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REVIEW

The origin and evolution of synaptic proteins – choanoflagellates lead the way

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ABSTRACT

The origin of neurons was a key event in evolution, allowing metazoans to evolve rapid behavioral responses to environmental cues. Reconstructing the origin of synaptic proteins promises to reveal their ancestral functions and might shed light on the evolution of the first neuron-like cells in metazoans. By analyzing the genomes of diverse metazoans and their closest relatives, the evolutionary history of diverse presynaptic and postsynaptic proteins has been reconstructed. These analyses revealed that choanoflagellates, the closest relatives of metazoans, possess diverse synaptic protein homologs. Recent studies have now begun to investigate their ancestral functions. A primordial neurosecretory apparatus in choanoflagellates was identified and it was found that the mechanism, by which presynaptic proteins required for secretion of neurotransmitters interact, is conserved in choanoflagellates and metazoans. Moreover, studies on the postsynaptic protein homolog Homer revealed unexpected localization patterns in choanoflagellates and new binding partners, both which are conserved in metazoans. These findings demonstrate that the study of choanoflagellates can uncover ancient and previously undescribed functions of synaptic proteins.

KEY WORDS: Metazoans, Synapse evolution, SNARE, Munc18, Postsynaptic scaffold, Homer

Introduction

Neurons are the key building blocks of our nervous system and are central to information processing and transport in the brain. Understanding how neurons evolved is key to reconstructing how metazoans evolved such vast biological and behavioral diversity. Neurons are specialized cells that transmit information from one cell to the other by electrical and/or chemical signaling. Chemical signaling occurs via synapses, specialized contact sites that allow neurons to exchange information rapidly. Upon Ca^{2+} influx, chemical signals (called neurotransmitters) are released from presynaptic synapses and diffuse across the synaptic cleft to react with receptors on postsynaptic synapses. This process involves an intricate network of synaptic proteins that forms the molecular machinery underlying neurotransmitter release from presynapses and activation of neurotransmitter receptors on postsynapses. Understanding when synaptic proteins first evolved and how they functioned promises to reveal core mechanisms underlying the evolution and function of neurons in metazoans.

Choanoflagellates form a sister group to Metazoa (Fig. 1A), making them a fascinating family of organisms for studying the origin and function of synaptic proteins. A close relationship between choanoflagellates and metazoans has long been postulated (James-Clark, 1868). Phylogenetic analyses based on sequence data have consistently demonstrated that choanoflagellates indeed represent the closest living relatives of metazoans (Fig. 1A) (Carr et al., 2008; King et al., 2008; Philippe et al., 2009; Ruiz-Trillo et al., 2008). These studies also confirmed that choanoflagellates and metazoans are independent lineages and it is now clear that metazoans and choanoflagellates form a monophyletic group. Choanoflagellates are a group of microbial eukaryotes that live in many different aquatic (both marine and freshwater) environments (Leadbeater and Thomsen, 2000). They are characterized by a remarkably uniform cell body morphology. This comprises a spherical to ovoid cell with a single apical flagellum surrounded by a collar of actin-filled microvilli (Fig. 1B,C). The undulation of the apical flagellum creates fluid currents that draw bacteria into the feeding collar for phagocytosis (Pettitt et al., 2002; Roper et al., 2013). Choanoflagellates share the same collar cell morphology as the feeding cells of sponges, so called choanocytes, indicating that choanocyte-like cells are likely to have been one of the earliest metazoan cell types (Nielsen, 2008; Nichols et al., 2009). Although all choanoflagellates have a single-celled phase in their life history, many species also form colonies composed of multiple cells (Fig. 1D) (Alegado et al., 2012; Dayel et al., 2011; Fairclough et al., 2010).

The genomes of the choanoflagellate *Monosiga brevicollis* and *Salpingoeca rosetta* were recently sequenced (Fairclough et al., 2013; King et al., 2008). Comparisons of the two choanoflagellate genomes with genomes from metazoans demonstrated their relevance to reconstructing the origin of protein families central to metazoan biology. For example, *M. brevicollis* contains homologs of metazoan receptor tyrosine kinases and cadherins, two protein families that had not been observed in non-metazoans before (King et al., 2003; King et al., 2008; Snell et al., 2006). Tyrosine kinase activity is required for *M. brevicollis* proliferation (King et al., 2003) and two cadherins from *M. brevicollis* localize to actin filaments in the collar of microvilli where bacterial prey is captured (Abedin and King, 2008). Moreover, septins and septin-regulating proteins, which stabilize intercellular bridges in metazoan neighboring cells, are significantly upregulated in *S. rosetta* colonies relative to single cells (Fairclough et al., 2013).

In addition, the study of choanoflagellates has begun to provide clues into the ancestry of metazoan synaptic proteins. Choanoflagellates do not produce synapses and neurons, but the genome of *M. brevicollis* encodes synaptic protein homologs, including cation channels similar to voltage-gated sodium channels (Liebeskind et al., 2011; Gur Barzilai et al., 2012) and the postsynaptic density (PSD) proteins Homer, Shank and PSD-95 (Alié and Manuel, 2010; Emes and Grant, 2012; Ryan and Grant, 2009; Sakarya et al., 2007). Moreover, homologs of various types of metazoan plasma membrane Ca^{2+} channels including the store-operated channel, ligand-operated channels, voltage-operated channels, second messenger-operated channels and transient

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The Journal of Experimental Biology

The Journal of Experimental Biology (2015) doi:10.1242/jeb.110247

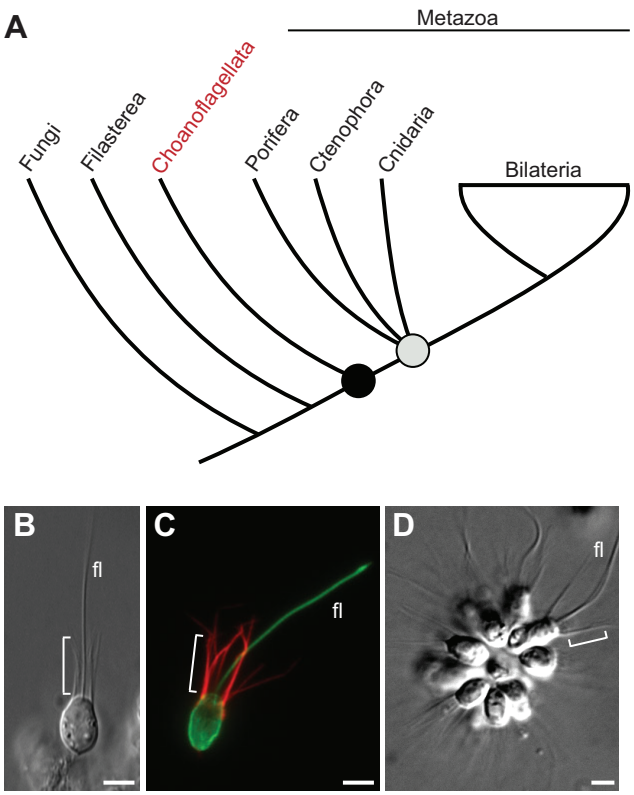

Fig. 1. Choanoflagellates: sister group of the Metazoa.
(A) Choanoflagellates are the closest living relatives of metazoans (Carr et al., 2008; King et al., 2008; Richter and King, 2013; Ruiz-Trillo et al., 2008). Black circle, Urchoanimal; gray circle, Urmetazoan. (B) Choanoflagellates, represented here by the choanoflagellate *Salpingoeca rosetta*, have a single apical flagellum (fl) surrounded by an apical collar of actin-filled microvilli (bracket). (C) *Salpingoeca rosetta* cells were stained with phalloidin (red) to visualize actin microfilaments and antibodies against β-tubulin (green) to highlight the cell body and flagellum. (D) Colonial life-history stage of *S. rosetta*. Scale bars: 2 µm.

receptor potential channel families were identified in the genome of *M. brevicollis* (Cai, 2008).

Diverse synaptic protein homologs in choanoflagellates

The identification of neuronal protein homologs in the choanoflagellate *M. brevicollis* led to an extended search for presynaptic and postsynaptic proteins in close relatives of Metazoa. Synaptic protein homologs that fall into five major functional categories: (1) neuronal exocytosis proteins, (2) active zone proteins, (3) adhesion and signaling proteins, (4) receptors and transmembrane proteins and (5) postsynaptic scaffolding proteins (Fig. 2A) were searched in the genomes of the filasterean *Capsaspora owczarzaki* and the choanoflagellates *S. rosetta* and *M. brevicollis*, and in addition in the genomes of the sponges *Amphimedon queenslandica* and *Oscarella carmela*, and the placozoan *Trichoplax adhaerens*.

In agreement with previous studies, most synaptic proteins were found to be seemingly restricted to metazoans and their closest relatives (Fig. 2B) (Sakarya et al., 2007; Ryan and Grant, 2009; Alié and Manuel, 2010). Those few synaptic proteins that have homologs in fungi and non-opisthokonts, such as SNAREs, Sec1/Munc18 (SM) and tomosyn, primarily function in the highly conserved process of exocytosis. The remaining functional categories are represented by no more than a single homolog in fungi (Fig. 2B).

In contrast with fungi, the earliest branching lineages of holozoans express homologs of diverse synaptic proteins belonging to each of the five functional categories of synaptic proteins (Fig. 2B). Proteins involved in neuronal exocytosis (category 1, e.g. complexin, synaptogyrin and synaptophysin), adhesion/signaling (category 3, e.g. LAR, PICK and CamKII) and postsynaptic scaffolding (category 5, e.g. Homer, Shank, PSD-95 and GKAP) were particularly well represented in the genomes of choanoflagellates and *C. owczarzaki*. Homologs of proteins in the active zone (category 2) and receptors/transmembrane protein (category 4) functional categories were relatively under-represented; only a few active zone proteins were found to be present in *C. owczarzaki* (e.g. CASKin) and in the two choanoflagellates *S. rosetta* and *M. brevicollis* (e.g. RIMbp).

During the rise of metazoans, the number of synaptic proteins in each of the five functional categories was greatly expanded. Notably, the number of receptors/transmembrane proteins, active zone proteins and adhesion/signaling proteins increased (Fig. 2B). For example, while absent in the genome of the sponge *A. queenslandica* (Srivastava et al., 2010), ionotropic glutamate receptors (iGluRs) were identified in the newly sequenced genome of *O. carmela* (Nichols et al., 2012; Burkhardt et al., 2014). Moreover, many of the active zone proteins are present in sponges (e.g. Erc/Cast, Mint and Liprin-α) and in placozoans (e.g. CASK and RIM), as are important adhesion molecules like ephrin receptors and neurexin (Burkhardt et al., 2014).

The analysis shows that many proteins essential for synaptic function were already present at the holozoan stem lineage (Fig. 2B), thus having an earlier origin than previously described. Moreover, in agreement with previous studies (Alié and Manuel, 2010; Emes and Grant, 2012; Sakarya et al., 2007; Suga et al., 2013), most synaptic proteins evolved before the origin of synapses and are probably pleiotropic. Only a few synaptic proteins seem to be restricted to metazoans with synapses and some of them are indeed dedicated to synapse formation (e.g. the adhesion molecules neuroligin and ephrin).

The rich repertoire of neuronal protein homologs in choanoflagellates and the close relationship to metazoans make choanoflagellates ideal candidates to study the origin and function of synaptic proteins.

The identification of a primordial neurosecretory apparatus in choanoflagellates

The presence of diverse synaptic protein homologs in choanoflagellates raises the possibility that central features of synaptic transmission evolved before the appearance of the first metazoans. Therefore, the question was asked: which molecular machinery was fundamental for the development of the neuronal communication apparatus (Burkhardt et al., 2011)? A key process in synapses is the rapid release of neurotransmitters from synaptic vesicles. The central machinery involved in this process is composed of members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment receptor) protein family. In vertebrates, this process is mediated by the neurosecretory SNARE proteins synaptobrevin 2, syntaxin 1 and SNAP-25. Their assembly between the vesicle and target membrane is thought to drive membrane fusion (Jahn and Fasshauer, 2012; Rizo and Rosenmund, 2008).

The neurosecretory SNARE machinery is organized and controlled by additional factors *in vivo*. Members of the cytosolic Sec1/Munc18-like (SM) family of proteins have been established as essential factors in neurosecretion. In the absence of the SM protein Munc18-1 (called Rop1 in *Drosophila melanogaster* and Unc18 in

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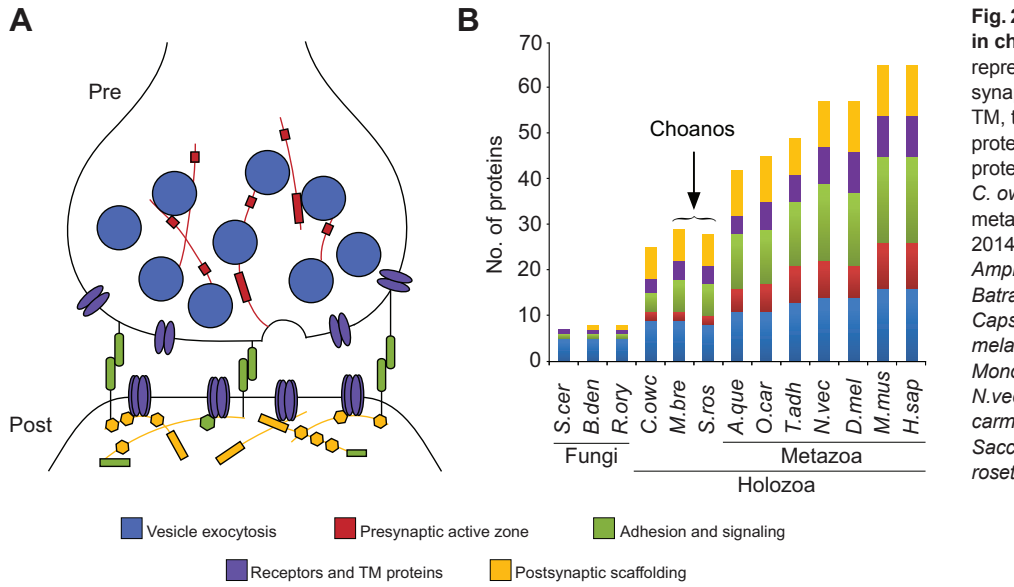

Fig. 2. Diverse synaptic protein homologs in choanoflagellates. (A) Schematic representation of a eumetazoan glutamatergic synapse. Protein types are defined in the key. TM, transmembrane. (B) Abundance of synaptic protein families in diverse eukaryotes. Many proteins evolved before the divergence of *C. owczarzaki*, choanoflagellates and metazoans (modified from Burkhardt et al., 2014). Choanos, choanoflagellates; *A.que*, *Amphimedon queenslandica*; *B.den*, *Batrachochytrium dendrobatidis*; *C.owc*, *Capsaspora owczarzaki*; *D.mel*, *Drosophila melanogaster*; *H.sap*, *Homo sapiens*; *M.bre*, *Monosiga brevicollis*; *M.mus*, *Mus musculus*; *N.vec*, *Nematostella vectensis*; *O.car*, *Oscarella carmela*; *R.ory*, *Rhizopus oryzae*; *S.cer*, *Saccharomyces cerevisiae*; *S.ros*, *Salpingoeca rosetta*; *T.adh*, *Trichoplax adhaerens*.

Caenorhabditis elegans), neurotransmitter release is blocked, indicating that this molecule has a positive role in neurosecretion (Hosono et al., 1992; Schulze et al., 1994; Verhage et al., 2000). However, *in vitro* studies showed that Munc18-1 binds with high affinity to syntaxin 1 (Pevsner et al., 1994). When bound to Munc18-1, syntaxin 1 adopts a 'closed' conformation, rendering it inaccessible to its partner SNAREs SNAP-25 and synaptobrevin 2, suggesting a negative role for Munc18-1 in neurosecretion (Burkhardt et al., 2008; Dulubova et al., 1999; Misura et al., 2000; Pevsner et al., 1994; Yang et al., 2000). In addition, studies on the *Saccharomyces cerevisiae* SM protein homolog Sec1 showed that Sec1 does not bind to the plasma membrane syntaxin Sso1p, but can instead bind to the assembled plasma membrane SNARE complex (Scott et al., 2004; Togneri et al., 2006). Thus, the secretion machinery of the choanoflagellate *M. brevicollis* was compared with that of metazoans, to be able to reconstruct the ancestral function of the Munc18-1/syntaxin 1 complex (Burkhardt et al., 2011).

The genome of the choanoflagellate *M. brevicollis* contains homologs of the secretory SNARE proteins synaptobrevin 2, SNAP-25 and syntaxin 1 (Fig. 3A), which are closely related to the those involved in regulated secretion in metazoans (Kloepper et al., 2008). In addition, a single Munc18 homolog was identified in *M. brevicollis*. The domain architecture and arrangement of the secretory SNARE proteins are conserved in metazoans and choanoflagellates (Fig. 3A). Moreover, SNAP-25 from *M. brevicollis* contains a stretch of cysteines at the C-terminal end of its first SNARE motif (Fig. 3A, highlighted in green). These cysteines are known to be palmitoylated and serve as a membrane anchor for SNAP-25 in metazoans (Veit et al., 1996). In addition, syntaxin 1 from *M. brevicollis* contains an N-peptide with a highly conserved DRL/TxxL-motif (Fig. 3A, highlighted in red). Notably, two amino acids of this motif, Arg (R) and Leu (L), are necessary for the binding of syntaxin 1 to Munc18-1 (Burkhardt et al., 2008). To test for direct interaction of the choanoflagellate SNAREs, proteins were expressed in *E. coli*. It was found that the three SNARE proteins SNAP-25, syntaxin 1 and synaptobrevin form an SDS-resistant complex, as the rat neuronal SNARE complex does (Fig. 3B).

The interaction of the choanoflagellate Munc18 with syntaxin 1 was studied using isothermal titration calorimetry (ITC), a biophysical technique to accurately measure the thermodynamics of an interaction (Burkhardt et al., 2011). In vertebrates, two regions of syntaxin 1 are known to contribute to the interaction with Munc18-1: the N-peptide, which binds to the outer surface of Munc18-1 domain 1, and the remainder of the syntaxin 1a molecule, which binds in a closed conformation to Munc18-1 (Fig. 3C) (Burkhardt et al., 2008). It was shown that *M. brevicollis* syntaxin 1 binds to Munc18 with a very high affinity and that a syntaxin 1 variant, in which the N-peptide was removed, showed a clearly reduced affinity to Munc18 (Burkhardt et al., 2011). Therefore, the data show that the N-peptide and the remaining portion of syntaxin 1 cooperate for high affinity binding to Munc18 and suggest that syntaxin 1 and Munc18 interact in *M. brevicollis* by mechanisms that are conserved with metazoans. Importantly, the crystal structure of the *M. brevicollis* Munc18/syntaxin 1 complex was solved (Burkhardt et al., 2011) and confirmed that two regions of syntaxin 1, the N-peptide and the remainder in closed conformation, bind to Munc18 simultaneously (Fig. 3D). Very similar to the vertebrate Munc18-1/syntaxin 1a complex, syntaxin 1 from *M. brevicollis* adopts a closed conformation in the Munc18/syntaxin 1 complex. The two structures (e.g. choanoflagellate Munc18/syntaxin 1 complex and rat Munc18-1/syntaxin 1a complex) can be superimposed with an overall main chain root mean square deviation of 2.0 Å. The biophysical and structural studies show that the Munc18/syntaxin 1 complex from *M. brevicollis* is structurally and functionally highly similar to the vertebrate complex, suggesting that it constitutes a fundamental step in the reaction pathway toward SNARE assembly (Burkhardt et al., 2011).

Recently it was discovered that in vertebrates, binding of the syntaxin 1a N-peptide to the outer surface of Munc18-1 serves as a switch to regulate SNARE complex assembly (Burkhardt et al., 2008). When the syntaxin 1a N-peptide is bound to Munc18-1, SNARE complex formation is blocked. Removal of the N-peptide enables binding of syntaxin 1 to its partner SNAREs SNAP-25 and synaptobrevin. To test whether this switch is present in the *M. brevicollis* Munc18–syntaxin 1 interaction, SNARE complex assembly in the presence of Munc18 was monitored using a fluorescence-based kinetic approach (Burkhardt et al., 2011). An almost complete block of SNARE complex formation was observed when syntaxin 1 was premixed with Munc18. This block was not observed when syntaxin 1 without the N-peptides was used instead, demonstrating that binding of the N-peptide to Munc18 is required to block SNARE complex formation. This suggests that Munc18

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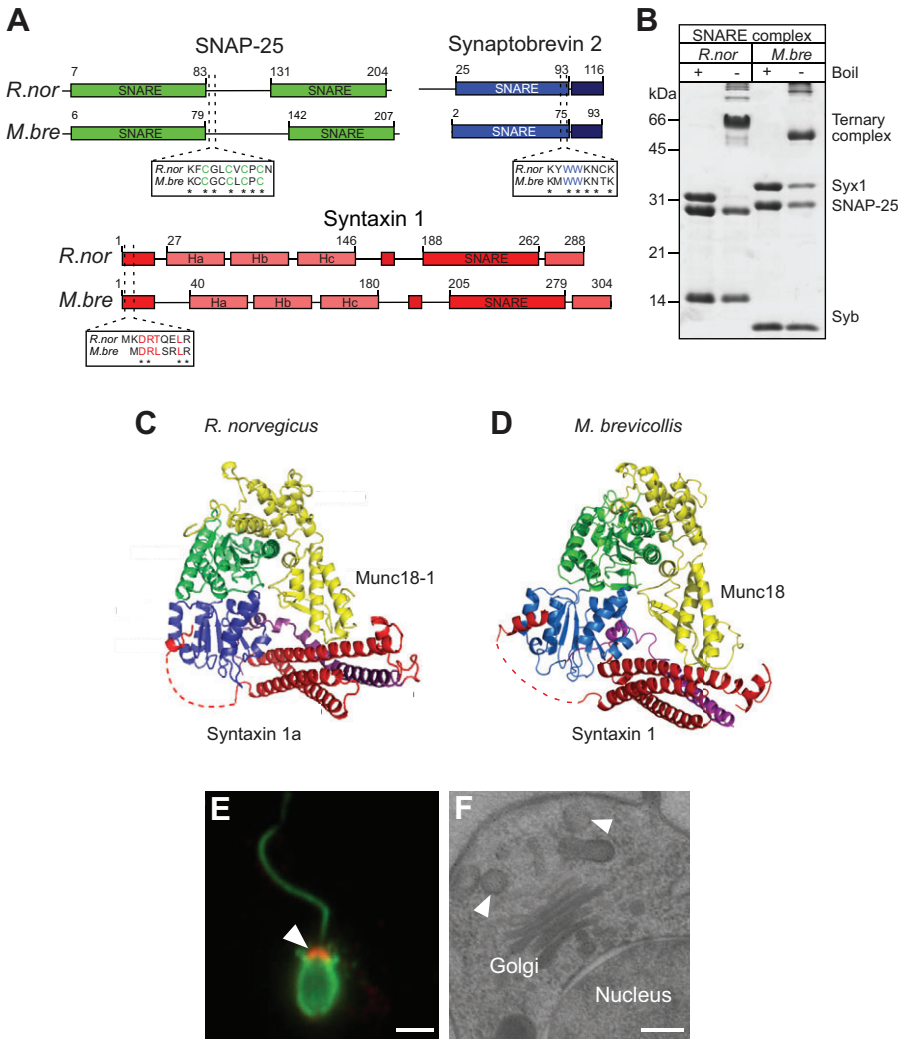

Fig. 3. Primordial neurosecretory apparatus in the choanoflagellate *Monosiga brevicollis*. (A) Conservation of domain architecture and key amino acids in *Rattus norvegicus* and *M. brevicollis* secretory SNARE proteins. SNAP-25 from *M. brevicollis* contains a stretch of cysteines (green) known to be palmitoylated in metazoans. Synaptobrevin from *M. brevicollis* contains two tryptophans (blue) known to mediate protein–lipid interactions in metazoans. In addition, syntaxin 1 from *M. brevicollis* contains an N-peptide with a highly conserved DRL/TxxL motif (red) necessary for the binding of syntaxin 1a to Munc18-1. (B) SNARE proteins from *M. brevicollis* form a highly stable, SDS-resistant complex (modified from Burkhardt et al., 2011). (C) Crystal structure of the rat Munc18-1/syntaxin 1a complex. Munc18-1 domains 1, 2 and 3 are colored blue, green and yellow, respectively. The SNARE domain of syntaxin is colored purple, and the regulatory Habc domain and N-peptide are colored red (modified from Burkhardt et al., 2008). (D) Crystal structure of the choanoflagellate Munc18/syntaxin 1 complex (domain colors as in C) (modified from Burkhardt et al., 2011). (E) A single choanoflagellate cell. The plasma membrane and flagellum are revealed by staining with antibodies to β-tubulin (green). Synaptobrevin (red) is detected near the apical membrane of the cell (arrowhead) (modified from Burkhardt et al., 2011). (F) Transmission electron microscopy of a thin section through a *M. brevicollis* cell reveals the presence of the Golgi apparatus and associated clear vesicles (arrowheads) at the apical membrane of the cell (modified from Burkhardt et al., 2011). Scale bars: 2 µm in E and 200 nm in F. *R.nor*, *Rattus norvegicus*; *M.bre*, *Monosiga brevicollis*.

controls the accessibility of syntaxin 1 to its partners in choanoflagellates and vertebrates.

To identify the subcellular localization of the secretory SNARE proteins and Munc18 in *M. brevicollis*, immunostaining experiments were performed (Burkhardt et al., 2011). First, a polyclonal antibody against the soluble region of synaptobrevin from *M. brevicollis* was raised and the protein was detected near the apical membrane of the choanoflagellate cell (Fig. 3E). In addition, several antibodies originally raised against the rat proteins syntaxin 1a, SNAP-25 and Munc18-1 specifically recognized their respective homologs from *M. brevicollis* and these proteins were also detected at the apical pole of the cell (Burkhardt et al., 2011). This region had been suggested to be involved in secretion (Laval, 1971). Transmission electron microscopy of *M. brevicollis* thin sections revealed a single Golgi apparatus beneath the nucleus, and numerous vesicles, which are located close to the apical plasma membrane (Fig. 3F). Together, the data suggest that the choanoflagellate *M. brevicollis* possesses a defined, yet relatively simple secretory machinery. This machinery may have served as a starting point that helped to develop a more complex secretion apparatus found in many metazoan cells, including neurons (Burkhardt et al., 2011).

The premetazoan ancestry of the neuronal protein Homer

The identification of a conserved neurosecretory apparatus in choanoflagellates raises the possibility that other building blocks of synapses evolved prior to the origin of neurons. For example, it is possible that a primordial synaptic-like scaffold evolved prior to the origin of the first metazoans as choanoflagellate genomes encode postsynaptic scaffolding protein homologs like Homer, Shank and PSD-95 (Alié and Manuel, 2010; Ryan and Grant, 2009; Sakarya et al., 2007). Understanding the ancestral role of postsynaptic scaffolding proteins could help reveal how and when a sophisticated signaling apparatus like the PSD evolved during metazoan evolution. Recent work has focused on reconstructing the ancestral function of Homer, one of the most abundant proteins in the PSD of excitatory synapses in metazoans (Cheng et al., 2006). Homer proteins are a major determinant of the size of dendritic spines and the PSD (Sala et al., 2001), and form large protein clusters with Shank proteins, metabotropic glutamate receptors and inositol trisphosphate receptors (Brakeman et al., 1997; Tu et al., 1998; Tu et al., 1999). They therefore play a critical role in activity-dependent changes in synaptic strength and, potentially, contribute to higher brain functions such as learning and memory (Thomas, 2002; Xiao et al., 1998). In addition to their neuronal roles, Homer proteins function in other, diverse metazoan-specific cell types, including regulating Ca^{2+} signaling and mechanotransduction in diverse muscle cells (Stiber et al., 2008; Worley et al., 2007), anchoring maternal effect transcripts in *D. melanogaster* oocytes (Babu et al., 2004), and acting as negative regulators of T cell activation (Huang et al., 2008).

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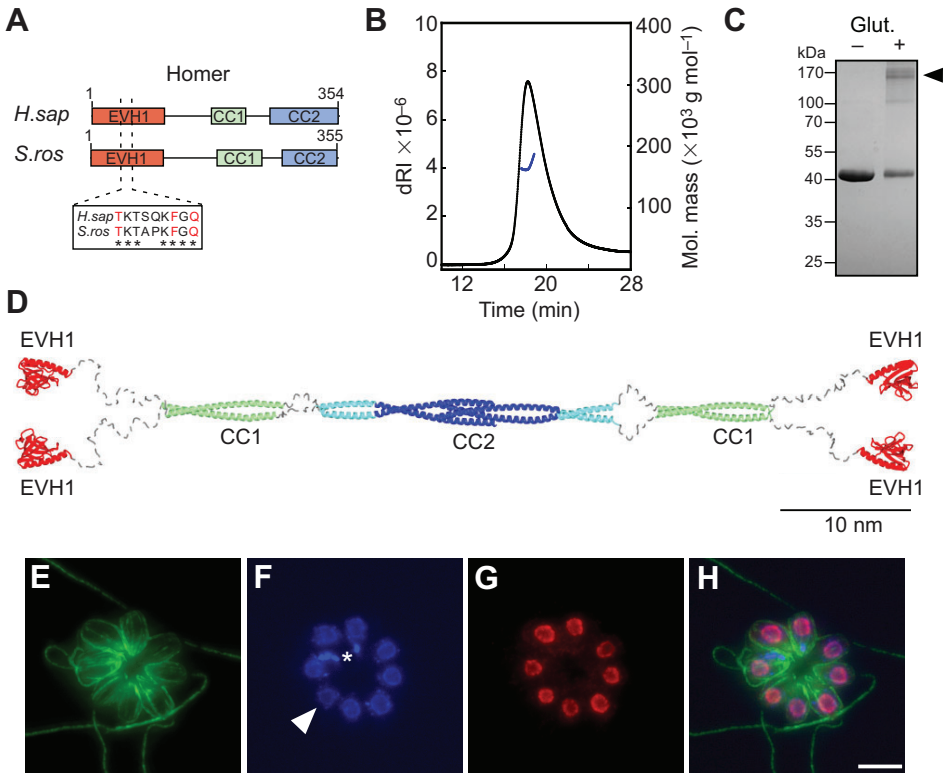

Fig. 4. Homer in the choanoflagellate *Salpingoeca rosetta*. (A) Conserved protein domain architecture of *Homo sapiens* and *S. rosetta* Homer proteins. Residues that are necessary for binding of vertebrate Homer proteins to Shank, metabotropic glutamate receptor, inositol trisphosphate receptor and Dynamin are conserved in *S. rosetta* Homer (highlighted in red). (B) Size-exclusion chromatography (SEC)-MALS reveals that full-length *S. rosetta* Homer exists predominantly as a tetramer (molecular mass, 167 kDa). The signal from the refractive index detector (dRI; left *y*-axis) is shown as solid, black lines. Molecular mass as calculated across the protein elution peak (right *y*-axis) is shown as a blue line (modified from Burkhardt et al., 2014). (C) Chemical cross-link of full-length *S. rosetta* Homer with glutaraldehyde (Glut.). The protein is shown before (–) and after (+) the cross-link reaction with the positions of molecular mass markers. Two prominent bands are visible on SDS-PAGE, with molecular masses estimated to be 41 and 165 kDa (black arrowhead). (D) Model of the tetrameric structure of full-length Homer illustrating how Homer proteins can act as large scaffolding molecules (modified from Hayashi et al., 2009). (E–G) A choanoflagellate rosette colony. Cells were stained with β-tubulin antibodies (E, green), DAPI (F, blue) and *S. rosetta* Homer antibodies (G, red). Arrowhead indicates a representative nucleus from one cell. DNA from prey bacteria (indicated with an asterisk) also stains with DAPI. (H) *Salpingoeca rosetta* Homer colocalizes with DNA in the nucleus (modified from Burkhardt et al., 2014). *H.sap*, *Homo sapiens*; *S.ros*, *Salpingoeca rosetta*; EVH1, enabled/vasodilator-stimulated phosphoprotein homology 1 domain; CC1/CC2, coiled-coil domain 1/2. Scale bar for E–H: 5 µm.

The protein domain architecture of Homer is conserved in humans and in choanoflagellates; Homer proteins from both of these lineages contain an N-terminal enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain and a Homer-specific C-terminal coiled-coil domain (Fig. 4A). Alignment of Homer amino acid sequences from choanoflagellates and metazoans shows a high degree of conservation (32% amino acid identity between human Homer 1 and *S. rosetta* Homer and 33% amino acid identity between human Homer 1 and *M. brevicollis* Homer), including residues that are necessary for binding to its known interaction partners Shank, metabotropic glutamate receptor and inositol trisphosphate receptor in metazoans (Fig. 4A, highlighted in red). While sequence comparison between Homer from both choanoflagellates, *M. brevicollis* and *S. rosetta*, shows that both proteins are equally well conserved with metazoan Homer proteins, it was found that not only is the *S. rosetta* Homer homolog more biochemically tractable than that of *M. brevicollis*, but *S. rosetta* also has different life history stages, including a multicellular stage called a rosette in which cells are attached to each other through fine intercellular bridges (Dayel et al., 2011).

In vertebrates, Homer tetramerization mediated by its C-terminal coiled-coil domain is required for the structural integrity of dendritic spines and for recruitment of specific proteins to synapses (Hayashi et al., 2009). Circular dichroism (CD) spectroscopy experiments revealed that the C-terminal coiled-coil domain of bacterially expressed *S. rosetta* Homer is indeed α-helical (Burkhardt et al., 2014), a prerequisite for the formation of a coiled-coil. To investigate whether full-length *S. rosetta* Homer can tetramerize, Multiangle Light Scattering (MALS) was used to accurately determine its molecular mass. Full-length Homer (amino acids 1–355, molecular mass 41 kDa) eluted with a molecular mass of 167±6 kDa (Fig. 4B), suggesting that *S. rosetta* Homer can form stable tetramers (Burkhardt et al., 2014). In addition, purified full-length *S. rosetta* Homer was chemically cross-linked with glutaraldehyde (Fig. 4C). The cross-linked fragment showed two prominent bands on SDS-PAGE, with molecular masses estimated to be 41 and 165 kDa, respectively, which can be explained as a monomer and a tetramer of 41 kDa protein fragments (Fig. 4C). This result also supports that *S. rosetta* Homer, like its vertebrate homolog (Fig. 4D), forms oligomers through its C-terminal coiled-coil domain. Therefore, Homer's polymerization activity likely evolved before the origin of metazoans and synapses.

Although choanoflagellates do not form synapses, it has been hypothesized that metazoan synapses evolved from protein machinery, such as the endocytic pathway, that allowed the Urmetazoan to interact with the environment (Ryan and Grant,

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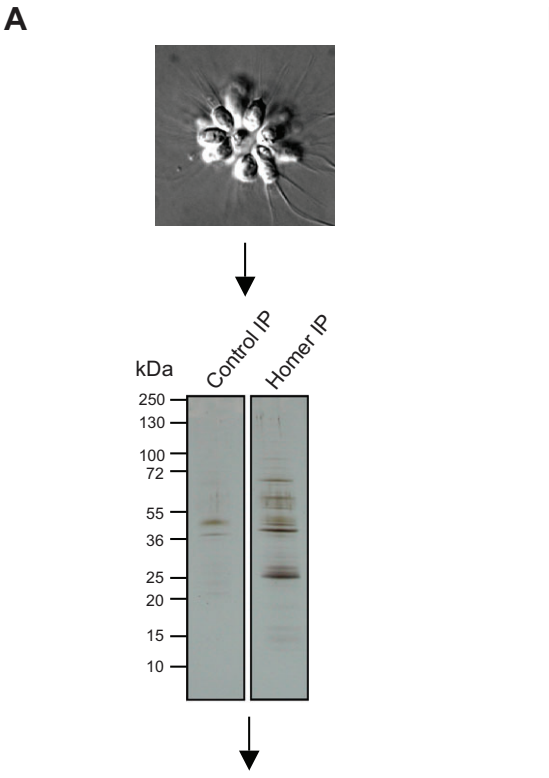
2009). To investigate whether Homer acts as a sub-cortical membrane scaffold in *S. rosetta* in a manner consistent with mediating interactions with the extracellular environment, its subcellular localization was investigated. Using affinity-purified and validated antibodies raised against *S. rosetta* Homer (Burkhardt et al., 2014), Homer co-localized with DNA was detected in the *S. rosetta* nucleus (Fig. 4E–H), in striking contrast to expectation based on its localization in neurons. Co-staining choanoflagellate cells with a nuclear pore complex (NPC) antibody confirmed that Homer is localized inside the nuclear membrane. In addition, immunogold-labeling experiments independently confirmed that Homer is predominantly localized to the nucleus (Burkhardt et al., 2014).

To gain insight into potential functions for Homer in the *S. rosetta* nucleus, researchers have sought to identify its native binding partners. Homer was immunoprecipitated from lysates of *S. rosetta* rosette colonies and multiple bound proteins were detected by SDS-PAGE and silver staining (Fig. 5A) (Burkhardt et al., 2014). Analysis of the immunoprecipitates by mass spectrometry revealed diverse known and potentially new Homer binding partners. As in metazoans, *S. rosetta* Homer likely interacts with the inositol trisphosphate receptor (IP3R), a calcium channel activated by IP3, and Dynamin, a GTPase involved in endocytosis and cytokinesis (Fig. 5A). Some other known Homer binding partners that are encoded by the *S. rosetta* genome (e.g. Shank) were not detected, although they may interact with Homer under conditions not tested in this experiment. Other previously unidentified binding partners include ATP-citrate lyase (ACLY), a key enzyme responsible for the synthesis of cytosolic acetyl-CoA with central roles in *de novo* lipid synthesis and histone acetylation in metazoans (Wellen et al., 2009), and Slit-Robo Rho GTPase-activating protein (SRGAP), a protein critical for neuronal migration in bilaterians (Guerrier et al., 2009) (Fig. 5A). Moreover, proteins with so far unknown functions were found in the immunoprecipitate; for example, EF-hand calcium-binding domain-containing protein 5, SNW domain-containing protein 1, Tetratricopeptide Repeat Protein 35 (TTC 35) and Neighbor of COX4 (COX4NB). It is interesting to note that TTC 35 was first identified in mouse nuclear envelope proteomes (Dreger et al., 2001) and later was shown to interact with COX4NB in a yeast two-hybrid screen (Boxem et al., 2008). However, the two most abundant detected binding partners were Flotillin 1 and Flotillin 2



Identity	Unique peptides
Homer*	34
Flotillin 2	25
Flotillin 1	22
EF-hand calcium-binding domain-containing protein 5	16
ATP-citrate lyase	13
SNW domain-containing protein 1	11
Tetratricopeptide repeat protein 35	11
WD repeat-containing protein 66	6
Neighbor of COX4	3
Dynamin*	3
SLIT-ROBO Rho GTPase-activating protein 1	3
Inositol 1,4,5-trisphosphate receptor type 1*	2

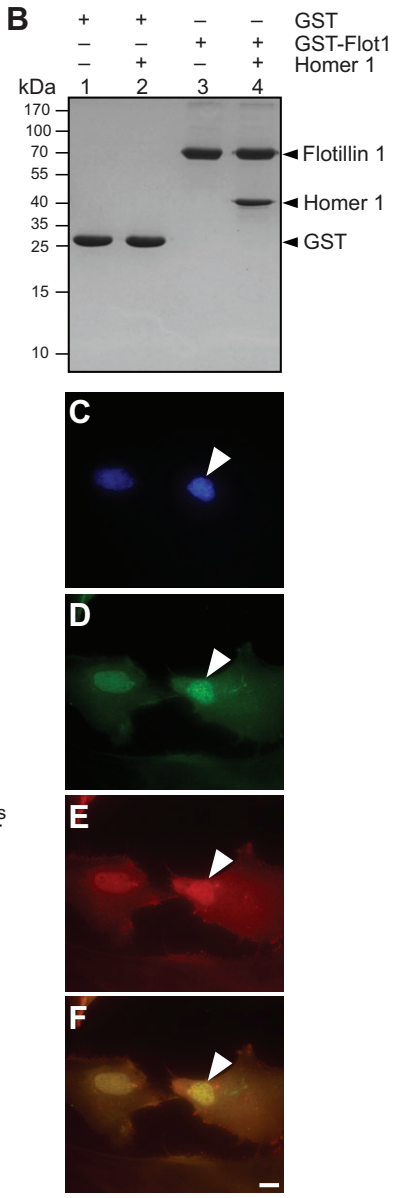


Fig. 5. Homer protein interactome in the choanoflagellate *Salpingoeca rosetta*. (A) In a co-immunoprecipitation experiment, choanoflagellate whole-cell lysate was incubated with antibody-coupled beads and diverse proteins were specifically pulled down by Homer antibodies, but not by control antibodies (modified from Burkhardt et al., 2014). Samples were analyzed by SDS-PAGE and silver staining. A select list of proteins identified by LC/MS-MS analysis of a Homer immunoprecipitate from whole-cell lysates is shown. Previously known Homer binding partners from metazoans are marked with an asterisk. (B) Bacterially expressed human Homer 1 binds to human GST-Flotillin 1, but not GST. Glutathione-Sepharose beads containing GST (lanes 1 and 2) or human GST-Flotillin 1 (lanes 3 and 4) were incubated with human Homer 1 (lanes 2 and 4) (modified from Burkhardt et al., 2014). Proteins eluted from the beads were separated using SDS-PAGE and visualized with Coomassie Blue. (C–F) Flotillin 1 and Homer 1 colocalize in rat astrocyte nuclei. Subcellular localization of Flotillin 1 (D, green), Homer 1 (E, red) and DAPI (C, blue) is shown. (F) The overlay of DAPI, Flotillin 1 and Homer 1 shows co-localization in the nucleus (white arrowheads) (modified from Burkhardt et al., 2014). Scale bar: 10 µm.

(Fig. 5A), which have not previously been shown to interact with Homer in metazoans.

Flotillins were originally discovered in neurons during axon regeneration (Schulte et al., 1997). Subsequently, they were independently identified as markers of lipid rafts in flotation assays and were hence named flotillins (Bickel et al., 1997). While flotillins are involved in the scaffolding of large heteromeric complexes that signal across the plasma membrane, their exact function remains somewhat elusive (Stuermer, 2010). Flotillins are characterized by a conserved N-terminal SPFH-domain, which harbors two conserved hydrophobic stretches that allow for membrane binding and a specific C-terminal Flotillin domain, important for hetero-dimerization of flotillins. An interaction between Homer and Flotillin has not been described before, so it was especially important to confirm that the detected interaction is physiologically relevant. To test whether *S. rosetta* Flotillin 1 is capable of interacting directly with *S. rosetta*, Homer GST pull-down experiments were performed. GST-Flotillin 1, immobilized on glutathione-Sepharose was incubated with Homer. It was found that Homer binds to Flotillin 1, but not to GST alone (Burkhardt et al., 2014). These experiments confirm the initial finding using mass spectrometry (Fig. 5A) and identify Flotillin 1 as a true Homer binding partner. If Homer and Flotillin are binding partners, it was hypothesized that in *S. rosetta*, Flotillin would localize to the nucleus. Indeed, Flotillin co-localizes with Homer in the *S. rosetta* nucleus (Burkhardt et al., 2014), providing additional evidence for their interaction and revealing an unexpected localization pattern for Flotillin, whose function is better characterized as a membrane scaffold.

The direct interaction between *S. rosetta* Homer and Flotillin and their co-localization in the *S. rosetta* nucleus led to the investigation of whether this unexpected interaction might be conserved in metazoans (Burkhardt et al., 2014). Human Flotillin 1 protein fused to GST (GST-Flotillin 1) was immobilized on GST and incubated with human Homer 1 protein. It was found that Homer 1 binds specifically to GST-Flotillin 1, but not to GST alone (Fig. 5B), revealing that the human proteins Homer 1 and Flotillin 1 are capable of interacting directly and suggesting that their interaction is evolutionary conserved. In addition, Homer 1 and Flotillin 1 co-localized in the nucleus of cultured rat hippocampal astrocytes (Fig. 5D–G). Together, the results indicate that Homer and Flotillin may have localized to the nucleus in the common ancestor of choanoflagellates and metazoans.

Summary and discussion

Most of our knowledge on the origin and evolution of metazoan synaptic proteins is at an early stage. It is now an exciting time to launch into this topic, a time with great promise for new insights into a pivotal event in metazoan history – the emergence of the first synapses and neurons. Choanoflagellates are beginning to emerge as model organisms to provide clues into the ancestry of synaptic proteins. The discovery in choanoflagellates (and in the filasterean *C. owczarzaki*) of protein family members that are integral to neuronal function in metazoans (Fig. 2B) underscores the relevance of these two groups to our understanding of the origin of synaptic proteins. While work on *C. owczarzaki* synaptic protein homologs is currently not available, the first studies on synaptic protein homologs have been conducted in choanoflagellates.

The neurosecretory SNAREs proteins syntaxin 1, SNAP-25 and synaptobrevin 2 and the SM protein Munc18 are essential for secretion of neurotransmitters from synaptic vesicles. It has been hypothesized that the binary Munc18-1/syntaxin 1a complex might be a specialization of the neuronal secretion apparatus (Rizo and Rosenmund, 2008; Shen et al., 2007; Shin, 2013; Südhof and Rothman, 2009), as this complex has been reported for neuronal tissues only. In this complex, Munc18-1 binds to a ‘closed’ conformation of syntaxin 1a, thereby occluding the binding site for the cognate SNARE partners SNAP-25 and synaptobrevin 2. This binding mode prompted the ‘negative regulator’ hypothesis according to which Munc18-1 sequesters syntaxin 1a and inhibits exocytosis. The strict structural conservation of the Munc18/syntaxin 1 complex in the choanoflagellate *M. brevicollis* reveals that the binary complex played an important role in the last common ancestor of choanoflagellates and metazoans. Both rat and *M. brevicollis* syntaxin 1 interact via a conserved binding mode that includes two spatially separate binding sites: the N-peptide and the remainder of syntaxin 1a in the closed conformation (Fig. 3C,D). Interestingly, when the N-peptide of syntaxin 1 is bound to the outer face of Munc18, syntaxin 1 cannot form a SNARE complex. When the N-peptide is released, however, Munc18-bound syntaxin 1 is able to bind to its partner SNARE proteins. The finding that this mechanism is conserved in *M. brevicollis* (Burkhardt et al., 2011) demonstrates its importance and reveals that choanoflagellates can assist in reconstructing the origin of synaptic proteins. Furthermore, using light and electron microscopy it was shown that in *M. brevicollis* components of the neurosecretory apparatus localize near the apical membrane of the cell.

These findings raise interesting new questions about the features of the secretory apparatus in choanoflagellates: which substances are secreted at the apical pole of a choanoflagellate cell? Are these substances used for some kind of communication between choanoflagellate cells? Which other proteins regulate the secretory machinery of choanoflagellates and is this machinery Ca^{2+} sensitive? It is important to note here that the genome of *M. brevicollis* encodes a homolog of complexin, but is apparently missing homologs of Munc13 and synaptotagmin 1. Munc13, a key component of the active zone of vertebrate synapses is believed to mediate the transition from the closed syntaxin–Munc18 complex to the SNARE complex (Ma et al., 2011). The fast Ca^{2+} sensor synaptotagmin 1 was reported to compete with complexin for SNARE-complex binding, thereby dislodging complexin from SNARE complexes in a Ca^{2+}-dependent manner (Tang et al., 2006). Comparisons of the neurosecretory apparatus from choanoflagellates with their counterparts from sponges and cnidarians will allow for further insights into how the neurosecretory apparatus in metazoans evolved. Taken together, the data support the hypothesis that the primordial secretion machinery of the common ancestor of choanoflagellates and metazoans was co-opted for synaptic roles during the rise of metazoans (Burkhardt et al., 2011).

In addition, the studies in the choanoflagellate *S. rosetta* provided unexpected insights into the postsynaptic protein Homer. In metazoans, Homer protein function is best understood in neurons, where it binds to other core scaffolding proteins like Shank to build the backbone of the PSD. Critical biochemical properties of Homer proteins were found to be conserved in choanoflagellates and metazoans. The ability of Homer to form tetramers, a key feature that is necessary for the structural integrity of the PSD and dendritic spines in neurons, is conserved in choanoflagellates and likely evolved before the origin of metazoans and synapses. Homer functions in metazoans as a scaffolding protein at the postsynaptic density. It was therefore surprising to find that Homer localized to the nucleus in the choanoflagellate *S. rosetta*, interacting with Flotillin, a protein more commonly associated with cell membranes. Significantly, Homer localization to the nucleus is conserved in some metazoan

cell types, as the protein localizes to the nucleus of cultured rat astrocytes and binds to Flotillin 1. The association between Homer and Flotillin in the nucleus has not previously been shown. Nevertheless, both proteins have been observed independently in the nuclei of metazoan cells: Homer in the nuclei of T cells, muscle fiber cells and cardiomyocytes (Chiarello et al., 2013; Ishiguro and Xavier, 2004; Volpe et al., 2004), and Flotillin in the nuclei of prostate and cervical cancer cells (Gómez et al., 2010; Santamaría et al., 2005). The functions of Homer and Flotillin in the nucleus remain to be elucidated, but nuclear-localized Homer in choanoflagellates, and possibly in astrocytes, could regulate nuclear Ca^{2+} entry and dynamics, as two Ca^{2+} channels – ryanodine receptor and IP3 receptor (which was detected as a potential binding partner of *S. rosetta* Homer) – are known to be activated by Homer and have been detected in the nuclear membranes of diverse metazoan cell types (Gerasimenko and Gerasimenko, 2004). It was inferred that the interaction between Homer and Flotillin is conserved and was present in the last common ancestor of choanoflagellates and metazoans (Burkhardt et al., 2014). The data suggest that the ancestral function of Homer likely differed from its well-known function as a scaffolding protein in metazoan neurons.

The co-immunoprecipitation experiments also revealed many novel potential Homer binding partners, some of which have been shown to localize to the nucleus in metazoan cells, e.g. ACLY (Wellen et al., 2009), SNW domain-containing protein 1 (also known as SKIP 1) (Folk et al., 2004) and srGAP (Guerrier et al., 2009). Whether these novel identified binding partners are true Homer interacting proteins *in vivo* and might cooperate will require further study. Surprisingly, an interaction between Homer and Shank was not detected in *S. rosetta*, although the residues that are necessary for binding are conserved in both proteins. It is possible that the two proteins might interact under other conditions tested in the co-immunoprecipitation experiments. For technical reasons, the analysis of Homer binding partners had to be restricted to rosette colonies. There is likely much more to be learned about Homer binding partners during different stages of *S. rosetta* life history [e.g. in thecate cells, solitary swimming cells and chain colonies (see Dayel et al., 2011)]. It is also possible that Homer and Shank interacted in the last common ancestor of choanoflagellates and metazoans but that the interaction was lost in *S. rosetta*. Alternatively, the core of the postsynaptic protein complex formed by Homer and Shank might have evolved after the divergence of the choanoflagellate and metazoan lineages. Additional studies on how proteins like Homer, Shank, GKAP and PSD-95 functioned at a molecular level in sponges and cnidarians will provide further insights into the evolution of the PSD and illuminate when these proteins were integrated to form a primordial synaptic-like scaffold.

The findings emphasize the untapped potential of choanoflagellates to yield important new insights into the core mechanisms underlying the origin and evolution of synaptic proteins. In the future, the advent of transgenic techniques in choanoflagellates will allow for additional insights into the premetazoan function of synaptic protein homologs. Currently, a major obstacle is the delivery of larger molecules (e.g. plasmids, proteins) into choanoflagellate cells, as many choanoflagellate species are covered by a thick extracellular matrix (Dayel et al., 2011). However, key advances have recently been made in establishing classical genetics in choanoflagellates, as the choanoflagellate *S. rosetta* produces morphologically differentiated gametes and engages in sexual reproduction (Levin and King, 2013; Levin et al., 2014). These studies together with the findings described here provide the first evidence that choanoflagellates may serve as a simple model for discovering ancestral functions of synaptic proteins.

Acknowledgements

This work was presented at the 'Evolution of the First Nervous Systems II' meeting, which was supported by National Science Foundation (NSF). The author thanks Peter Anderson for organizing a wonderful meeting.

Competing interests

The author declares no competing or financial interests.

Funding

The author was supported by the Graduate Program 521 'Protein-Protein Interactions During Intracellular Transport of Macromolecules' and a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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