REVIEWS

The origin and evolution of synapses

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Darwin200

Abstract | Understanding the evolutionary origins of behaviour is a central aim in the study of biology and may lead to insights into human disorders. Synaptic transmission is observed in a wide range of invertebrate and vertebrate organisms and underlies their behaviour. Proteomic studies of the molecular components of the highly complex mammalian postsynaptic machinery point to an ancestral molecular machinery in unicellular organisms — the protosynapse — that existed before the evolution of metazoans and neurons, and hence challenges existing views on the origins of the brain. The phylogeny of the molecular components of the synapse provides a new model for studying synapse diversity and complexity, and their implications for brain evolution.

Postsynaptic proteome
The complete set of proteins currently identified at the postsynaptic side of the synapse.

MAGUK
Membrane-associated guanylate kinase (MAGUK) proteins act as scaffolds for the clustering of receptors, ion channels and associated signalling proteins at postsynaptic sites.

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The synapse is an intensely studied and highly specialized cellular site yet surprisingly little is known about its origins and evolution. A century of electrophysiological and pharmacological studies have demonstrated the importance of synaptic function as a universal property of neural circuits. During the past two decades, molecular studies have provided us with an understanding of the fundamental mechanisms of synaptic transmission and plasticity, and have shown that these are remarkably conserved across a range of animal species[1].

It remains unknown whether there are central behavioural mechanisms shared by all organisms and whether these share common ancestry. For example Hebb's learning model proposed in the 1940s[2] applies only to animals with neural circuits. Broader concepts of behaviour traversing unicellular organisms to humans were stated in the nineteenth century. In 1891, C. L. Morgan observed that "the primary end and object of the receptions of the influences (stimuli) of the external world or environment, is to enable the organisms to answer or respond to these special modes of influence, or stimuli. In other words, their purpose is to set agoing certain activities. Now in the unicellular organisms, where both the reception and the response are effected by one and the same cell, the activities are for the most part simple, though even among these protozoa there are some which show no little complexity of response"[3].

Scholarly attempts to investigate or draw theories on the evolution of the nervous system did not until recently address the question of the origin and evolution of the synapse itself. This is not surprising, given the lack of tools available to explore the synapse in an evolutionarily meaningful way, as the traditional methods to study synapse structure and function, which rely on microscopy and electrophysiology, do not serve this purpose.

To examine the synapse through an evolutionary framework, experimental methods are required that allow the identification of the specific adaptations that mediated the diversification and natural selection of synapses between species. Recent systematic approaches to study synapse evolution are based on advancements in proteomics and genomics and are coupled with molecular phylogenetic approaches. Proteomics has provided the key step in the understanding of this field by identifying the protein constituents of synapses and their interactions. The postsynaptic proteome of mice has ~1,500 proteins and the presynaptic proteome and synaptic vesicles also comprise hundreds of proteins[4,5], demonstrating the complexity of mammalian synapses.

The postsynaptic density (PSD) is composed of a set of multiprotein complexes assembled from diverse protein classes, such as ion channels, receptors, cell adhesion and cytoskeletal proteins, kinases and phosphatases, scaffolding proteins and signalling molecules[4,6] (see Supplementary information S1 (table)). Multiprotein complexes that are associated with postsynaptic neurotransmitter receptors (such as glutamate, serotonin and acetylcholine receptors) as well as potassium channels have also been extensively characterised at the PSD because of their key role in synaptic transmission[5]. *N*-methyl-D-aspartate (NMDA) receptors, and their interacting MAGUK (membrane-associated guanylate kinase) proteins, are particularly well studied and discussed in detail in this Review. Interestingly, a large proportion of the MAGUK associated signalling complexes (MASC) proteins has been implicated in synaptic plasticity and/or learning phenotypes in mutant mice, or associated with psychiatric disorders in humans[5–8]. The general concept of multiprotein signalling complexes as 'signalosomes' has now been widely accepted, and is

illustrated by examples in immunology (the mammalian T cell receptor)[9] and vision research (the *Drosophila melanogaster* InaD complex)[10].

Complementary to charting the proteome of synapses, the flood of data from genome sequencing projects allows the use of comparative genomics to examine how and when synaptic proteins originated and diversified. Comparative genomics has revolutionized evolutionary biology, bolstering our understanding of the relationships between species, and also into the mechanisms of molecular evolution[11]. By combining proteomic and genomic approaches, the postsynaptic proteome provides unprecedented opportunities for probing and mapping the evolution of the synapse across species. Here we review such attempts and present the evidence accumulated so far as a working model of synapse molecular evolution, based primarily on the postsynaptic machinery, which has been more extensively studied. We also make suggestions as to how this topic may be addressed in the future.

The origin of synapses

The question of origin is central to the study of evolution[12]. When did synapses first form? What was the identity of the organism that necessitated this evolutionary step? Were the first synapses capable of plasticity? How are the origins of neurons and synapses related? Knowing the proteomic composition of synaptic structures allows the use of comparative genomics to question what the minimal, ancestral components of the synapse might have been. If we can deduce the composition of the last common ancestor of all synapses, the ursynapse, then we should be in a position to address the question of how and why the first synapse originated.

We approach the question of the composition of the ursynapse by taking synaptic proteins identified in vertebrate model organisms and searching for orthologues in the genomes of two categories of organism. The first category includes unicellular eukaryotes and multicellular metazoans that lack a nervous system, and provides a means of identifying synaptic components that were present before the evolution of the nervous system. Such proteins, which make up the protosynapse, originated before the emergence of classical morphological synapses and have been co-opted for synaptic roles. The second category is composed of non-bilaterian multicellular metazoans that have a nervous system, which allows the identification of synaptic components present in primitive synapses, giving us insight into the composition of the synapse at a relatively short time after it originated (FIG. 1).

Protosynaptic organisms. The most evolutionary ancient synaptic protein families are conserved in unicellular eukaryotes such as the yeast *Saccharomyces cerevisiae* and the amoeba *Dictyostelium discoideum*. The calcium transporter PMCA (plasma membrane calcium ATPase) and protein kinase C (PKC) are found in both these unicellular species and also have fundamental synaptic roles in animals[13–15]. More broadly, when the mammalian MASCs and larger PSD gene sets were compared against the *S. cerevisiae* genome, over 21% of the MASC genes and 25% of the PSD genes were found to have direct orthologues in protosynaptic organisms, labelling them as protosynaptic proteins of pre-metazoan ancestry[16]. Whether protosynaptic components in yeast form functional multiprotein complexes in a similar manner to which they do in neurons is unknown, but loss-of-function studies in yeast show that many of these genes are involved in regulating the cell's response to the environment (TABLE 1)[16], including vesicular trafficking and cytoskeletal regulation in response to nutrients, ions and pheromones from the environment[16]. A portion (15%) of yeast PSD orthologues functions in signal transduction pathways that mediate environmental response. Yeast orthologues of the mammalian RAS regulator neurofibronin 1 (NF1; that is, yeast IRA2), the extracellular signal-regulated kinase 2 (ERK2; also known as MAPK1; that is, yeast FUS3), and the G-protein guanine nucleotide binding protein 5 (GNB5; that is, yeast STE4) regulate cell morphology, stress response and cell proliferation induced by pheromone signalling. Strikingly, quintessential players that mediate changes dependant on neuronal activity during synaptic plasticity are also essential components of the yeast's response to environmental changes. The calcium binding protein calmodulin that activates calcium/calmodulin-dependent protein kinase II (CaMKII) and the protein phosphatase calcineurin (which has been associated with Schizophrenia[17]) both regulate calcium influx at the cell membrane and are implicated in postsynaptic signalling pathways in neurons and in environmental responses in yeast cells[18].

Yeast is an excellent organism for the study of genetics and genomics, but is a distant outgroup of metazoans and is unlikely to hold a quasi-comprehensive set of protosynaptic proteins. To understand the origin of the synapse it is desirable to consider a unicellular organism that is closely related to multicellullar Metazoa. Choanoflagellates fulfil this role. These unicellular eukaryotes are the closest unicellular relatives of multicellular metazoans on account of their cell body structure and phylogenetic analyses of nuclear and mitochondrial DNA[19,20] (FIG. 1). The genomes of choanoflagellates have assisted in reconstructing the genome of the last unicellular ancestor of animals, and could also be useful for elucidating the origin of the synapse[21]. Multicellular Porifera (sponges), which lack neurons, are further candidates for the identification of protosynaptic components[22].

Bioinformatic analyses of choanoflagellate genomes using comprehensive PSD gene sets as a starting point have yet to be reported. However, pioneering studies of candidate genes have demonstrated the presence of synaptic molecules in choanoflagellates that are absent in all other non-metazoans, including numerous tyrosine kinases of the choanoflagellate *Monosiga brevicollis*[23–26]. Rapid phosphorylation of tyrosine kinase substrates was observed following treatment of starved *M. brevicollis* cells with seawater and *Enterobacter* spp., which demonstrates that the tyrosine kinase pathway has a role in the environmental response[24]. Inhibition of tyrosine kinases delayed progression of *M. brevicollis* cultures into logarithmic growth, wherease a SRC family tyrosine kinase

Ursynapse
The last common ancestor of all synapses. This was the platform from which diversity of synaptic proteins between different organisms and different synapse types evolved.

Orthologues
Homologous genes that separated due to a speciation event.

Protosynapse
Those synaptic components that were present before the emergence of synapses and most likely contributed to their evolution.

Bilaterians
Animals belonging to the phylum Bilateria. These are a clade of animals with bilateral symmetry that possess complex nervous systems. They are divided into protostomes and deuterostomes.

Outgroup
A group of organisms that serves as a reference group for determination of the evolutionary relationship between monophyletic groups of organisms.

Choanoflagellates
Organisms belonging to the phylum Choanoflagellata. These are unicellular eukaryotes that can exist in both free-living and colonial forms, and are multicellular metazoans considered to be the closest unicellular relative of multicellular metazoans.

Porifera
Phylum of multicellular animals (poriferans or sponges) that lack a nervous system.

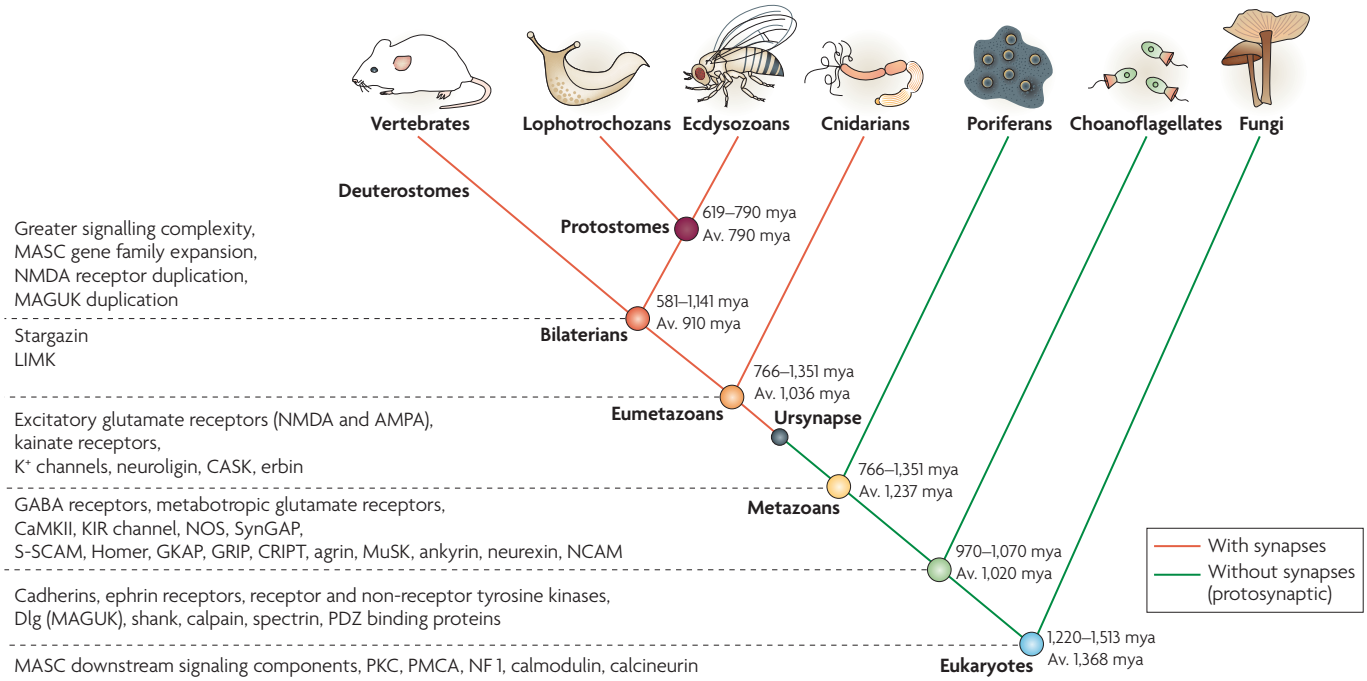

Figure 1 | **Phylogenetic tree depicting taxons of current relevance to synapse evolution.** An extant model organism of each clade is displayed at the top of the phylogeny (see Supplementary information S2 (Box) for additional details on the phylogenetic placing of Porifera relative to Cnidaria). Nodes on the phylogeny represent the divergence points of various clades and are presented by coloured circles. The red node represents Urbilateria (the last common ancestor of all bilaterians). The small grey circle represents the ursynapse (last common ancestor of all synapses). Beside each node the range of published estimations of the given divergence time are given in mya (millions of years ago), as well as the average (av) estimated divergence time based on published studies (available through the public resource for knowledge on the timescale and evolutionary history of life, Timetree[43,104]). Superimposed on the phylogeny are notable proteins that are involved in synapse formation and/or function, showing at what intervals in evolutionary history various synaptic components arose. See Supplementary information S3 (Box) for additional details on the possible origins of GABA and metabotropic glutamate receptors. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaMKII, calcium/calmodulin-dependent protein kinase II; CASK, calcium/calmodulin dependent serine protein kinase; CRIPT, cysteine-rich PDZ-binding protein; Dlg, discs, large homolog; GABA, γ-aminobutyric acid; GKAP, guanylate kinase associated protein; GRIP, glutamate receptor interacting protein; KIR channel, inwardly rectifying potassium channel; LIMK, LIM domain kinase; MAGUK, membrane-associated guanylate kinase; MASC, MAGUK associated signalling complex; MuSK, muscle specific kinase; NCAM, neural cell adhesion molecule; NF1, neurofibromin 1; NMDA, *N*-methylase-D-aspartate; NOS, nitric oxide synthase; PKC, protein kinase C; PMCA, plasma membrane calcium transporting ATPase; Shank, SH3 and multiple ankyrin repeat domains; S-SCAM, membrane associated guanylate kinase, WW and PDZ domain containing 2; SynGAP, synaptic Ras GTPase activating protein.

inhibitor abolished cell proliferation[24]. Given the central role of SRC family kinases in plasticity at excitatory synapses, the establishment of this apparatus and mechanism before the evolution of synapses is of significance[27,28].

Molecules that have been implicated in synaptogenesis such as cadherins are also present in choanoflagellates, and co-localize with actin at the cell's apical collar membrane, surrounding the singular flagellum[29]. Homophilic cadherin interactions are essential for excitatory synapse formation in mammals and in *D. melanogaster*, in which cadherin interacts directly with actin at the postsynaptic terminal[30]. Cadherins may therefore be important for cytoskeletal rearrangement in choanoflagellates and may represent a precursor to synapse formation. It is conceivable that the first protein–protein interaction that led to synaptogenesis would be homophilic, as the same molecules would be required by both cells, whereas heterophilic transynaptic protein interactions probably evolved later.

Comparisons between the PSDs of Bilateria and sponges have allowed the identification of genes that were present in a multicellular ancestor of Metazoa that immediately preceded the emergence of organisms with synaptic junctions[13,31]. Additional cell-signalling and adhesion molecules that have roles in synaptogenesis have been identified in the sponge *Oscarella carmela*, including ankyrin, neurexin and tyrosine kinase signalling components [32]. We postulate that these molecules originated before the evolution of synapses, in a metazoan/choanoflagellate common ancestor, and therefore were instrumental to the development of the first synaptic junction. Transgenic experiments in unicellular organisms and sponges may prove useful in testing this idea.

Many MASC components that have been implicated in synaptic plasticity in mammals are also present in the demosponge *Amphimedon queenslandica* (TABLE 1)[13]. Various interaction domains of postsynaptic proteins,

Demosponge
Organism belonging to the primary class of Porifera. Demosponges account for ~90% all sponge species.

Table 1 | **The origin and ancestral functions of protosynaptic proteins**

Molecule	Clade of origin	Organism reported	Ancestral function
PKA	Fungi	*S. cerevisiae*[16]	Nutrient induced cell proliferation[106]
NF1	Fungi	*S. cerevisiae*[16]	Stress response[107]
Calmodulin	Fungi	*S. cerevisiae*[16]	Ca^{2+} dependant stress response[18]
Calcineurin	Fungi	*S. cerevisiae*[16]	Ca^{2+} dependant stress response[108]
ERK2	Fungi	*S. cerevisiae*[16]	Pheromone induced cell proliferation[109]
GNB5	Fungi	*S. cerevisiae*[16]	Pheromone induced signalling[110]
SNAP-25	Fungi	*S. cerevisiae*[111]	Vacuolar morphogenesis and trafficking[111]
Syntaxin	Fungi	*S. cerevisiae*[16]	Vacuolar morphogenesis and trafficking[111]
TRP channels	Fungi	*S. cerevisiae*[112]	Osmolarity stress response[112]
Cadherin	Choanoflagellata	*M. brevicollis*[24]	Unknown, co-localizes with actin filaments at the apical collar[29]
SRC kinase	Choanoflagellata	*M. brevicollis*[113]	Regulation of cell proliferation[24]
RAF kinase	Choanoflagellata	*M. brevicollis*[24]	Unknown
Ephrin receptors	Choanoflagellata	*M. brevicollis*[26]	Unknown
Calpain	Choanoflagellata	*M. brevicollis*[114]	Unknown
Spectrin	Choanoflagellata	*M. brevicollis*[115]	Unknown
Dlg (MAGUK)	Choanoflagellata	*M. brevicollis*[13,116]	Unknown
Shank	Choanoflagellata	*M. brevicollis*[13,116]	Unknown
Agrin	Porifera	*O. carmela*[31]	Unknown
MuSK	Porifera	*O. carmela*[31]	Unknown
Ankyrin	Porifera	*O. carmela*[31]	Unknown
Neurexin	Porifera	*O. carmela*[31]	Unknown
NCAM	Porifera	*O. carmela*[31]	Unknown
GABA receptors	Porifera	*A. queenslandica*[13]	Unknown
mGluR receptors	Porifera	*A. queenslandica*[13]	Unknown, activity modulates Ca^{2+} influx[35]
KIR channels	Porifera	*A. queenslandica*[13]	Unknown
CaMKII	Porifera	*A. queenslandica*[13]	Unknown
NOS	Porifera	*A. queenslandica*[13]	Unknown
SynGAP	Porifera	*A. queenslandica*[13]	Unknown
S-SCAM	Porifera	*A. queenslandica*[13]	Unknown, located in epithelial cells[117]
Homer	Porifera	*A. queenslandica*[13]	Unknown, located in epithelial cells[13]
GKAP	Porifera	*A. queenslandica*[13]	Unknown, located in epithelial cells[13]
GRIP	Porifera	*A. queenslandica*[13]	Unknown, located in epithelial cells[13]
CRIPT	Porifera	*A. queenslandica*[13]	Unknown, located in epithelial cells[13]

A. queenslandica, *Amphimedon queenslandica*; CaMKII, calcium/calmodulin-dependent protein kinase II; CRIPT, cysteine-rich PDZ-binding protein; Dlg, discs large homolog; ERK2, extracellular signal-regulated kinase 2; GKAP, guanylate kinase associated protein; GNB5, guanine nucleotide binding protein β5; GRIP, glutamate receptor interacting protein; KIR channel, inwardly rectifying potassium channel; *M. brevicollis*, *Monosiga brevicollis*; MuSK, muscle specific kinase; NCAM, neural cell adhesion molecule; NF1, neurofibromin 1; NOS, nitric oxide synthase; *O. carmela*, *Oscarella carmela*; PKA, protein kinase A; *S. cerevisiae*, *Saccharomyces cerevisiae*; Shank, SH3 and multiple ankyrin repeat domains; SNAP-25, synaptosome-associated protein of 25,000 daltons; S-SCAM, membrane associated guanylate kinase, WW and PDZ domain containing 2; SynGAP, synaptic Ras GTPase activating protein; TRP channel, transient receptor potential channel.

including PDZ binding domains, are almost completely conserved between the sponge and the Bilateria, arguing strongly in favour of the existence of an assembled multiprotein structure. Five of the postsynaptic proteins are present and co-expressed in epithelial flask cells of *A. queenslandica* (discs large homologue (Dlg), guanylate kinase-associated protein (GKAP), glutamate receptor interacting protein (GRIP), Homer and cysteine-rich PDZ-binding protein (CRIPT)), implying that they may form a protosynaptic complex involved in sensing environmental stimuli and that they could represent an evolutionary precursor to synaptic sites[33]. Importantly, a number of channels and receptors that have crucial roles at synapses are also expressed in the sponge, including inhibitory GABA receptors, the K^+ channel KIR, and metabotropic (G-protein coupled) glutamate receptors

Table 2 | **Synaptic proteins present in early organisms with a nervous system**

Molecule	Clade idenified	Organism	Synaptic function
NMDA receptors	Cnidaria	*N. vectensis*[13]	Induction of synaptic plasticity[118]
AMPA receptors	Cnidaria	*N. vectensis*[13]	Fast synaptic transmission and plasticity[119]
Kainate receptors	Cnidaria	*N. vectensis*[13]	Modulation of synaptic transmission and plasticity[120]
Shaker channel	Cnidaria	*N. vectensis*[13]	Synaptic homeostasis[121]
Neuroligin	Cnidaria	*N. vectensis*[13]	Synapse formation[42]
Erbin	Cnidaria	*N. vectensis*[13]	Modulation of voltage dependant calcium channels[122]
CASK	Cnidaria	*N. vectensis*[13]	Regulation of neurotransmitter release[123]

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CASK, calcium/calmodulin-dependent serine protein kinase; NMDA, *N*-methyl-D-aspartate; *N. vectensis*, *Nematostella vectenis*.

(mGluRs)[13,34]. mGluR activity has been demonstrated in cells cultured from the sponge *Geodia cydonium*, which responds to the presence of glutamate by rising its intracellular calcium concentration[35]. Indeed, application of electrical or tactile stimuli to the sponge *Rhabdocalyptus dawsoni*, causes it to propagate an electrical impulse that controls water flow through the organism[36]. As no excitatory, ionotropic glutamate receptors have been identified in sponges it seems that inhibitory GABA receptors evolved in the common ancestor of Porifera and Bilateria, and importantly, preceded the origin of excitatory ionotropic glutamate receptors.

The above evidence not only demonstrates the existence of MASC components and electrically active ion channels in sponges, but implies that there is a co-localized protosynaptic complex expressed in an anatomical region that has a role in environmental adaptation, and this complex emerged around the same time, in evolutionary terms, as the first synaptic channels and receptors. Of course, many of these proteins have also evolved non-neuronal functions in animals and indeed their presence in unicellular organisms and sponges demonstrates their fundamental roles in intracellular signalling. For example PI3K (phosphoinositide 3-kinase) is a protooncogene that regulates cell division, and MAGUK family members are known regulators of mammalian T cell activation[9,37,38]. Nevertheless, these pleiotropic proteins have evolved discrete synaptic functions and it is compelling to see that a substantial number of synaptic proteins regulate environmental adaptations in unicellular organisms such as yeast and choanoflagellates, and co-localize in the sensory cells of sponges[13,37,39]. Protosynaptic proteins represent therefore a pre-adaptation that later co-opted for synaptic function and may have contributed to the development of the first synapse.

Primitive metazoans with synapses. The complimentary approach to investigate the origins of the synapse is to search the genomes of primitive metazoans with visible synapses, such as the cnidarian *Nematostella vectensis*, which phylogenetically branched off ~200 million years before the bilaterians (FIG. 1) and has a rudimentary nervous system[13]. Common features of cnidarians and bilaterians are representative of a common ancestor that would have existed following the origin of the synapse in early metazoans (TABLE 2). The most notable feature of cnidarians is the emergence of postsynaptic ionotropic glutamate receptors including NMDA receptors and AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors), which mediate synaptic plasticity in the vertebrate CNS[40,41].

Additionally, the postsynaptic transmembrane protein neuroligin originated in a common ancestor of *N. vectensis* and bilaterians, and interacts transynaptically with the presynaptic and protosynaptic protein neurexin during synaptogenesis[42]. The neurexin–neuroligin interaction has an essential and general role in the formation of both excitatory and inhibitory synapses. It is noteworthy that ectopic expression of neurexin or neuroligin in non-neuronal cells co-cultured with neurons is sufficient to induce synaptic differentiation of the non-neuronal cells[42]. All of the ion channels that emerged after the cnidarian–poriferan split interact with protosynaptic intracellular scaffold proteins that are expressed in sponges and which therefore evolved earlier. In effect, upstream transmembrane receptors plug into a pre-existing intracellular machinery and this provides a framework for the emergence of a higher complexity of signalling pathways (FIG. 2).

Comparing vertebrates and invertebrates

Owing to the pioneering studies of the molecular mechanisms of synaptic plasticity in the lophotrochozoan *Aplysia californica* (sea slug) and the parallel mechanisms subsequently discovered in mice[1], one might assume that an invertebrate synapse is essentially identical to a vertebrate synapse. Recent evidence challenges this paradigm, highlighting the degree of divergence that has occurred between the synaptic proteomes of vertebrates and invertebrates[16]. Bilaterians comprise two major clades: the protostomes and the deuterostomes. Protostomes include among other phyla the invertebrate Arthropoda (arthropods) and Nematoda (nematodes) (FIG. 1), whereas deuterostomes include the subphylum Vertebrata (vertebrates). The time of divergence of protostomes and deuterostomes is estimated at ~910 million years ago[43]. The hypothetical last common ancestor of all protostomes and deuterostomes is referred to as Urbilateria, which gave rise to the vast majority of animal diversity that exists in nature today[44]. On the one

Cnidarian
Animal belonging to the phylum Cnidaria. Cnidarians are animals with radial symmetry including jellyfish, coral, hyrda and anemones. Cnidarian nervous systems consist of diffuse neuronal net-like structures.

Clade
An evolutionary group consisting of a given single common ancestor and all of its descendants.

Protostomes
Animals belonging to the phylum Protostomia, an animal clade that includes the superphyla Ecdysozoa (arthropods and nematodes) and Lophotrochozoa.

Deuterostomes
Animals belonging to the superphylum Deuterostomia that includes the subphylum Vertebrata.

hand, comparisons between protostomes and deuterostomes allow inferences as to the features of the urbilaterian, because whatever is common to protostomes and deuterostomes was present in the common ancestor. On the other hand, comparisons between protostomes and deuterostomes can identify features that have evolved specifically within each clade[16].

Few direct comparisons have been made for synapses at the morphological level. One anecdotal difference is that *D. melanogaster* dendrites can develop from the primary neurite (that normally give rise to axons) as well as from the neuronal body, whereas in deuterostomes dendrites have only been reported to arise independently from axons[45]. At the molecular level, the urbilaterian would have possessed many of the canonical neurotransmitter receptors, including acetylcholine, GABA, glycine, dopamine and serotonin receptors, as these are found in both protostomes and deuterostomes (FIG. 1)[46,47]. The voltage-dependant calcium channel subunit stargazin (FIG. 2) is a canonical urbilaterian synaptic protein, as it is present in all bilaterians and is absent in cnidarians. When protostome genomes including *D. melanogaster*, *Caenorhabditis elegans* and *Apis mellifera* were searched using the mouse PSD and MASC gene sets, 44.8% and 46.2%, respectively, had orthologues in *D. melanogaster*[16]. This shows that the PSDs of protostomes and deuterostomes share a core set of homologous proteins that has expanded divergently since the urbilaterian ancestor, and that in vertebrates the PSD and MASC complexes have more components. Genomic comparisons can identify which mouse PSD proteins are absent from protostome genomes and are therefore specific to vertebrate synapses. However, such analyses cannot identify the actual components of the protostome PSD or MASC complexes. Indeed, the *D. melanogaster* PSD could include numerous protostome specific proteins. Comparative proteomics can address this problem. Mass spectrometric analysis of the *D. melanogaster* MASC detected 220 proteins, showing that *D. melanogaster* possesses a MASC similar in size to that of mouse (186 proteins). However, it is the content of the MASC that differs between the two organisms, and this feature cannot be observed at the level of genomic sequence comparison. When the isolated *D. melanogaster* MASC was compared across species in the same manner as for the mouse, 71% of the *D. melanogaster* MASC components were found to have orthologues in yeast, whereas mice MASC components only had 21.2% yeast orthologues, showing that the majority of *D. melanogaster* MASC proteins are ancestral, being common to all eukaryotes and not of invertebrate or metazoan origin[16]. Therefore, the mouse MASC is enriched for recently evolved proteins, relative to that of invertebrates. Additionally, when the components of the mouse MASC were classified according to gene ontology categories, over 60% were found to be 'upstream' signalling components, such as receptors, scaffolding proteins and signal transduction molecules (FIG. 3). Conversely, only 25% of the *D. melanogaster* MASC falls into this category, with the majority pertaining to the category of 'downstream' components, such as metabolic enzymes, chaperones and mitochondrial proteins. These numbers suggest that the deuterostome MASC has evolved a significantly greater degree of signalling complexity.

The paradigm of the NMDA receptor carboxy-terminus and its interactions. Besides the evolution of synaptic complexity, there is evidence that the organization of these proteins, mediated by their interaction domains, has also differentially evolved. For example, GKAP (guanylate kinase associated protein) and shank (SH3 and multiple ankyrin repeats domain) are both postsynaptic scaffolding proteins that regulate synapse assembly in animals and are present in sponges[13,48,49]. However, the GKAP PDZ domain necessary for its interaction with shank is deuterostome specific, and is likely to influence synaptogenesis[13,50]. The significance of domain evolution at the synapse is exemplified by the intracellular carboxy-terminal domains of the NMDA receptor, which interacts with proteins of the MASC[51,52]. Comparison of the amino acid sequence of various excitatory and metabotropic glutamate receptors across species revealed a significant vertebrate–invertebrate dichotomy in the protein size of NMDA receptors[51]. Specifically, the vertebrate NMDA receptor NR2 (NMDA receptor 2) subunits possess intracellular carboxy-terminal domains five times larger than all known invertebrate, protostome NR2 protein sequences (FIG. 4). This evolutionary dichotomy is unique to the NR2 subunit as no differences of this magnitude were observed for any other neurotransmitter receptor subunit. Within the protostome and deuterostome clades the NR2 carboxy-terminal domains retain a relatively conserved size of ~100 amino acids in protostomes and ~600 amino acids in deuterostomes. The larger, deuterostome NR2 carboxy-terminus contains many protein interaction sites and phosphorylation sites that are not present in protostomes, arguing strongly in favour of a higher complexity of NMDA receptor interactions in vertebrates (FIG. 4). A high degree of sequence conservation of the carboxy-terminal domain is seen in the deuterostome clade, demonstrating functional significance of this domain, which has been elegantly demonstrated with respect to hippocampal synaptic plasticity and memory formation by *in vivo* genetic studies[53].

NR2 duplication and carboxy-terminal divergence. Two rounds of whole genome duplication have occurred in a common ancestor of chordates in the deuterostome clade[54]. As a result many *D. melanogaster* genes have up to four orthologues in mouse. Synaptic gene families that have undergone chordate specific expansion include sodium and potassium channels, calcium channels, transient receptor potential (TRP) channels, glutamate and GABA receptors, ephrin receptors, protein kinase C (PKC), plasma membrane calcium ATPase (PMCA), cadherin, neuroligin, Dlg, nitric oxide synthase (NOS), calcium/calmodulin-dependent protein kinase II (CaMKII) and GKAP[13,55]. NMDA receptors have evolved further in vertebrates due to gene duplications of the NR2 subunit that have resulted in four distinct paralogues (NR2A–NR2D), which have

Homologues
Set of genes or proteins that are related by descent, that is, they share a common ancestor.

Genome duplication
Duplication of an entire genome that results in an abundance of duplicated genes, most of which are lost. Two rounds of genome duplication are believed to have occurred at the base of the chordate lineage.

Gene duplication
Duplication of a given gene owing to replication errors and resulting in two redundant copies of the original gene.

Paralogues
Homologous genes that separated because of a gene duplication event.

diverged a great extent with respect to their spatio-temporal expression patterns[56]. The four NR2 subunits have also diverged at the level of protein sequence, but primarily in their intracellular domains — with no motif being conserved between the four paralogues except the terminal PDZ binding domain that interacts with the MAGUKs[51]. Therefore although the NR2 amino-terminal domains that contain the extracellular

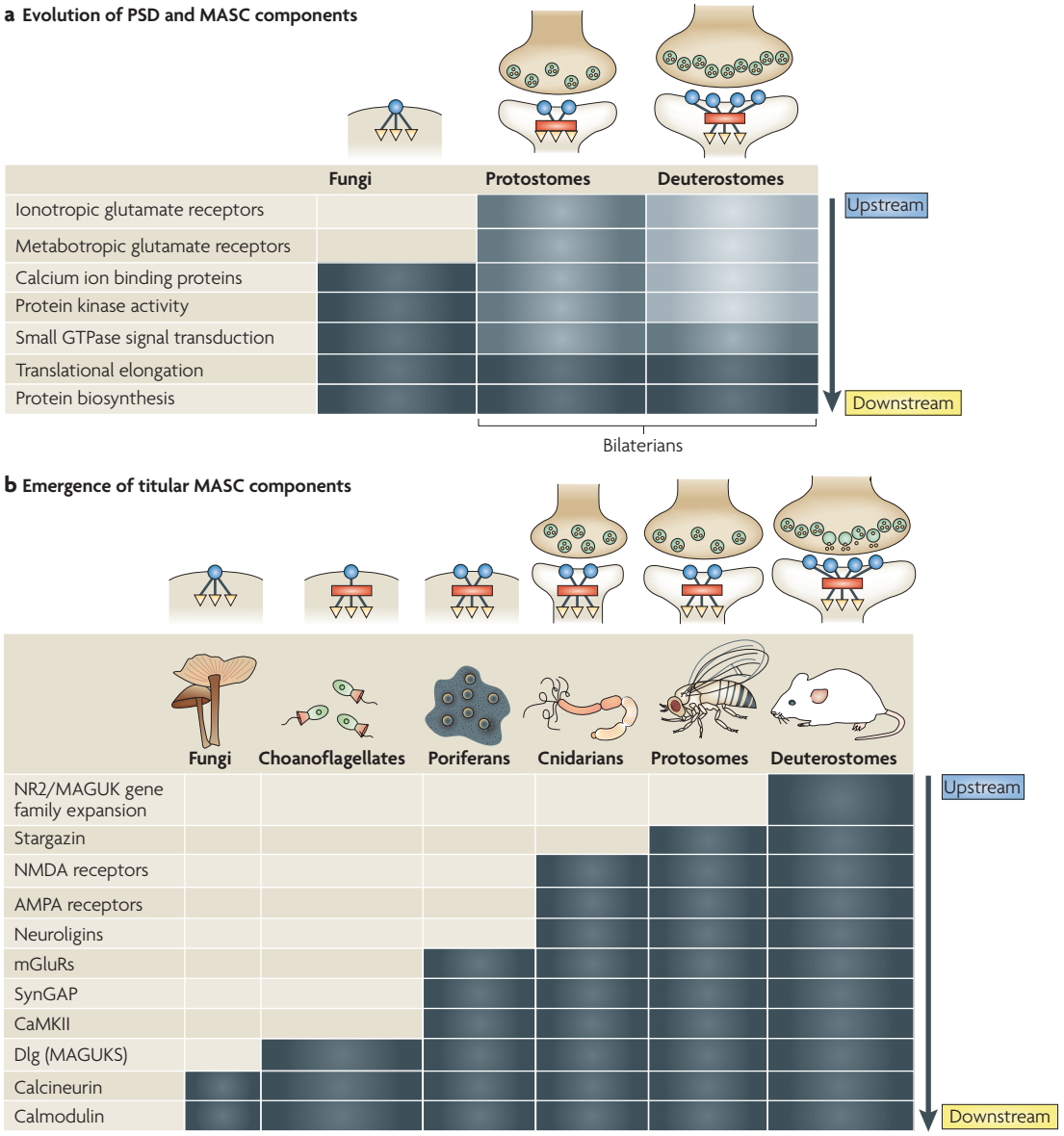

Figure 2 | **Evolution of postsynaptic components. a** | Various protein types (left column) that constitute the postsynaptic density (PSD) and membrane-associated guanylate kinase (MAGUK) associated signalling complexes (MASCs) in unicellular eukaryotes (fungi), protostomes and deuterostomes are ordered based on whether they have 'upstream' or 'downstream' signalling roles. Non-coloured fields represent the absence of a given protein. Dark grey rectangles represent presence of protein. Grey rectangles represent enrichment of a protein type in protostomes, or protostomes and deuterostomes when compared with unicellular eukaryotes. Light grey rectangles represent enrichment of a protein type in deuterostomes when compared with protostomes. The diagrams above each column represent the molecular assembly of MASC, in which upstream proteins (blue circles) are connected to downstream proteins (yellow triangles) through intermediate signalling proteins (red rectangle). The relative proportions of these proteins in eukaryotes, protostomes and deuterostomes is therefore illustrated. **b** | The emergence of titular MASC components across clades is illustrated. Proteins are ordered based on whether they are located 'upstream' or 'downstream' in synaptic signal transduction pathways[16]. Non-coloured fields represent the absence of a given protein, whereas dark grey rectangles denote its presence. Diagrams of MASC structure are placed above each clade, along with an illustration of a representative model organism. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaMKII, calcium/calmodulin-dependent protein kinase II; Dlg, discs large homologue; mGluRs, metabotropic glutamate receptors; NMDA, *N*-methyl-D-aspartate; NR2, NMDA receptor 2; SynGAP, synaptic Ras GTPase activating protein. Diagrams in part **a** are modified, with permission, from REF. 16 © (2008) Macmillan Publishers Ltd. All rights reserved.

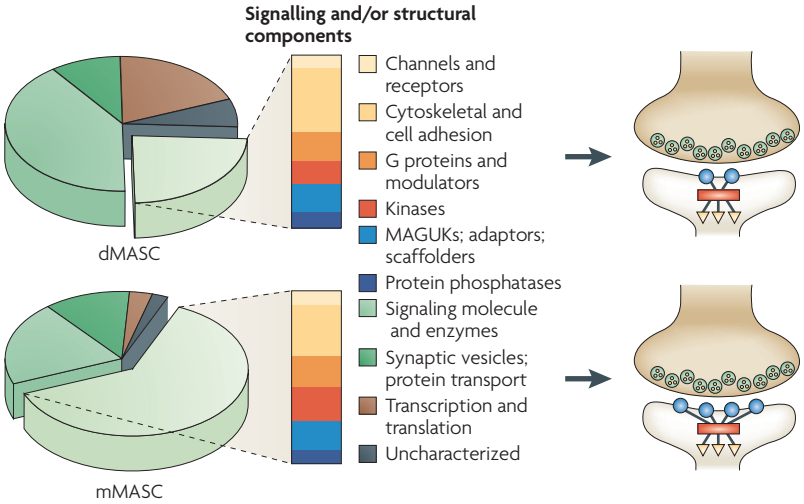

Figure 3 | **Comparative proteomics of mouse and *Drosophila melanogaster* MASC.** Pie charts showing the proportion of various functional classes of proteins in the isolated *Drosophila melanogaster* membrane-associated guanylate kinase (MAGUK) associated signalling complex (dMASC) and mouse MASC (mMASC). The mMASC is enriched for 'upstream' signalling components (blue circles in right panels). Figure is modified, with permission from REF. 16 © (2008) Macmillan Publishers Ltd. All rights reserved.

ligand-binding and transmembrane domains, are largely conserved throughout vertebrate evolution, selection has acted primarily on the intracellular domains of the NR2 proteins. The evolutionary divergence of the NR2 carboxy-terminal domains has resulted in different MASC sets being recruited by different NMDA receptors depending on the particular NR2 subunit present. Experimental evidence has shown that a number of NMDA receptor interacting proteins, such as SynGAP (synaptic Ras GTPase activating protein)[57], adaptor protein 2 (AP2)[58], calcineurin[59], cyclin dependant kinase 5 (CDK5)[60], PSD95 and SAP102 (REF. 61) interact specifically or preferentially with particular NR2 subunits.

NMDA-receptor–MAGUK interactions. MAGUKs are intracellular scaffolds that anchor and traffic NMDA receptors and AMPA receptors to the synapse[39,62]. They are composed of PDZ domains, a SRC homology 3 (SH3) domain and a guanylate kinase domain. The MAGUK family has 22 members classified into 7 subfamilies (MAG1, CASK, MPP, ZO, DLG, CCNB and CARMA)[52], all of which originated in metazoans: 4 subfamilies are of ancient origin and are present in Porifera (MAGI, MPP, DLG, DLG5); 2 subfamilies (ZO, CACNB) emerged later in cnidarians and bilaterians; and the CARMA (CARD–MAGUK) subfamily, which functions in signal transduction at immunological synapses[63], is deuterostome specific. Although most MAGUK subfamilies are common to both protostomes and deuterostomes, five show deuterostome specific gene expansion thereby bolstering the number of MAGUKs available. For example, Dlg has only been duplicated in vertebrates, giving rise to four paralogues: DLG1–DLG4 (also known as SAP97, PSD93, SAP102, and PSD95, respectively), and mutations in DLG1–DLG4 in mice result in distinct behavioural phenotypes[64–66]. Deuterostomes have single NR2 and Dlg genes whereas chordates have four paralogues of each, thus the number of potential chordate NR2–Dlg interactions has expanded to be 12-fold greater[67] (FIG. 4b). This particular interaction serves as an example for the combinatorial complexity at the level of protein interactions that has evolved for chordate synaptic proteins.

GABA and metabotropic glutamate receptor evolution. Inhibitory GABA receptors also display deuterostome specific expansion, with two primary clades of genomically clustered subunits. The first contains α, γ and ε subunits, the second includes ρ, β, σ, θ and π subunits[68]. The two groups diverged within the deuterostome clade, in a common ancestor of chordates and urochordates[68]. Protostomes by contrast possess a small compliment of GABA-receptor-like subunits[69]. GABA receptor subunits have also undergone mammalian specific gene duplication and loss. The GABA receptor ε and τ subunits evolved by duplication of the γ and β subunits, respectively, and the β4 and γ4 subunits were eliminated during evolution[68,70]. Positive selection has since acted on the protein coding region of the θ subunit, whereas the ε has displayed evidence of relaxation of constraint (neutral evolution)[68]. The functional significance of these evolutionary events is unknown, but may lie in ligand binding affinity or receptor sensitivity.

Metabotropic glutamate receptors, also have an ancient origin at the base of Metazoans, and show substantial expansion in vertebrates[13,71]. mGluRs are classified into three groups (I–III) based on sequence homology. Whereas vertebrates have two to three members in each group, invertebrates have one[71,72].

Presynaptic proteins. Comparative genomics has made progress in examining the evolution of presynaptic proteins across bilaterians. One study addressed the conservation of 120 presynaptic proteins between vertebrates and insects[73]. This protein set showed strong but variable conservation, and interestingly the degree of conservation for exocytotic proteins (such as synaptobrevin 2 (VAMP2)) was found to correlate with the number of interacting partners. This is consistent with reports demonstrating that protein connectivity generally correlates with protein conservation, that is, the more interactions a given protein has, the more likely it is to have its sequence conserved throughout evolution[74]. However, for endocytic proteins (such as synaptojanin) the degree of conservation did not correlate with the number of protein interactions. The reason for this dichotomy is unknown but may lie in group specific protein size and domain structure.

Another study selected 150 primarily presynaptic genes and studied them with respect to conservation of their coding sequences and adjacent non-coding genomic elements across 8 vertebrate species including human and mouse[75]. The vast majority of the gene set was found to be under strong purifying selection, with non-synonymous nucleotide substitution to synonymous nucleotide substitution ratios (dN/dS ratios) over fivefold lower than the genomic average. This outcome is consistent with reports of genes expressed in the brain being under more conservative selective pressure than genes expressed in other tissues[76].

Immunological synapse
A region that can form between two cells of the immune system in close contact. The immunolgical synapse originally reffered to the interaction between a T cell and an antigen-presenting cell.

Positive selection
Positive selection is said to occur when a given genetic variant rises to prevalence in a population by increasing the reproductive fitness of the organism in a given environment. Positive selection at the level of amino acid sequence is identified by the dN/dS ratio.

Non-synonymous nucleotide substitution
A nucleotide substitution in the coding sequence of a gene that alters the amino acid sequence of the protein.

Synonymous nucleotide substitution
A nucleotide substitution in the coding sequence of a gene that does not alter the amino acid sequence of the protein.

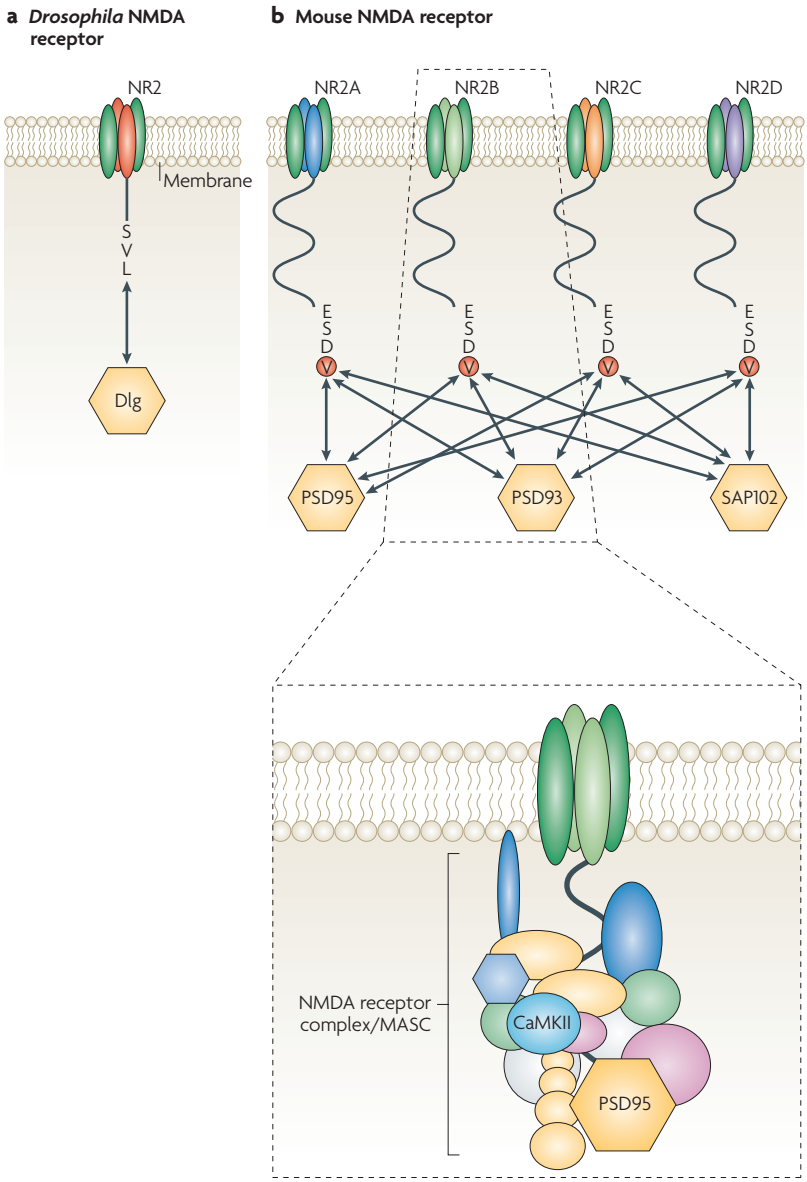

Figure 4 | **NMDA receptor carboxy-terminal evolution.** Schematic comparing the *Drosophila melanogaster* NR2 containing NMDA receptor (**a**) and the mouse NR2 containing NMDA (*N*-methyl-D-aspartate) receptors (**b**). The PDZ binding domains at the carboxy-terminus of both *D. melanogaster* NMDA receptor 2 (NR2; SVL) and mouse NR2 (ESDV) are indicated. Note that the mouse NR2 intracellular domain is five times larger than that of *D. melanogaster*. The diagrams compare the evolution of NMDA receptor signalling complexity in *D. melanogaster* and mouse showing protein–protein interactions at the NR2 carboxy-termini. The only established protein interaction site on the *D. melanogaster* NR2 carboxy-terminus is the interaction of *Dlg* (discs large homologue; SAP97 orthologue) (Bayes A. and S.G.N.G., unpublished data) through the PDZ binding domain. The vertebrate NR2B carboxy-terminus has numerous established primary and secondary interacting proteins (zoom out) and therefore a greater degree of NMDA receptor signalling complexity. Furthermore, the number of potential interactions of the NMDA receptor with MAGUK (membrane-associated guanylate kinase) components differ between protostome and deuterostome synapses. In the case of protostomes only one such interaction can occur, between NR2 and the protostome MAGUK, *Dlg*. Because of gene family expansion in chordates there are four available NR2 subunits (NR2A–NR2D) and four MAGUKs (PSD95, SAP102, PSD93 and SAP97). Three of the MAGUK paralogues can interact with any of the four NR2 subunits, making twelve potential deuterostome NR2–MAGUK interactions. As NMDA receptors are considered to be tetramers that contain two NR2 subunits, the existence of tri-heteromeric NMDA receptor channel further increases this combinatorial complexity[105].

Synapse diversity

Variability of synaptic protein composition is visible not only between different organisms. There is also a parallel between the evolutionary origin of synaptic genes and their expression pattern in the mammalian brain. In general terms, the evolution of synaptic genes at the eukaryote–metazoan and metazoan–chordate boundaries preceded their expression in different populations of neurons and synapses and thereby allowed diversity of function in nervous systems that have emerged later, in evolutionary terms, and that are generally larger[16]. For example, MASC mRNA expression has been compared between 22 regions of the mouse brain, and this has shown that the genes of most variable expression originated since the deuterostome common ancestor, whereas genes of low expression variability are of ancient, pre-metazoan origin (FIG. 5). It is likely that the relative expression of MASC components results in different combinatorial versions of MASC components in separate brain regions, contributing to synaptic and neuronal diversity.

Innovative transgenic methods have contributed to our knowledge of synapse diversity, showing that expression pattern of synaptic proteins is an identifier of neuronal subsets. One study conducted whole genome expression analysis on mRNA isolated from 12 discrete green fluorescent protein (GFP)-tagged GABAergic or glutamatergic neuronal populations of the mouse brain, to produce a phylogeny of neuronal subtypes[77]. Using this method it was shown that among genes that exhibited variable expression between brain regions, synaptic and axonal components were significantly over-represented and that there was more expression diversity in GABAergic neuronal subpopulations than in glutamatergic ones. Importantly, genes expressed in the brain with heterogeneous expression patterns are enriched for paralogues, which lends credence to the hypothesis that gene duplication leads to the subfunctionalisation of duplicates by divergence of expression patterns[77,78]. Indeed, the excitatory glutamate receptors and MAGUKs fall into the highly variable region and have undergone chordate specific gene duplication[16]. The resolution of such profiling studies will increase in the future as larger sets of genes are studied in more discrete anatomical regions[79]. The development of sophisticated transgenic technologies, such as those using tagged ribosomal proteins expressed in discrete neuronal populations, will facilitate such studies[80,81].

Human synapse evolution

What makes us human is one of the oldest and most tantalising questions in biology[82–84]. The most striking feature of humans is our increased cognitive capacity. Little is known about the genetic events that led to the emergence of human specific behaviour but it is widely assumed that the enlarged and convoluted neocortex is the basis for our increased mental abilities (see article by Pasko Rakic in this issue)[85]. Here, we posit that the evolution of the synapse complement might have enabled the increase of neuronal cell types and, in turn, the neuronal network complexity of the human brain. Due to difficulties in obtaining appropriate tissue samples for proteomic work the investigation of human synaptic complexes is

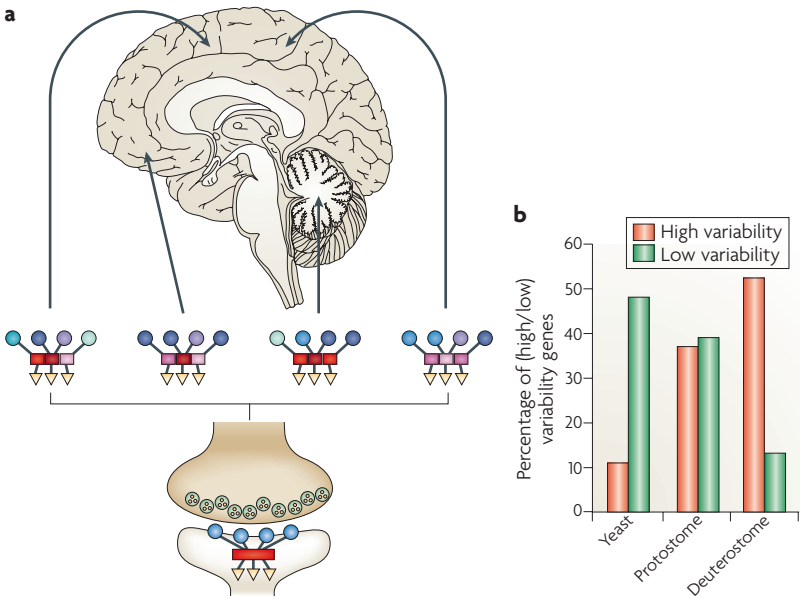

Figure 5 | **MASC signalling diversity within the brain. a** | Model depicting expression variation of MASC (membrane-associated guanylate kinase (MAGUK) associated signalling complex) components throughout the deuterostome brain where different combinations of MASC proteins are found in different synapses. Four different MASC complexes comprised of various combinations of discrete upstream (coloured circles), intermediate (red rectangles) and downstream (yellow triangles) proteins are expressed in different regions of the brain (arrows). **b** | MASC components of ancient pre-metazoan origin (present in yeast), protostome origin and deuterostome origin are displayed in a histogram that shows their percentage composition of genes of high expression variability and low expression variability. MASC genes of deuterostome origin are enriched for genes of high expression variability in the mouse brain: ancient genes are uniformly expressed and recent genes are most variable. Figure is modified, with permission from REF. 16 © (2008) Macmillan Publishers Ltd. All rights reserved.

not straightforward. In the mean time, there are examples of positive selection (accelerated evolution) of particular synaptic, primate and human proteins, including receptors for acetylcholine, oxytocin, kainate, dopamine and glutamate receptor subunits[86]. Of particular interest is the NR2A subunit, which shows evidence of positive selection in primates and happens to be the NR2A paralogue that is expressed postnatally in mammals.

The human genes of GABA(A) receptor subunits display a disproportionate number of mutations in microRNA target recognition sites, indicating alterations in human GABA receptor gene expression[87]. MAOA (monoamine oxidase A), an enzyme that catabolises neurotransmitters, has been shown to be under positive selection in humans[88]. Within populations, genetic variations of MAOA have also been implicated in antisocial behaviour in humans[89,90]. Additionally, dopamine receptor subunit D4 (DRD4) and GABA(A) receptor β2 subunit (GABRB2) variants are under positive selection in human populations, and are associated with attention deficit hyperactivity disorder, and schizophrenia, respectively[91–96]. It is possible that positive selection on synaptic proteins has contributed to the evolution of human behaviour at the cost of increased vulnerability to psychiatric conditions[97–99].

Comparison of protein expression levels in the brains of human and non-human primates has led to the identification of numerous proteins, including synaptic proteins such as CaMKII, that are upregulated specifically in the human brain[100,101]. Currently, we can only speculate about the function of the human specific variants of synaptic proteins and the reasons why their selection sometimes correlates with 'disease' phenotypes. Cell culture studies allied with transgenic 'humanization' of genes in model organisms may help to elucidate the role of these genes in primate and/or human brain evolution.

dN/dS ratio
The ratio of non-synonymous nucleotide substitutions to synonymous nucleotide substitution for a given protein-coding gene. A dN/dS ratio of < 1 implies purifying selection or conservative evolution, ~0 implies relaxation of constraint or neutral evolution, > 1 implies positive selection or adaptive evolution. This measure is based on Kimura's theory of molecular evolution, which argues that the vast majority of nucleotide sequence changes are functionally neutral.

Conclusions

In this Review we discuss how the synapse, a fundamental structural and functional unit of the nervous system, is a fertile platform with which to explore the evolution of the brain. Many mammalian synaptic components existed before the appearance of synapses, and some of these may have been pivotal to the origin of the ursynapse. It is conceivable that synapses would have evolved before axons and dendrites, as an organism with a small number of closely associated neurons would not need axonal projections for neuron to neuron communication. By the same logic, without the synaptic specifications, axons or dendrites would have no relevance. Similarly, synapse formation would have evolved before other stages in neural development including neuronal migration. Therefore we posit that synapse formation is one of the exceptions to the trend that ontogeny recapitulates phylogeny as, although it is the final stage of neural development, it necessarily evolved before the earlier developmental stages. We consider the emergence of the synapse to be a crucial step in the origin of the nervous system. Indeed, one could argue that the evolution of synapses led to the evolution of neurons, as a neuron is defined by synaptic connections. We refer to this model of the origins of the brain as the 'synapse first' model. It may prove useful to profile the expression of PSD and MASC genes during synaptic development with respect to phylogenetic origin, in order to test the 'ontogeny recapitulates phylogeny' evo–devo paradigm in the context of synaptogenesis[102,103].

Studies focusing on synapse evolution are at early stages, but it is now an intellectually and technologically opportune time to launch into this topic. The focus has been on excitatory glutamate receptor associated postsynaptic proteins, as this family of proteins is well known and of central importance to synaptic function. These studies should be expanded to include all presynaptic and postsynaptic proteins. Most of our knowledge on the topic of synapse evolution is based on data derived from comparative genomics and proteomics. It will be necessary to expand on these studies with thoughtful functional experiments. Comparing the physiological and behavioural importance of ancient and mammalian specific synaptic genes through loss-of-function experiments in mice may aid in identifying the functional roles for which they were selected. Transgenic gain-of-function studies in organisms without synapses might establish what combinations of synaptic proteins resulted in the origin of the synapse. Finally, methods already applied to mice should be applied to humans to see to what extent the evolution of the synapse has contributed to the evolution of the human brain.

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DATABASES

UniProtKB: http://www.uniprot.org
CRIPT | Dlg | ERK2 | GKAP | GNB5 | GRIP | Homer | NF1 | PKC | PMCA

FURTHER INFORMATION

Seth G. N. Grant's homepage:http://www.sanger.ac.uk/Teams/faculty/grant/
Timetree: http://www.timetree.org/

SUPPLEMENTARY INFORMATION

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Biographies

Tomás Ryan received a B.A. in human genetics from the University of Dublin, Trinity College (Ireland). He was awarded a Wellcome Trust Ph.D. Studentship to study in the research group of Seth Grant at the Wellcome Trust Sanger Institute and Darwin College, University of Cambridge (UK), where he was a final year graduate student at time of writing. His thesis research focused on the generation and analysis of transgenic mouse models of NMDA (*N*-methyl-D-aspartate) receptor molecular evolution. He is currently a Junior Research Fellow at Wolfson College, University of Cambridge.

Seth Grant is a neuroscientist best known for his work using mouse genetics and synapse proteomics to study plasticity and learning. This work has uncovered unexpectedly high molecular complexity in synapse protein complexes and the postsynaptic density providing avenues to study synapse evolution and brain disease. He received degrees in physiology, medicine and surgery from the University of Sydney, Australia, and postdoctoral training at Cold Spring Harbor Laboratory, USA, and with Eric Kandel at Columbia University, USA. He currently heads the Genes to Cognition Programme at the Wellcome Trust Sanger Institute in Cambridge UK and is Professor of Molecular Neuroscience at Edinburgh and Cambridge Universities, UK.

Online summary

- The molecular composition of the synapse has recently been proved to be useful for studying the evolution of the brain.
- Synapse proteomics data sets, such as those of the postsynaptic density (PSD) and associated protein complexes when combined with comparative genomics have provided unprecedented insights into the evolution of synapses.
- The PSD that is found in organisms with nervous systems has evolved from an ancient protosynaptic core that exists in unicellular organisms and multicellular organisms without nervous systems.
- Comparisons of vertebrate PSD and synaptogenesis genes with orthologues from sponges and cnidarians open an avenue for speculating as to what may have contributed to the origin of the first synapse.
- Comparative proteomics has shown that vertebrate excitatory synapses have evolved to be significantly more complex than invertebrates.

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