mass spectrometry profiling of whole synapses, pre- and postsynaptic fractions, and subcellular structures including synaptic vesicles and signaling complexes (reviewed in Bayes & Grant 2009). In this review we separate discussion of the presynaptic proteome (PreSP) from the postsynaptic proteome (PSP) because most evolutionary research has been performed on the PSP. This is also a useful distinction with regard to function because these proteomes have separate origins in unicellular organisms: The presynaptic release machinery is composed largely of the vesicular release machinery used by unicellular organisms to release chemicals or output information into their environment, whereas the postsynaptic machinery is the point on the cell surface at which information from the environment is received, sensed, or input to the cell.

The mammalian PreSP comprises hundreds of proteins centered around the vesicular release of the neurotransmitter, which occurs in response to the invasion of the action potential into the presynaptic terminal. The ternary complexes formed by synaptobrevin, synaptotagmin, and SNAP25 in mammals form a core structure derived from invertebrate and eukaryotic proteins. Purification of the rodent PreSP coupled with prediction of interacting partners identified 117 core proteins including 32 proteins involved in trafficking [including adapter protein (AP)] complex, syntaxins, synapsins, and synaptotagmins), 22 signaling molecules (including G proteins and 14-3-3 proteins), and 23 cytoskeletal proteins (including actins, septins, and tubulins) (Abul-Husn et al. 2009). Proteomic analysis of the presynaptic active zone with docked synaptic vesicles identified 240 proteins including many plasma membrane and synaptic vesicle proteins (Morciano et al. 2009). Although much is known about the function of many of the individual proteins in neurotransmitter release, by contrast with the PSP relatively little is known about the organization of presynaptic molecular networks and how their complexity is functionally integrated. The PreSP appears to be less complex than the PSP, perhaps because its function is simpler—to reliably release signals when instructed by the arriving action potential—whereas the PSP needs to decode a wide variety of signals in the patterns of neurotransmitter release and other signals from the extracellular environment.

More than 1000 proteins have been identified in the PSP of mammalian brain excitatory synapses (Bayes et al. 2010; Cheng et al. 2006; Collins et al. 2006; Dosemeci et al. 2006, 2007; Fernandez et al. 2009; Hahn et al. 2009; Jordan et al. 2004; Peng et al. 2004; Satoh et al. 2002; Selimi et al. 2009; Trinidad et al. 2005,

Presynaptic proteome (PreSP): complete set of identified proteins in the presynaptic terminal of the synapse

Postsynaptic proteome (PSP): complete set of identified proteins in the postsynaptic terminal of the synapse

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Postsynaptic density (PSD): a dense structure observed beneath the postsynaptic membrane of vertebrate synapses using electron microscopy

MASC: MAGUK-associated signaling complex containing ionotropic NMDA and metabotropic subtypes of glutamate receptors

MAGUK: membrane-associated guanylate kinase

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2008; Walikonis et al. 2000; Yoshimura et al. 2004). Less than 10% of these proteins are neurotransmitter receptors, which highlights that the majority of PSP proteins are not directly involved in electrophysiological functions and instead perform a plethora of signaling and regulatory roles. It has therefore been of great importance to understand how this high numerical complexity can be simplified, understood, and represented in a logical framework. Toward this objective, tools that classify individual proteins into their respective functional types by structure, protein domain composition, and organization and interactions with other proteins have been used (Bayes et al. 2010; Emes et al. 2008; Pocklington et al. 2006a,b). These generate molecular networks that reveal an architecture or organization with several key features. From the membrane, where receptors and channels reside and information is first received by the neuron, to the most downstream of cytoplasmic signaling pathways is a hierarchy (upstream to downstream) of highly complex networks (Coba et al. 2009). Within these networks are pathways, modules or groups of functionally similar proteins, and proteins that are highly connected (hub proteins), many of which are scaffolding proteins that assemble other proteins into multiprotein signaling complexes.

This architecture suggests that activation of a neurotransmitter receptor orchestrates a multitude of intracellular proteins via protein interactions and that these are in many classes of effectors: ion channels, receptors, and structural, biosynthetic, metabolic, and signaling enzymes. This network model of signaling was supported by phosphoproteomic experiments such as those showing that the activation of the *N*-methyl-D-aspartate receptor (NMDAR) in mouse hippocampus slices produced simultaneous changes in phosphorylation of more than 130 postsynaptic density (PSD) proteins on >200 phosphorylation sites (Coba et al. 2009). Moreover, the detailed study of the phosphorylation sites and their network relationships showed seven types of phosphorylation building blocks that are used in combination on different proteins to perform regulatory roles (Coba et al. 2009). These studies emphasize that the PSP is a molecular system employing an elaborate multidimensional interrelationship of hundreds of proteins that require mathematical methods to represent their structure and function. Thus in considering the evolution of the synapse, one must draw attention not only to the origins of the proteins, their domains, and regulatory sites, but also to their organization and physical interrelationships into higher-order structures.

Examples of such structures found within the PSP are the signaling complexes, which play a central role in detecting and processing the information that arrives at the postsynaptic terminal (**Figure 1**). They have also served as more manageable sets of proteins for experimental manipulation. The prototype postsynaptic complex is known as MASC (MAGUK-associated signaling complex) comprising ~10% of all vertebrate PSP proteins. MASC can be physically isolated from the brain using affinity purification methods (Fernandez et al. 2009, Husi et al. 2000). MAGUK proteins are scaffold proteins in the membrane-associated guanylate kinase family and have no enzymatic activity but contain protein-binding domains that allow receptors and enzymes to act in close proximity (Good et al. 2011, Nourry et al. 2003). MASC contains the principal postsynaptic machinery involved in synaptic transmission and synaptic plasticity: ionotropic and metabotropic glutamate receptors, potassium channels, cell-adhesion proteins, and MAGUK and other scaffold proteins as well as their associated signaling enzymes and structural proteins (Bayes et al. 2010; Collins et al. 2005, 2006; Emes et al. 2008; Husi et al. 2000; Pocklington et al. 2006b). The functional importance of the prototypical MAGUK protein called postsynaptic density 95 (PSD-95) was shown using knockout mice, which had impairments in synaptic plasticity, learning, and other forms of behavioral adaptation (Migaud et al. 1998). Mice carrying mutations in other MASC proteins also show impairments in synaptic plasticity and adaptive behaviors, indicating

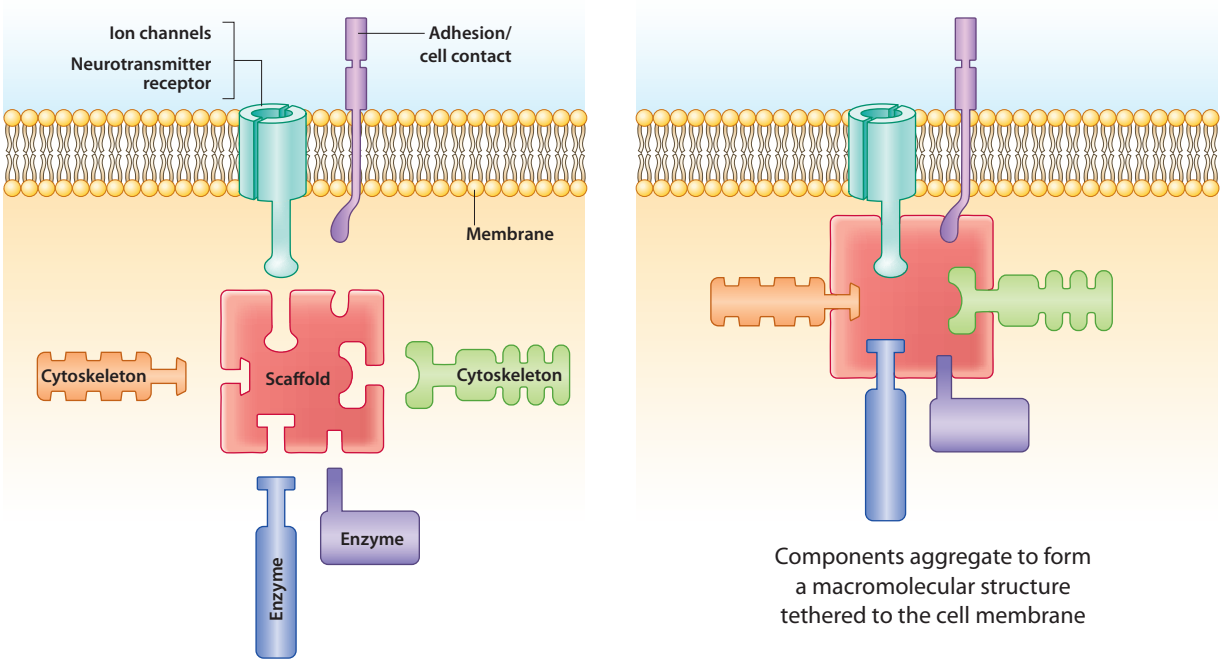

Figure 1

Organization of signaling complexes. The physical interaction of multiple types of proteins builds multiprotein complexes to allow signals to be received from the environment and communicated to intracellular biological processes and pathways. The left panel shows individual types of proteins illustrated with specific binding sites that attach to the scaffold protein, and the right panel shows them assembled into the aggregate multiprotein complex.

that these adaptive responses are an emergent property of this set of interacting proteins.

GENOME EVOLUTION AND ITS ROLE IN SYNAPSE EVOLUTION

Understanding the origins of synapses or any other aspect of brain evolution is inextricably interlinked with understanding the evolution of genomes (reviewed in Lynch 2007). Here we remind the reader of some basic principles and identify the types of genomic mutation that played key roles in synapse evolution.

The major forces affecting genome evolution are mutation, duplication, and deletion. Mutation of DNA can occur as single nucleotide events, as single nucleotide polymorphisms (SNPs) in populations of individuals, or as larger insertion or deletion (indel) events. These changes in the DNA may be silent or affect mRNA abundance, whereas if they occur in the coding sequence, they may affect the structure and hence function of the encoded protein. At the level of the gene, two major processes are associated with evolution: gain and loss. The de novo formation of a new gene from noncoding DNA is relatively rare. However, duplication of genes from existing genes or exons is more common. The formation of gene duplicates is associated with relaxation of selection pressure, allowing potential for rapid diversification. The fate of a duplicated gene will be to undertake a related role (subfunctionalization), to develop a new function (neofunctionalization), or to become nonfunctional by accumulation of deleterious mutations (pseudogenization) (Hurles 2004). More dramatic evolutionary events such as whole-genome duplication can also occur such as the two rounds of duplication in the Cambrian period (between 500 and 600 Mya) prior to the divergence of the vertebrate animals (Van de Peer et al. 2009).

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Synaptome: the complete protein complement of the synapse. The synaptome is the sum of the PreSP and PSP

The comparison of genes from different organisms provides a wealth of information on which genes are shared and hence which functions we may predict for different species. The central tenet of these methods is to look for similarities between gene or protein sequences that are greater than we would expect by chance. If we detect sequence similarity, the most parsimonious explanation is to infer homology between compared sequences and predict that the genes or proteins share a common ancestor. Additional methods may compare domain composition between genes and genomes. Most protein domains are ancient and are found either at the origin of the eukaryotes or are even shared between different kingdoms (Ekman et al. 2007). However, the number of domain combinations or domain architecture dramatically increased in eukaryotes in a process termed domain accretion (Koonin et al. 2000). For example, Chothia et al. (2003) reported that vertebrate genomes contain 2.5 fold more domains per protein family compared with invertebrates. Protein domains conserved in the eukaryote PSP (present in metazoa and yeast but not detected in prokaryotes) show the incorporation of proteins containing domains involved in signal transduction, vesicle-mediated and intracellular protein transport, and ATP synthesis coupled proton transport (**Figure 2**). Molecular comparisons using these methods dominate the field of phylogenetics, which attempts to construct representations of the relationship between genes or species. This is not a trivial task, and the abundance of molecular data has revealed the complexity of gene transfer, duplication, and loss, making the drafting of a universal tree of life for all genes a complicated endeavor. However, a generally accepted relationship of species has emerged, and we show a subset of the tree of life representing species discussed within this review with some of their synaptic proteome features (**Figure 2**).

For ease of comprehension, this review describes our understanding of the origins of synapse genes and the accumulating complexity of the PSP in a step-wise manner from the most distant ancestral prokaryote organisms to a range of invertebrate and vertebrate organisms with nervous systems. We are not implying that there was a direct evolutionary trajectory from prokaryotes to vertebrate synapses by climbing the *scala naturae*. This approach is useful to identify conserved proteins and protein domains in different groups of organisms and to expand our understanding of the composition of the genetic toolkit for synapse construction. Thus one can examine the components available in the common ancestor of two groups and examine the evolution of the synaptome in different lineages.

PROKARYOTE ORIGINS OF SYNAPSE PROTEINS AND CORE PATHWAYS

Comparative genomics of the synapse has used two complementary strategies: studies of specific protein classes [e.g., glutamate receptors or scaffold proteins (Kosik 2009, Ryan et al. 2008)] or more comprehensive studies of the multiple classes of proteins comprising the range of proteins that constitute the synapse proteome (Bayes et al. 2010, Emes et al. 2008, Kosik 2009, Pocklington et al. 2006b, Ryan et al. 2008, Ryan & Grant 2009). This latter approach has tackled the problem of identifying the most ancient elements of the PSP: those conserved between vertebrates and prokaryotes (**Figure 2**). A novel method was applied to compare each mammalian PSP gene to a set of 28 bacterial and archaeal genomes (Emes & Grant 2011). By comparing species from the three superkingdoms of eukaryota, bacteria, and archaea, we identified conserved genes predating the last eukaryotic common ancestor. Using this sensitive method to identify homologs, 28.5% of genes encoding the human PSP were conserved in all superkingdoms. These represent a diverse range of 65 family types including enzymes, ribosomal proteins, and kinases. Among these, 61 genes were conserved across all the bacterial and archaeal species tested. Some of these are potentially spurious, resulting from horizontal gene

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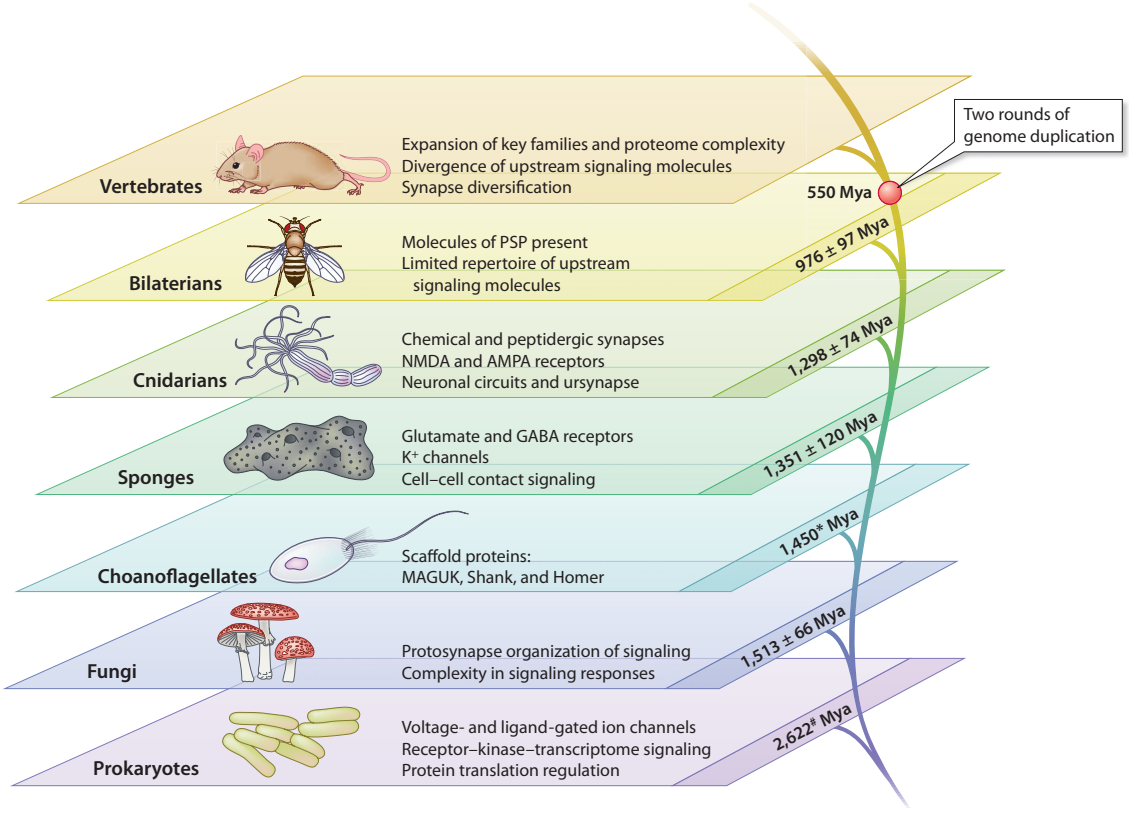

Figure 2

Cladogram of taxonomic groups and origins of PSP components. A generalized cladogram showing the groups discussed and the types of molecules and functions found in the mammalian PSP are indicated (modified from Ryan & Grant 2009). These represent the genetic toolkit for synapse construction. Dates indicate divergence time in millions of years +/− error estimates of divergence (Hedges et al. 2004). *Estimate of divergence based on midpoint of adjacent nodes (Hedges et al. 2004). #Weighted average divergence time of vertebrates and eubacteria as calculated by time tree (Hedges et al. 2006).

transfer (HGT) events between prokaryotes and eukaryotes (Koonin 2003, Lawrence & Hendrickson 2003) such as synthetase genes (Koonin et al. 2001, Wolf et al. 1999). However, ribosomal proteins, translation elongation factors, lyases, chaperone proteins, and the G protein OLA1 are monophylogenetic without obvious evidence of HGT and are therefore candidates for PSP homologs shared since the last common ancestor of prokaryotes and eukaryotes (Emes & Grant 2011). To determine the conserved biological functions underlying the detection of homologous genes, it is useful to investigate the conservation of protein domains. Owing to the functional information known about conserved domains (Finn et al. 2010, Marchler-Bauer et al. 2005, Schultz et al. 2000), we can link domains to biological processes shared between prokaryotes and eukaryotes. The domains conserved in all three superkingdoms reflected basic biological processes, such as translation, carbohydrate metabolic process, glycolysis, and tRNA aminoacylation for protein translation. Comparison of eukaryotic PSP biochemical pathways with a representative prokaryote identified ten pathways that had a significantly greater number of prokaryote-eukaryote

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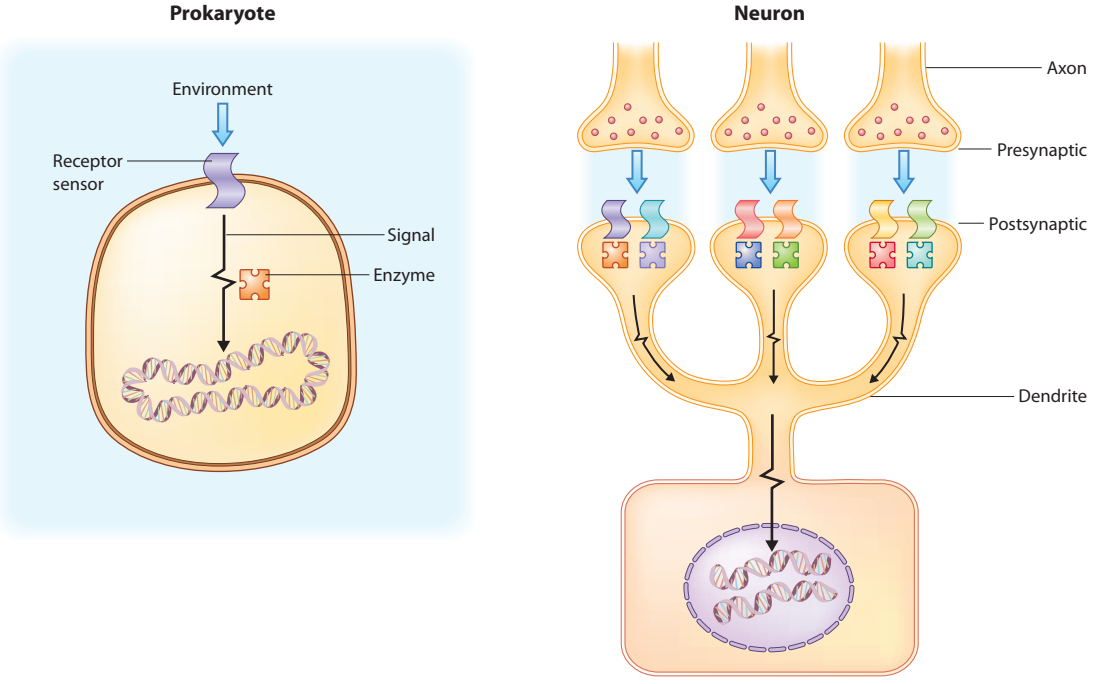

Figure 3

Sensing of the environment by prokaryotes and mammalian synapses. In the prokaryote (*left panel*) the external environment (*blue*) may be a nutrient or diffusible signal that stimulates membrane proteins (receptor sensor) that can trigger intracellular kinases (enzyme) that regulate transcription. Similar mechanisms are conserved in mammalian synapses (*right panel*); however, the sensing proteins are on the postsynaptic terminal where they respond to pulses of neurotransmitter released from the presynaptic terminal, which in turn is receiving action potentials from sensory organs. Multiple varieties of mammalian receptors and intracellular enzymes produce synaptic diversity.

homologs than expected by chance. Pathways involved in energy generation [e.g., the tricarboxylic acid (TCA) cycle] and fatty acid biosynthesis are conserved in a near complete state. In addition to the specific information about the conserved domains and proteins, these data provide evidence that some of the organization between the proteins in prokaryotes is conserved: a proportion of the homologs detected were interacting proteins prior to the divergence of prokaryotes and eukaryotes (Emes & Grant 2011).

In recent years these biosynthetic proteins and pathways have been found to be important in vertebrate and invertebrate mechanisms of synaptic plasticity. It is also important to note that there is conservation of plasticity mechanisms seen in environmental sensing proteins (**Figure 3**). The *Escherichia coli* ArcAB two-component signal transduction system is involved in responses to anaerobic environments; the chemotaxis protein CheA of the chemotactic signal transduction system and the sensor kinase ArcB (a membrane-associated protein) were all conserved. Moreover, the bacterial membrane has many voltage- and ligand-gated ion channels, which control energy production, mechanosensation, motility, and resting membrane potential (Martinac et al. 2008). Furthermore, Kralj et al. (2011) recently showed that the membrane potential of *E. coli* is dynamic and shows electrical spiking, albeit slower than in neurons. There is also evidence of prokaryote ancestry to the neurotransmitter receptors that initiate the postsynaptic response in the mammalian brain. For example, the key structural features of ligand-gated ion channels are conserved

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(Bocquet et al. 2007, 2009; Nury et al. 2011), and in the case of the glutamate receptors, there is evidence of conservation of glutamate binding domains (Janovjak et al. 2011, Nakanishi et al. 1990, Sprengel et al. 2001).

This conservation from receptor-to-transcriptome signaling is interesting to consider in terms of environmental sensing and adaptive behaviors (**Figure 3**). In the case of the unicellular prokaryote, the sensing is initiated at the cell's surface, where it is in direct contact with the environment. In the case of the brain, the environment of the outside world is detected by sensory end organs (e.g., eyes, ears), which convert information into patterns of action potentials that are transmitted by nerve conduction to the synapses in the brain. These action potentials stimulate releases of pulses of neurotransmitters into the local extracellular environment where the receptors and signaling systems in the PSP are activated. The sets of synapse proteins comprising receptors and their signaling and biosynthetic pathways arose in prokaryotes, and their role in enabling the prokaryotic organisms to respond and adapt to changing environments appears to be broadly the same role they perform in the brain.

EUKARYOTE INNOVATIONS AND THE PROTOSYNAPSE

The emergence of eukaryotic cells was marked by larger and more complex genomes, linear chromosomes requiring capping with telomeres, and multiple replication origins. The increase in complexity of the transcriptome was marked by a shift from prokaryotic operons to splicing and novel RNA regulator machinery programming the proteome of complex subcellular structures, including membrane-enclosed organelles and the cytoskeleton. The vesicular machinery (which later evolved into neurotransmitter release machinery in metazoans) allowed the active movement and engulfing of material by phagocytosis, and this was likely a key step in the origins of the eukaryote: Predation of aerobic bacteria by an ancestral eukaryote cell resulted in the symbiosis of the genomes of engulfed bacteria to form the mitochondria as well as contributing expansions to the genome of the stem eukaryote (Lynch 2007). It is fascinating to consider that the vesicular mechanism that might have been responsible for eukaryotic genomic complexity, and thus the complex biology of eukaryotes, also underpinned the mechanisms of neurotransmitter vesicle recycling, which is a central function of the presynaptic terminal.

A comparison of mouse PSP orthologs with 19 eukaryote species (fungi, invertebrates, nonmammalian vertebrates, and mammals) revealed extensive conservation of the components of the synapse across the eukaryota (Emes et al. 2008). Approximately 23% of genes tested had a detectable ortholog in the yeast *Saccharomyces cerevisiae*, and this rose to ~45% having detectable orthologs in *Caenorhabditis elegans* or *Drosophila melanogaster* (these numbers should not be directly compared with those reported for the prokaryote above because here we are describing identified orthologs and hence expect to see a relatively lower percentage than that for all homologs detected by the method described above). With this finding it is evident that the genome of the common ancestor of mammals and *S. cerevisiae* that obviously lacked a nervous system or morphological synapse harbored many of the genes used to encode the constituents of the functional synapse.

As with the conserved prokaryote genes, many conserved eukaryotic genes in the PSP encode environment-sensing mechanisms driving signal transduction pathways and basic cellular functions such as protein synthesis and degradation enzymes controlling turnover of synaptic proteins (Emes et al. 2008). The detection of yeast orthologs of *NF1* (*ira2*), *PKA* (*tpk2*), *Erk2* (*fus3*), and *GNB5* (*ste4*) members of canonical pathways regulating transcription, cell morphology, and adhesion downstream of nutrient- and pheromone-sensitive GPCRs (Elion et al. 2005; Erdman & Snyder 2001; Harashima et al. 2006; Palecek et al. 2002) suggests that these components of synaptic pathways regulating protein synthesis and structural plasticity in mammals conduct analogous roles

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Protosynapse: complex of synaptic proteins present in early metazoans without a defined nervous system

in unicellular responses to environmental cues (ions, nutrients) and cell-cell communication.

Although a complete analysis of PSP homologs in choanoflagellates (free living unicellular flagellates) or sponges is lacking, analysis of key PSP genes in these organisms has proved informative in understanding the organization of the networks of interacting proteins at the base of the eukaryotes. The scaffold proteins such as the MAGUKs were present in the Opisthokont common ancestor: They are detectable in the demosponge *Amphimedon queenslandica* (Sakarya et al. 2007) and in the choanoflagellate *Monosiga brevicollis* (Alie & Manuel 2010). In addition to these, sponges express GABA (γ-aminobutyric acid) receptors, K^+ channels (KIR), and metabotropic G protein–coupled glutamate receptors (MGluR). Notably absent are the NMDA ionotropic glutamate and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors essential for mammalian synaptic plasticity (Richards et al. 2008); however, they are present in the cnidarian *Nematostella vecensis*, which possesses a nerve net (Sakarya et al. 2007). Additionally, multiple cnidarian species contain genes for Na_v family ion channels (Liebeskind et al. 2011).

Together these data suggest that the organization of proteins that is typical of postsynaptic multiprotein complexes—scaffold proteins, receptors, and enzymes—were present in choanoflagellates, Cnidaria and Porifera. This set of proteins in these organisms and other unicellular eukaryotes has been referred to as the protosynapse (Emes et al. 2008) because it comprises the organization of sets of proteins that confer key signaling properties on synapses (**Figure 1**). This physical organization is highly relevant in eukaryotic biology because the multiprotein complexes assembled by scaffold proteins are important for controlling the flow of cellular information in a multitude of settings (Good et al. 2011). In line with the observations on the interactomic and phosphoproteomic networks of the PSP (Coba et al. 2009, Pocklington et al. 2006b), the assembly and partitioning of proteins into complexes produce modularity and higher-order regulatory mechanisms in information processing such as amplification and forms of switching, and again these emergent properties of multiprotein signaling complexes are found in unicellular eukaryotes (Good et al. 2011).

The ancestral function of the protosynapse is thought to have provided the link between Ca^{2+} signaling and actin cytoskeleton regulation (Alie & Manuel 2010). Additionally, analysis of *M. brevicollis* has revealed the presence of multiple tyrosine kinases involved in the response to environmental stimuli (King et al. 2003, King & Carroll 2001, Manning et al. 2008, Pincus et al. 2008). This finding, again, suggests that the functional roles of the protosynapse may have been sensing and responding to a changing environment; in doing so, it can be engaged and driven by different classes of receptors and hence respond to different kinds of environmental signals. The proteomics of mammalian MASC complexes also shows that multiple types of receptors can connect to the scaffold protein complexes (Fernandez et al. 2009).

Complementary to the power of comparative genomics is the need to perform comparative proteomics on synapse proteins and complexes. In the case of humans, mice, and rats, it is clear that similar sets of proteins comprising the PSD can be isolated with an overall complexity of between 1 and 2000 proteins (Bayes et al. 2010; Cheng et al. 2006; Collins et al. 2006; Dosemeci et al. 2006, 2007; Fernandez et al. 2009; Hahn et al. 2009; Jordan et al. 2004; Peng et al. 2004; Satoh et al. 2002; Selimi et al. 2009; Trinidad et al. 2005, 2008; Walikonis et al. 2000; Yoshimura et al. 2004). The first example of the isolation of an invertebrate synapse proteome was the isolation of the synaptic MASC from *Drosophila* (fMASC) and its direct comparison with its mouse counterpart (mMASC) (Emes et al. 2008). Surprisingly, 220 fMASC proteins were identified showing that the MASC of fly and mouse [mMASC 186 proteins when isolated using a similar technique (Collins et al. 2006, Husi et al. 2000)] was of

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comparable size. However, although mMASC and fMASC are approximately equal in size, major differences in the types of proteins were identified. By functional annotation investigators revealed that upstream signaling/structural components (receptors, scaffolds, signal transduction molecules) accounted for ~25% of the fMASC proteome compared with >60% of the mMASC. When the composition of the fMASC was compared with yeast, 71% of fMASC genes were also found in the yeast *S. cerevisiae*, and hence only 29% appeared to be of metazoan origin. Thus the majority of downstream components were present in yeast, while the upstream signaling/structural components of fMASC and mMASC showed lineage-specific functional expansions. Thus the core functionality of the protosynapse machinery that comprises MASC evolved in unicellular eukaryotes and lineage expansion of upstream signaling molecules (such as receptors and their directly associated cytoplasmic proteins) in metazoans increased the molecular complexity of this machinery (**Figures 1** and **4**). Together the comparative proteomic and genomic studies reveal that invertebrates evolved synapses with highly complex molecular composition built around the protosynapse.

COMPLEXITY AND DIVERSITY IN METAZOAN SYNAPSES

The molecular phylogeny described above indicates that the core functionality in protein components, pathways, and organization in mammalian synapses was present prior to the first morphologically visible synapses, such as synapses in Cnidaria that evolved ~900–1400 Myr ago (**Figure 2**). The protosynapse machinery with its specialized environmental sensing capacity and regulation of transcriptome responses for adaptive changes evolved before this and was incorporated into the ursynapse. The simple nervous systems of Cnidaria were the precursors of the highly elaborate and diverse nervous systems that characterize the multitude of invertebrates and vertebrates that subsequently arose. In these anatomically complex brains, some with enormous numbers of synapses, there is considerable anatomical diversity in the postsynaptic dendritic spines. It is therefore of great interest to understand how diversity in populations of synapses arose and if this was relevant to the molecular organization of the protosynapse.

Ursynapse: last common ancestor of all morphological synapses from which all extant synapses evolved

A major event in the diversification of biological functions that characterize chordates and thus all vertebrates was the 2 complete genome duplication events ~550 Myr ago (Van de Peer et al. 2009) (**Figure 4*b***). This period corresponded with the Cambrian explosion, when there was a dramatic increase in the diversity of animal life in the fossil record, which was presumably accompanied by a diversification in nervous system complexity. Comparison of 13 vertebrate genomes (including primate, rodent, fish, chicken, and opossum genomes) showed a step-wise expansion from invertebrates in the number of conserved PSP homologs to ~85–90% conservation (Emes et al. 2008). This PSP expansion in complexity event coincides with the predicted genome duplication, where these two rounds of genome duplication are thought to have occurred prior to the divergence of the hagfish and lampreys (Holland 2009). The two rounds of genome duplication typically expanded gene families to four copies; however, some gene families have lost copies and other gene families have gained further copies by individual gene duplication events (**Figure 4*b***) (Van de Peer et al. 2009). Further evidence for the importance of gene duplication or gene retention following whole genome duplication comes from the comparison of protein domains. The number of domain types did not increase to the same extent that gene number did, which suggests that the synapse proteome expansion seen in vertebrate genomes does not represent a recruitment of proteins containing new domain types but rather the expansion of protein types already present in the PSP of early branching metazoans such as flies and worms.

Examining the expansion in the vertebrate PSP further showed that the protein families that had the greatest expansion were the upstream signaling proteins (receptors, adhesion proteins in the membrane, and their proximal

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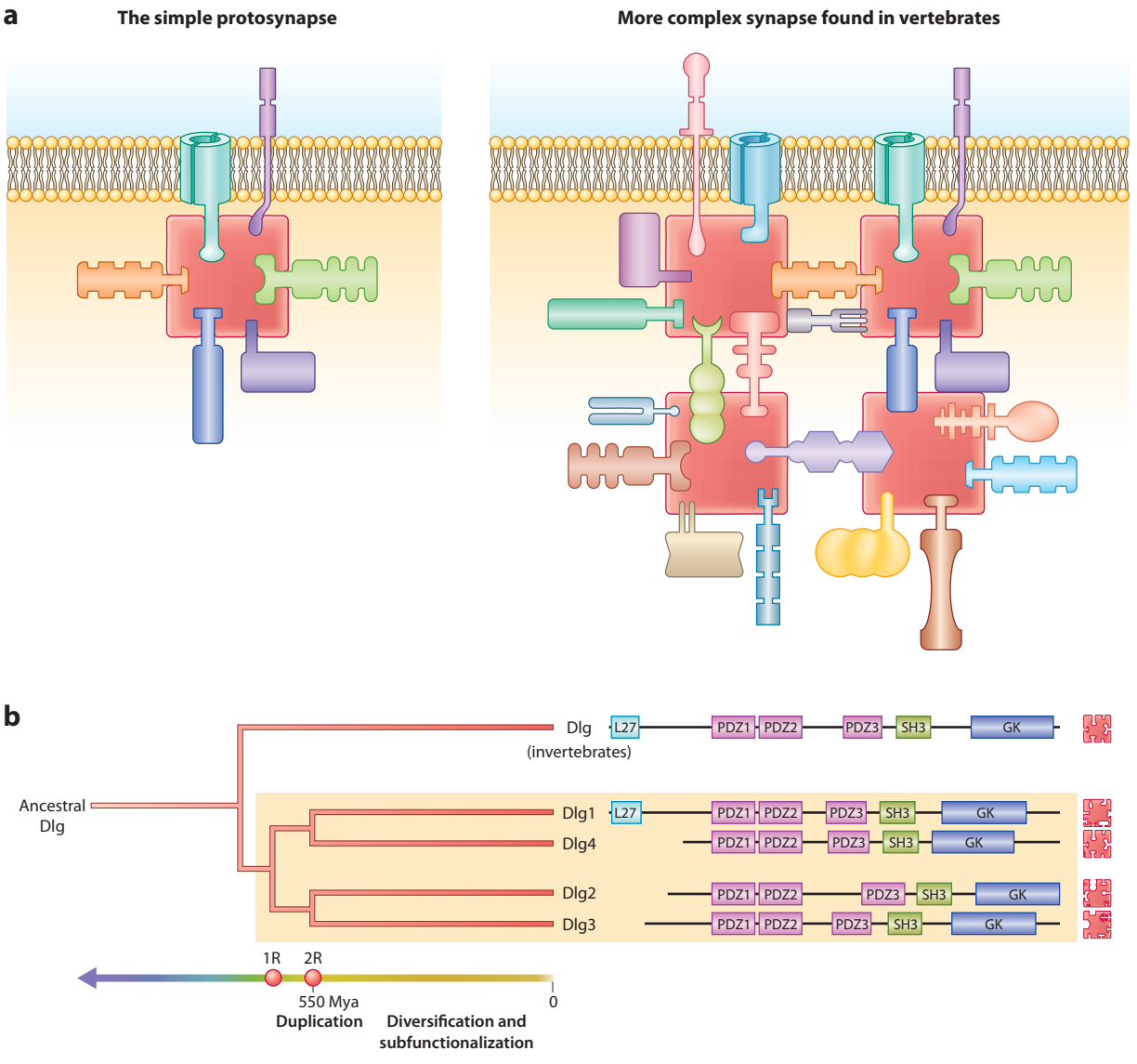

Figure 4

Genome duplication expands protosynapse complexity. (*a*) The basic components of the core signaling complexes are found in eukaryotes (*left panel*) have been multiplied by the process of gene duplication (see panel *b*), producing greater varieties of component proteins for vertebrate synapses (*right panel*). (*b*) Gene duplication of a MAGUK scaffold protein. The ancestral *Dlg* gene found in invertebrates was duplicated twice (1R and 2R) around 500–600 Mya resulting in 4 vertebrate paralogs. Following duplication, the accumulation of sequence diversity in each paralog results in functional and structural diversification of each Dlg protein. The conserved domain structure of the invertebrate Dlg and mammalian Dlg1–4 is shown (PDZ, SH3, and GK domains illustrated). The red shapes (*adjacent to each Dlg protein on right*) indicate that Dlg encodes the core scaffold proteins shown in **Figures 1**, **4*a***, and **5** with the same shape. Genome duplication similarly increased the complexity of many other PSP gene families in vertebrate lineages.

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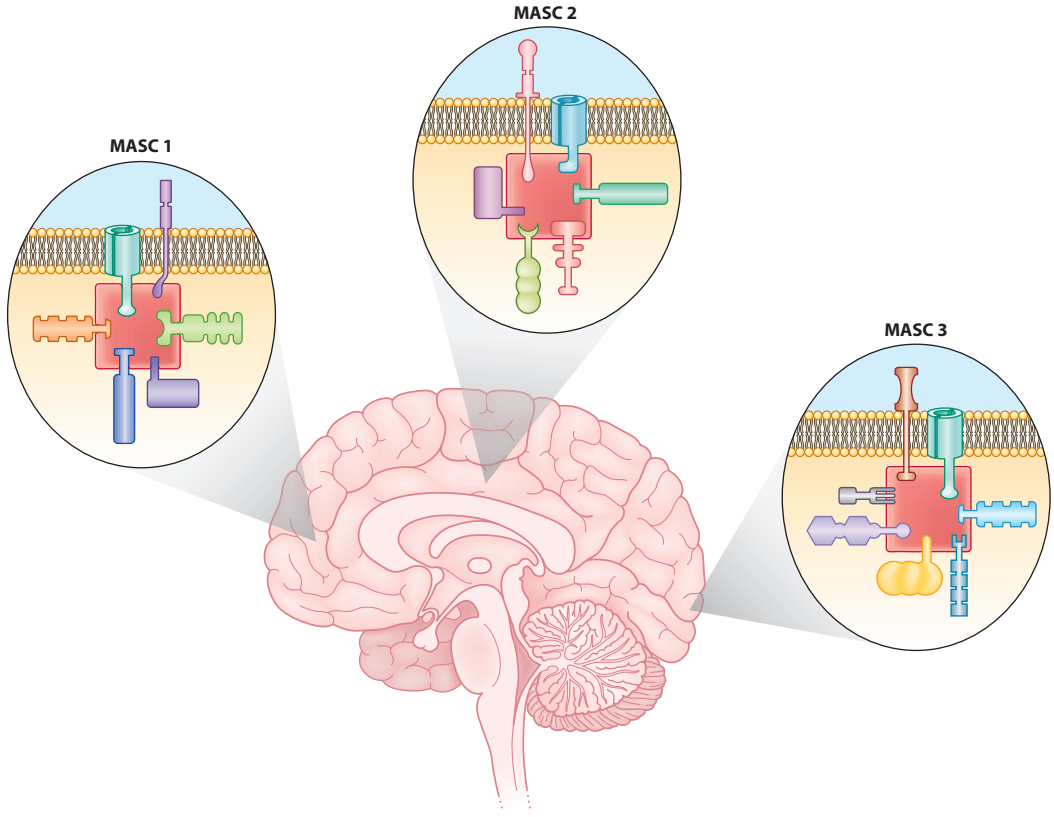

Figure 5

Synapse diversity in the mammalian brain is generated by combinations of protosynapse and MASC proteins. Three varieties of MASC complexes (labeled MASC1, 2, 3) comprising central scaffold proteins bound to receptors and adhesion protein, enzymes, and cytoskeleton are shown as in **Figure 1**. The variation in shapes of the components between the three complexes indicates that they are paralogs in expanded vertebrate gene families arising from duplication of the ancestral genes. The paralogs arising in early vertebrate evolution played a major role in diversifying neuroanatomical function.

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associated proteins), and the families maintaining equal numbers of genes were those encoding the downstream cytoplasmic signaling proteins (Emes et al. 2008). This finding shows that the hierarchy of the PSP network was subject to differential expansion, and these upstream protein families were presumably retained and potentially diversified by sub- or neofunctionalization since their duplication.

The expansion in upstream proteins specifically indicates that there was adaptive advantage in the diversity of neurotransmitter receptors and adhesion proteins in the vertebrate nervous system. Obviously this diversity could generate different MASC and PSP combinations, which could ultimately be expressed in different synapse types (**Figure 5**). This mechanism of synapse diversity also produces synapses with different signaling and adhesion specificity with the potential to connect to varieties of presynaptic terminals that are also distinguished by their varying molecular compositions. A good example of how this duplication and diversification in chordate genomes resulted in signaling diversity and differential expression is in the MAGUK proteins of the Dlg family and the NMDAR, the GluN2 subunit for which directly binds to Dlg (Ryan & Grant 2009). Invertebrate genomes encode a single GluN2 and Dlg and thus assemble a single type of

MASC complex, whereas mammals have 4 genes of each, potentially producing 16 types of MASC complexes. If one considers the other proteins that are in these complexes, including the many upstream proteins that were retained with the duplication events, an astronomical number of MASC and PSP types are available to diversify the synaptic types of vertebrates.

It is now important to highlight the connection between the generation of chordate synaptic complexity from genomic duplication events and the anatomical evolution of their nervous systems. Of note is that the genomic duplication events preceded the evolution of the large and anatomically diverse nervous systems of most vertebrates. The expansions in synaptic genes provided a molecular tool kit for generating a virtually limitless number of synaptic and neuronal types that could be used to generate diversity within the brain and also between different species. Direct evidence that this mechanism of diversity has indeed produced neuronal and synaptic diversity was observed in analyzing expression patterns of PSP genes in the mouse brain (Emes et al. 2008). The expression of PSP mRNAs and proteins were examined in many regions of the mouse brain, and those proteins that showed the greatest regional and neuronal type variation were more likely to be encoded by the expanded upstream families of vertebrate genes. Moreover, the set of proteins that was most ubiquitously and uniformly expressed in all regions was that of the ancestral protosynapse, indicating that a patterning in the diversity of synapse types in the mammalian brain arose from the expansion in complexity generated by gene duplications around the protosynapse (**Figure 5**).

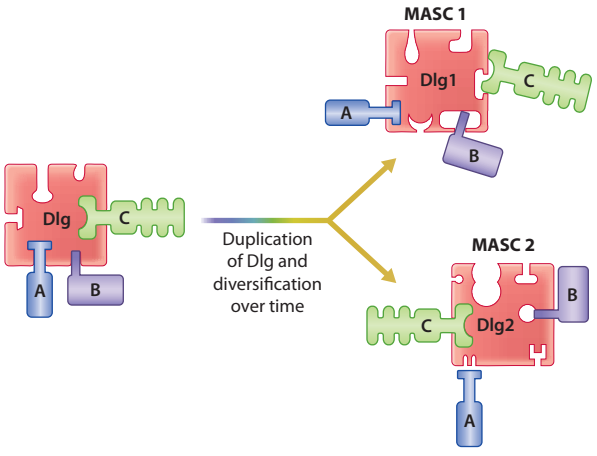

Figure 6

Duplication and diversification can lead to reorganization in signaling complexes. Duplication of scaffold proteins and diversification in their sequence modifies the organization and diversifies the molecular networks in MASC. The left-hand side shows an interaction network between Dlg and 3 binding partners (A, B, C) as in the ancestral protosynapse. As a result of gene duplication of *Dlg*, two paralogs (*Dlg1* and *Dlg2*) organize MASC1 and MASC2. With subsequent accumulated mutations in *Dlg1* and *-2*, their diversified protein binding affinity (e.g., different shapes of slots in jigsaw piece) alters the strength of interaction with binding partners so that MASC1 and MASC2 are different. The net effect is to produce diversity in MASC complexes, which can be expressed in different synapses to provide different physiological properties such as forms of synaptic plasticity.

In addition to generating a more diverse set of vertebrate synaptic genes with variation in structure and expression pattern, the organization of MASC protein networks is impacted in several important ways by genome duplication. First, as mentioned above there are multiplicative increases in the number of MASC variants produced by duplicates in its components (**Figure 4*a***). Second, a duplication in a scaffold protein that interacts with several others can lead to a rewiring of the molecular interaction network (Dreze et al. 2011), which alters the flow of signals in the network (**Figure 6**). A key organizational principle of these synaptic diversity mechanisms is that they utilize combinations of synaptic proteins in two distinct ways: the combinations of types of proteins that build up the core functionality of the protosynapse (scaffolds, receptors, enzymes, etc) and then the combinations of paralogs arising from duplication. The diversity arising from varied compositions of MASC complexes provides a way to categorize different MASCs (MASC1, MASC2, etc) and thus different classes of synapses with different physiological properties.

The NMDA receptors of the metazoan provide a prime example of the process of duplication and diversification of upstream signaling components. The NMDARs are glutamate-gated ion channels located at the surface of the postsynapse. Two rounds of duplication of

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the NMDA receptors in the vertebrate lineage resulted in four extant sequences. With this expansion, a dramatic change in the intracellular C-terminus of the NMDA proteins occurred. The C-terminus is the location of the phosphorylation-dependent interaction with scaffold and signaling molecules e.g., Fyn, CamKII, P85 PI3K, and PSD95. This C-terminal region (which is encoded by a single exon) is almost absent in invertebrate homologs, and hence so is the potential for multiple protein interactions in these species (Ryan et al. 2008). Thus the evolution of the intracellular portion of the NMDARs in the vertebrate lineages was likely a key stage in the link between sensing and cellular response to environmental stimuli.

INSIGHTS FROM THE HUMAN SYNAPSE

What sets humans apart from the rest of the animals, and what is the basis of human disease? The first comprehensive profiling of the human PSP and detailed study of its evolution showed that the genes of the PSP over the past 100 Myr are evolving under very strong purifying selection compared with the rest of the genome or other neuronal proteins and subcellular organelles (Bayes et al. 2010). This constraint was observed in primate and rodent lineages and shown to correlate with structural, physiological, and behavioral functions. The most conserved subset of the PSP was MASC, reinforcing its centrality in PSP function. The conserved functions of mouse and human PSP proteins were identified by systematic phenotype mapping of mutations and showed cognitive and motor functions, including learning and memory, and social functions were highly conserved. These findings again highlight the importance of adaptive behaviors as central and ancestral functions of MASC and the PSP. Another insight that arose from the proteomics of the human PSP was to identify that ~200 genes are involved with Mendelian diseases of which 130 were brain diseases (Bayes et al. 2010). Many of these diseases arose from gene duplication events, indicating that the cost of the evolution of paralogs was susceptibility to disease. A number of the MASC proteins in humans have been identified as mutated in patients with schizophrenia and other cognitive disorders (Fernandez et al. 2009, Kirov et al. 2011). Linking the genetic disease phenotypes to the observed constraint in vertebrate PSP evolution indicates that reduced fitness from PSP mutations is observed as strong and pervasive purifying selection.

Less is presently known about the changes in the PSP that are unique to the human lineage after it diverged from other primates around 6–8 Myr ago. Using methods to identify punctuated periods of adaptive evolution, eight genes of the PSP (*Cybrd1*, *SirpA*, *Ank2*, *Ca2*, *Cox5A*, *Pclo*, *Ndufb6*, and *Psd3*) show significant evidence of positive selection along the primate (including human) compared with the nonprimate lineage (R.D. Emes and S.G.N. Grant, manuscript in preparation). These genes may be candidates that underlie clade-specific adaptive evolution and may underpin more subtle differences in primate synapse function and hence in cognitive ability as well.

A MODEL FOR THE EVOLUTION OF THE POSTSYNAPTIC PROTEOME

Taken together, the comparative studies to date suggest a consistent model for the evolution of the PSP and its contribution to synaptic diversity and behavior. Elements of environmental sensing from surface receptor to transcriptome and core components associated with basic cellular life such as translation, energy generation, and fatty acid biosynthesis identified in bacteria and archaea were present prior to the divergence of the common ancestor of eukaryotes and prokaryotes. A number of constituents are conserved in prokaryotes as interacting proteins, suggesting that these were co-opted into the protosynapse as an interacting complex and remain in the extant PSP. In the fungi we observe homologs of the signal transduction pathways, including

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increased repertoire of protein kinases and the important role of scaffold proteins assembling and organizing signaling machinery. The presence of most types of synapse proteins in unicellular eukaryotes such as fungi and choanoflagellates and Porifera highlights that the evolution of the fundamental synaptic components predates the origins of identifiable neurons in metazoans. The ion channels incorporated after the cnidarian–poriferan divergence therefore would interact with a preexisting scaffold of intracellular proteins. The expanding transmembrane receptors would also plug into this preexisting network to expand rapidly the signaling complexity of the synapse (**Figure 4*a***). By comparing the predicted PSP of invertebrate metazoan species (fly, worm, bee, and mosquito) to vertebrates and by directly isolating the fly MASC component of the PSP, it is clear that the majority of protein classes were present in the invertebrates. In addition, specialization and division of labor were expanded by differential gene expression, providing combinations of proteins in different synapses (**Figure 5**). Following the divergence of the vertebrates from other deuterostomes, the driving force was of rapid expansion by duplication and diversification, particularly in the upstream signaling components such as receptors and signal transduction molecules. The expansion of the synapse proteome, therefore, predates the development of anatomically enlarged brains. This model in which the development of the synapse is a necessary step prior to the expansion and development of an enlarged nervous system has been proposed in the "synapse first" model of brain evolution (Ryan & Grant 2009).

One prediction of this model is that the synapse developed before axons and the branching network of dendrites. The increase of neuronal connectivity produced by neuronal branching rapidly increases the number of synapses and multiplies the computational power and diversity of synapses and overall signaling complexity. This may have driven the form of the nervous system we see today. Support for the theory that gene repertoire may predate the enlargement or increase in encephalization of the brain has recently emerged. Encephalization, the development of relatively excess brain size, is often measured by comparing the encephalization quotient (EQ, the log brain size versus log body size) and has been proposed as a measure of information-processing capacity or intelligence (Jerison 1977, 1985). By using high-resolution X-ray computed tomography, Rowe et al. (2011) recently showed that the particular enlargement of cerebral hemispheres and cerebellum associated with mammals occurred in a step-wise manner and was associated with expansion of key gene families. Expansion of the brain, especially the olfactory bulb and olfactory cortex, is seen in the skull of *Morganucodon oehleri*, a basal member of the mammaliaforms from the early Jurassic (~199–175 Mya). This postdates the expansion of the PSP we predict to have occurred in the ancestor of the vertebrates. A second wave of encephalization was seen with *Hadrocodium wui*, where expansion of the olfactory bulb and olfactory cortex accounts for the increase in EQ to within that seen in extant crown group mammals. Rowe et al. propose that the first wave of EQ expansion was driven by increase in olfaction and tactile sensitivity. These were then further amplified by an olfactory expansion owing to expression of the expanded olfactory receptor genome. We propose that, like the olfactory receptors, the expansion of gene repertoire in the PSP by gene duplication and expansion at the base of the vertebrates was a driver rather than a consequence of an increase in EQ. Like the exploding bubbles when uncorking champagne, mutations leading to change in brain size released the potential of the expanded PSP repertoire.

With the model that additional protein interactors plugged into an existing scaffold, why should the PSP complex expand by increasing interacting partners? The selective advantage of accumulating interacting proteins by scaffold protein binding proposes an intuitive adaptionist theory for the evolution of the synapse. The scaffold proteins provide a means to localize interacting proteins among

the multifarious soup of proteins in a cell and orchestrates the flow of information in a cell (Good et al. 2011). For example, the signaling complex of mating pheromones in *S. cerevisiae* is tethered by a scaffold protein (Ste5) that acts to increase signal transduction efficiency. At low total protein concentration, the resulting colocalization will increase local concentration and hence the probability of interaction. Additionally, the scaffold proteins containing varying architectures of domains promoting protein-protein interactions (e.g., the PDZ domains of PSD-95) can act as a means to allow the rapid evolution of new pathways by changing binding specificity and hence interacting partners. This type of universal port allows different interacting proteins to plug into a preexisting network of downstream effectors.

This theory suggests that adaptive evolution by natural selection of beneficial mutations has driven the aggregation of synapse proteins and other protein complexes. This mechanism could have expanded the MASC into the even greater complexity of PSD. However, Fernandez & Lynch (2011) recently proposed a nonadaptive theory to explain the trend of protein complex development seen in eukaryotes. They suggested that the small population size seen in eukaryotes compared with the prokaryotes allows the accumulation of mildly deleterious mutations by genetic drift in key proteins (drift is less dominant in larger populations owing to stronger selection coefficients). The accumulation of these mutations in turn drives the accumulation of protein complexes to stabilize individual proteins (Fernandez & Lynch 2011). Therefore, the growth of the synapse proteome with time may simply be due to the selection pressure to maintain protein function following neutral mutations.

CONCLUSIONS

Studies of synapse proteomes have shed light on the organization of molecular networks and macromolecular complexes in synapses and enabled the first systematic studies on synapse evolution. These studies reveal that synapses evolved from humble beginnings in prokaryotes and the earliest forms of cellular life. The realization that the primary role of the nervous system in sensing and responding to the environment arose in the organized protein architecture of signaling complexes or protosynapses in unicellular organisms, prior to the first neurons in any multicellular organism, opens new paths to understand the origins of behavior and the evolution of the behavioral repertoire of animals. It was the organization of this molecular machinery, primarily through combinatorial use of preexisting building blocks, that was exploited to generate the remarkable synaptic diversity found in invertebrates and vertebrates. These observations suggest that to understand the function of the brain we should aim to understand the evolution in the complexity of synaptic molecular systems. How behavioral diversity arose in organisms with large and complex brains remains mysterious, and it may be that the diversity or repertoire of their

THE EVOLUTION OF CELL-CELL ADHESION

The essence of multicellularity is the adhesion of cells via cell-cell junctions. The most basic of these is the adherens junction containing the cadherin domains identified in sponge (Fahey & Degnan 2010) and choanoflagellate proteins (King et al. 2003, 2008). Junctions that allow cell-cell communication by passing small molecules such as gap junctions are predicted to have evolved later with cnidarian (for review, see Abedin & King 2010). Cells mixed from two species of sponge will reform as species-specific clumps (Wilson 1910) via a proteoglycan ligand for a cell-surface receptor (Dunham et al. 1983), suggesting cell-cell signaling coupled to cell adhesion is an ancient process. Cell-adhesion molecules, including cadherins, are key to synapse formation and function. These molecules are not limited to the neuronal synapse: the interaction of mammalian T-cells and antigen-presenting cells utilizes these proteins and is known as the immunological synapse (Dustin 2009, Dustin et al. 2010, Paul & Seder 1994). Moreover, presynaptic proteins such as SNARE, VAMP, and SNAP proteins are found at the immunological synapse (Griffiths et al. 2010), supporting a general model for the evolution of synaptic mechanisms in the biology of many neuronal and nonneuronal cells.

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adaptive behaviors was shaped by the evolutionary mechanisms discovered in the synapse. The framework of the evolution and composition of the synaptome provides a path to investigate these problems and perhaps lead to a truly unified understanding of synapse biology.

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