The remembrance of the things past: Conserved signalling pathways link protozoa to mammalian nervous system

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Abstract

The aim of the present article is to analyse the evolutionary links between protozoa and neuronal and neurosecretory cells. To this effect we employ functional and topological data available for ciliates, in particular for Paramecium. Of note, much less data are available for choanoflagellates, the progenitors of metazoans, which currently are in the focus of metazoan genomic data mining. Key molecular players are found from the base to the highest levels of eukaryote evolution, including neurones and neurosecretory cells. Several common fundamental mechanisms, such as SNARE proteins and assembly of exocytosis sites, GTPases, Ca2+-sensors, voltage-gated Ca2+-influx channels and their inhibition by the forming Ca2+/calmodulin complex are conserved, albeit with different subcellular channel localisation, from protozoans to man. Similarly, Ca2+-release channels represented by InsP2 receptors and putative precursors of ryanoide receptors, which all emerged in protozoa, serve for focal intracellular Ca2+-signalling from ciliates to mammalian neuronal cells, eventually in conjunction with store-operated Ca2+-influx. Restriction of Ca2+ signals by high capacity/low affinity Ca2+-binding proteins is maintained throughout the evolutionary tree although the proteins involved differ between the taxa. Phosphatase 2B/calcineurin appears to be involved in signalling and in membrane recycling throughout evolution. Most impressive example of evolutionary conservation is the sub-second dynamics of exocytosis-endocytosis coupling in Paramecium cells, with similar kinetics in neuronal and neurosecretory systems. Numerous cell surface receptors and channels that emerge in protozoa operate in the human nervous system, whereas a variety of cell adhesion molecules are newly "invented" during evolution, enabled by an increase in gene numbers, alternative splice forms and transcription factors. Thereby, important regulatory and signalling molecules are retained as a protozoan heritage.

1. Introduction

The fundamental parallels in behaviour of unicellular organisms and metazoans have been advocated at the beginning of 20 s century by Jennings [1] in his remarkable book “Behavior of the Lower Organisms”. On a molecular level several cardinal signalling cascades critical for the nerve cells function emerged in protozoa, where they provided for excitation and behavioural response. In the present narrative we shall extend this reasoning further to recognise essential similarities and divergences, as we shall present pivotal proteins and protein-based mechanisms that are conserved from protozoa up to humans.

Mammalian neurones and neuroendocrine cells share the following properties: (i) They possess an electrically excitable cell membrane. (ii) Generation of cytosolic Ca2+ signals relies upon a complement of voltage-gated and other plasmalemmal Ca2+ channels and intracellular Ca2+ release channels (CRCs; represented by ryanoide receptors, RyRs, and InsP2 receptors, InsP2Rs). (iii) Cytosolic Ca2+-binding proteins (CaBP) localise Ca2+ signals, while Ca2+-ATPases/pumps, together with cation exchangers ascertain homeostatic recovery of [Ca2+]. (iv) Transmitter vesicles are delivered to the cell surface via microtubular rails. (v) Membrane proteins for targeted delivery and docking at the cell membrane include GTPases, SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors), H+-ATPase and actin. (vi) A Ca2+-sensitive fusogenic protein, synaptotagmin, mediates exocytotic transmitter release and membrane fusions. (vii) Ca2+-dependent cascades provide for internalisation and recycling of the membranes of emptied vesicle. Thus intraneuronal communications, as well as integrative processes that occur in pre- and post-

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synaptic compartments are regulated by ionized Ca\(^{2+}\) [2–4].

In this review we consider proteins and protein-based mechanisms in neuronal and neuroendocrine cells, which have originated in protozoa. We discuss peripheral neurones and neuromuscular junction, neuroendocrine cells, as well as neurones of the central nervous system. We also examine fundamental aspects of exocytosis of clear or dense core-secretory vesicles. Despite their widely different structure and molecular endowment, clear and dense vesicles share many similarities at the organelar and molecular level. For instance, both types of vesicles operate in nociceptive neurones [4,5], which release glutamate and peptides of different molecular weight [6]. Similarly, neuroendocrine cells, such as adrenal medullary cells, contain large dense core vesicles (“chromaffin granules”) for release of catecholamines [7], together with clear vesicles for release of acetylcholine [8]. Neuropeptides, e.g. those delivered from the hypothalamus to the pituitary gland are frequently packaged in large dense core vesicles [5]. Since comparison of anatomical features does not appear feasible we rather concentrate on examples which have proved easily accessible for cell biological investigation. We present arguments which allow to trace evolutionary origins of key proteins and protein-based mechanisms in the different neuronal and neurosecretory cells to protozoa. Choanoflagellates and their close relatives, the filamentous, are closest to the evolutionary roots of metazoa (Fig. 1 and [9]). Several insights into the early evolution of molecular components of neuronal cells are derived from data mining of choanoflagellate databases, although functional data are still rather limited. In contrast, ciliates provide considerable information about complexity, function and intracellular localisation of a variety of proteins relevant for Ca\(^{2+}\) signalling, vesicle trafficking and exocytosis [10–13]. Experimental analysis of ciliates is based on electrophysiology, cell fractionation, light and electron microscopy, gene silencing etc. Therefore, the current survey is contemplated not only to complement studies focusing on functions predicted for choanoflagellates based on sequencing data, but to elaborate on aspects known already in considerable detail from other protozoa, notably ciliates, and in part also from the myxamoeba Dictyostelium, Ca\(^{2+}\)-binding proteins (CaBPs) being an example [14]. On this background, we may attempt to trace some characteristics to protozoa. The emphasis will be on two genera of ciliated protozoa, such as *Paramecium* and *Tetrahymena*, notably *P. tetraurelia* and *T. thermophila*, for which substantial data are available.

Choanoflagellates, together with myxamoebae, and ciliates (ciliophora) are respective representatives of two main evolutionary lineages, monokonts and bikonts. Despite some significant differences between the two lineages, there are also remarkable similarities [12,15]; for example, Ca\(^{2+}\) as a key regulatory molecule for vesicle trafficking and mechanisms of exocytosis/endocytosis are conserved from early eukaryotes onwards [15,16].

Progressing through the phylogeny we find key players in different structural and functional context, as evolution is driven not only by duplication and recombination of a common toolkit, but also by re-localisation and re-functionalisation of proteins [17,18]. Proteins engaged in Ca\(^{2+}\) regulation and signalling seemingly evolved more dramatically than many other cell components [19]; some examples of such proteins in protozoa are summarised in Table 1. Some other key proteins, such as SNAREs, are present in comparable basic forms and numbers in *P. tetraurelia* (disregarding “ohnologs” from recent whole genome duplications [20]) and in mammals [21,22].

2. Similarities and differences between ciliates, neurones and neuroendocrine cells

2.1. Biogenesis and transport of secretory organelles

The molecular machinery required for formation and release of clear vesicles and dense core-vesicles is essentially the same: SNAREs are needed, as are GTPases and a Ca\(^{2+}\) sensor protein, synaptotagmin, though in different isoforms [23,24]. Dense core-secretory organelles are called chromaffin granules/vesicles in neurosecretory chromaffin cells (Fig. 2A), trichocysts in *Paramecium* (Fig. 2B and C) and mucocysts in *Tetrahymena*. The trichocysts originate in part in the Golgi complex and subsequently are transported by saltyatory movement along microtubules, emanating from ciliary basalbodies [25,26] to the cell membrane for stimulated exocytosis. There are more forms of dense core-secretory organelles in protozoa, with different (ultra)structure, cargo

![Fig. 1. The tree of life.](image-url)
and composition, although their functional role requires further scrutiny [26]. Targeting, docking and release of vesicles depends on GTPases, as shown in *Tetrahymena* [27], on SNAREs, as shown in *Paramecium* [13,28–30], and, indirectly, on H⁺-ATPase [31], as over-viewed elsewhere [13,32]. Empty vesicular ghosts are internalised after exocytosis, but not recycled. How and in which sequence steps required to achieve intracellular (vesicle and other) targeting may have evolved remains debatable [33,34]. Some secretory components contained in secretory organelles selected for occurrence up to mammalian neuronal systems. For details, see also *Paramecium* database at ParameciumDB (http://paramecium.cgm.cnrs-gif.fr) and *Tetrahymena* database at ftp://ftp.ncbi.nih.gov/pub/TraceDB/tetrahymenathermophila.

<table>
<thead>
<tr>
<th>Type of protein</th>
<th>References</th>
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<tr>
<td>Ca²⁺-release channels (CRC): <em>P. tetraurelia</em></td>
<td>[169]</td>
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<tr>
<td>inositol 1,4,5 triphosphate receptor (InsP₃R)</td>
<td>[102]</td>
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<tr>
<td>ryanodine receptor-like proteins (RyR-LP)</td>
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<tr>
<td>Ca²⁺-ATPases/pumps: <em>P. tetraurelia</em></td>
<td>[223]</td>
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<tr>
<td>plasmamembrane Ca²⁺-ATPase, PMCA</td>
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<tr>
<td>sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA)</td>
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<td>Ca²⁺-bindings proteins: <em>P. tetraurelia</em></td>
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<tr>
<td>calmodulin and calmodulin-LP</td>
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</tr>
<tr>
<td>centrin</td>
<td>[186]</td>
</tr>
<tr>
<td>copiyres</td>
<td>[119,120]</td>
</tr>
<tr>
<td>SNAREs and SNARE-binding proteins: <em>P. tetraurelia</em></td>
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<tr>
<td>Syntaxobrevin</td>
<td>[28,29]</td>
</tr>
<tr>
<td>vesicle-associated membrane protein-associated protein(VAMP-AP)</td>
<td>[28,60]</td>
</tr>
<tr>
<td>syntaxin</td>
<td>[30]</td>
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<td>α-SNAP; DNA sequence only</td>
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<td>N-ethylmaleimide-sensitive factor, NSF (SNARE chaperone)</td>
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<td>GTPases</td>
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<td>All Rab types: <em>T. thermophila</em></td>
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<td>Rab 7</td>
<td>[226]</td>
</tr>
<tr>
<td>GAPs, GEFs, GIP: <em>P. tetraurelia</em> (identification only)</td>
<td>[79]</td>
</tr>
</tbody>
</table>

### Vesicle budding

Clathrin and adaptor protein: *T. thermophila* | [156] |
| Adaptor proteins (Ap): *P. tetraurelia* (identification only) | [156] |
| Dynamin | [156] |

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<thead>
<tr>
<th>Type of protein</th>
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<tr>
<td>H⁺-ATPase (V-ATPase)</td>
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<tr>
<td>A-, C-, D-, E-, F-, H-subunit: <em>P. tetraurelia</em></td>
<td>[31,78,227]</td>
</tr>
<tr>
<td>B-subunit: <em>P.multimicronucleatum</em></td>
<td>[228]</td>
</tr>
<tr>
<td>A-, B-, C-, D-, E-, F-, H-subunit: <em>P. tetraurelia</em></td>
<td>[31,78,227]</td>
</tr>
</tbody>
</table>

| Protein kinases and phosphatases: *P. tetraurelia* | |
| cGMP-dependent protein kinase | [229] |
| cAMP-dependent protein kinase | [230,231] |
| casein kinases | [232–234] |
| CDPK (Ca²⁺-dependent protein kinase) | [39] |
| calcineurin | [169] |

### Varix: different species

actin isoforms and actin-related proteins (arp): *P. tetraurelia* | [158,159,235] |
| protein disulphide isomerase: *P. tetraurelia* (gene sequencing only) | [79] |
| phosphopase C. *T. thermophila* | [166,167] |
| phosphopase C. *P. tetraurelia* | [167,168] |

Although secretory contents of trichocysts and mucocysts share some common characteristics, the organelles themselves differ in composition, appearance, and function. In concert with membrane fusion, trichocyst release is executed rapidly within < 1 ms (Fig. 3) due to recrystallisation of the paracrystalline secretory materials as soon as it comes in contact with extracellular Ca²⁺ through the exocytosis pore [13,26]. Thus, trichocysts can serve for predator defence, in contrast to mucocysts, which produce endurable cysts. Sporadic claims of chromogranin or calmodulin in trichocysts have not been confirmed [26]. In mammalian systems, the contents of dense core-neuroendocrine vesicles are more complex than in clear neurotransmitter vesicles. In chromaffin vesicles (Fig. 2A), noradrenalin is bound, together with ATP, Ca²⁺ and chromogranin in a ternary storage complex [7,42], in which Ca²⁺ serves as a stabiliser – in contrast to trichocyst contents where Ca²⁺ causes physiological destabilisation for cargo extrusion [13,26]. In conclusion, contents are widely different in the proto- and metazoan dense core vesicle systems analysed so far.

To summarise, as far as secretory organelles pathway to their site of release and the underlying machinery involved are concerned, there are significant similarities between neuronal/neurosecretory and ciliate protozoan systems. Essential differences are observed in the secretory contents and the directionality of transport.

#### 2.2. Formation and composition of exocytosis sites

To drive fast secretion (which underlies neurotransmitter release from synaptic terminals), Ca²⁺ entry spots must be closely co-localised with docking sites for transmitter vesicles [52]. As far as secretory organelles pathway to their site of release and the underlying machinery involved are concerned, there are significant similarities between neuronal/neurosecretory and ciliate protozoan systems. Essential differences are observed in the secretory contents and the directionality of transport.

The principal pathway of biogenesis and intracellular transport of secretory organelles in ciliates resembles that of neuronal/neuroendocrine vesicles [36,37], with several essential differences: (i) In ciliates, microtubules originate from nucleation sites at ciliary basal bodies; here, microtubules originate from the cell periphery [25]. Conversely, in neurons and neuroendocrine cells vesicles are transported along microtubules originating from the cell centre and elongating toward the periphery [37]. Thus, directionality is not maintained throughout evolution. Of note, in some non-neuronal mammalian cells, such as epithelia and T-lymphocytes, microtubule polarity is similar to that in ciliates [25,38]. (ii) Only neurotransmitter vesicles undergo recycling. (iii) Neither actin nor any other cortical filaments interfere with tri-chocyst docking, in contrast to neuronal/neuroendocrine cells. (iv) Ciliates do not express true "CaM-kinease" (activated by a complex of Ca²⁺ and calmodulin (CaM)); instead they possess "Ca²⁺-dependent protein kinases" (CDPK) with a CaM-like domain integrated in their carboxy-terminal part [39], probably due to an early gene fusion [40]. One gene encoding a genuine CaM kinase has been found in the monokont, *Dictyostelium* [41].
a complex of RIM- and Munc-type proteins is assembled; this complex links a Rab-type GTPase on the surface of a transmitter vesicle and a Ca\(^{2+}\)-influx channel in the cell membrane [53]. This is suggested to represent the molecular background of fast reaction in an “active zone.” However, no such phenomena are known in protozoa, as they perform fast exocytosis according to another principle.

As shown below, ciliates work by co-assembly of exocytosis sites and cortical Ca\(^{2+}\) stores, which produce the initial Ca\(^{2+}\) signal superimposed by secondary Ca\(^{2+}\) influx. In axonal terminals Ca\(^{2+}\)-influx channels and exocytosis sites are strictly linked by co-assembly of molecules mediating Ca\(^{2+}\) influx and membrane fusion. Neurosecretory cells, however, are devoid of such co-assembly, thus operating by merely stochastic coordination. These differences account for the spatial and temporal precision required to react to a stimulus.
Machinery required for the docking and fusion is based on R-and Q-SNARE proteins. In all taxa this refers to R (arginine) or Q (glutamine) as the central amino acid in the characteristic α-helical SNARE domain [54]. During docking and fusion, SNAREs of opposite side close like a zipper toward their membrane anchor [55,56]. The same process operates in *Paramecium*, but with deviations in R-SNAREs some of which may also possess an extra “longin” domain.

In trichocysts, the R-SNARE inserted in the organelle membrane is most likely PtSyb5 [29]. Of note, due to difficulty subtyping because of extra long-domains in most “synaptobrevins”, the prefix Pt is added to indicate *P. tetraurelia* specific nomenclature. Also note that, besides the predominant “brevin” forms, some longin-type R-SNAREs occur in mono- and bikont protists as well as in mammals [57]. The Q-SNARE syntaxin 1 relevant for exocytosis is similarly conserved from ciliates to mammals [30]. In *Paramecium* this Q-SNARE is diffusely scattered over the cell membrane; its silencing greatly reduces the number of exocytosed trichocysts [30]. The SNAP-25 is another component relevant for establishing docking/fusion sites in *Paramecium* [60].

Does the number of SNAREs increase during evolution? *P. tetraurelia* contains ∼40 genes for functionally different SNAREs (disregarding structurally and functionally very similar ohnologs) [22]. This is very similar to the 39–41 SNARE genes identified in humans [21,61] and many more than the 23 SNAREs identified in *Dictyostelium* [61]. The number of SNAREs in the ur-eukaryote was estimated at ∼20, while in
Calmodulin is mandatory for the assembly of functional exocytosis sites; in chromaffin cells, such as PC12 and cells isolated from the adrenal medulla, CaM mediates the correct arrangement of synaptobrevins [68]. According to immuno-electron microscopic localisation [69] and molecular biology-based functional studies [70], CaM is similarly mandatory for the assembly of trichocyst exocytosis sites.

The specificity of membrane-to-membrane interaction and fusion in neurones is mediated by GTPases [71,72]; the same pathway operates in protozoa where Rab-, Rac- and Rho-type GTPases have been identified [73]. The Rab-type GTPases are activated by GTP hydrolysis to GDP, under control of GAPs (GTPase activating proteins), GEF (guanosine nucleotide exchange factors) and GDI (guanosyl nucleotide dissociation inhibitor). According to more recent data D. discoideum has 54, T. thermophila 56, and H. sapiens > 63 Rab-encoding genes [27], again highlighting their importance for vesicle trafficking since early eukaryote evolution.

In ciliates, the presence of GTPases has been initially shown by radioactive GTP binding on gels [74] and subsequently by genomic data mining [75]. Expression of fluorescent translation products revealed the presence of specific GTPase molecules at mucocyst docking sites in T. thermophila [27]. In mammalian cells, binding of GTPases to vesicles requires the multimeric H+-ATPase which, by its V0 part protruding into the cytosol. A conformational change of the H+-ATPase molecule, in response to luminal acidification, enables binding of GEF, thus allowing activation of specific GTPases [76]. H+-pumps are contained in neurotransmitter vesicles where they regulate the exocytotic machinery [77]. In Paramecium's trichocyst membrane, an H+-pump has not been identified and these organelles are not remarkably acidic [31], possibly because of permanent consumption of the ΔH+ . However, silencing of specific H+-ATPase subunits considerably affects trichocyst biogenesis [78]. This suggests that a general mechanism for H+-ATPase and GTPase activation of membrane interaction emerged already in protozoa.

In conclusion, protozoa contain a substantial representation of SNAREs and GTPases when compared to humans. Although there are only preliminary observations about GAP, GDI and GEF in Paramaecium [79], the assembly of exocytosis systems evidently requires a very similar set of proteins in unicellular and mammalian systems. Flooding of exocytosis sites with Ca2+ upon stimulation, however, is achieved in rather different ways, as discussed below.

2.3. Ca2+ signals in synaptic terminals and in neurosecretory cells vs. ciliates

In synaptic terminals in the peripheral and in the central nervous
system, local Ca2+ signals regulate or trigger several processes which include: (i) Priming of vesicle docking sites. (ii) Triggering the transmitter release by exocytotic membrane fusion through the C2-type CaBP, synaptotagmin [56]. (iii) Regulating the exocytosis-coupled endocytosis of empty vesicles that employs dynamin as a “pinchase” after dephosphorylation by phosphatase 2B (PP2B)/calcineurin [80,81]. (iv) Mobilising new vesicles from terminal interior by release from F-actin via phosphorylation of synaptoin by a Ca2+/CaM activated CaM kinase [62]. These multiple roles of Ca2+ have been documented in many neuronal systems [83].

In axonal terminals Ca2+ is provided, upon depolarisation, by influx through voltage-gated Ca2+-channels, which are coordinated with exocytosis sites [50]. This is in striking contrast to chromaffin cells isolated from the bovine adrenal medulla; these chromaffin cells express voltage-gated Ca2+-channels, which, however, are not coordinated with exocytosis sites [49]. Here, [Ca2+] shares a similar mechanism as in neurones [120]. Copines are assumed to serve for membrane domains [104], which thus can be contained in the fragmented form of this CRC variant.

In conclusion, exocytosis of dense core-vesicles in Paramecium is regulated by Ca2+ release from cortical stores (alveolar sacs) and a store-operated Ca2+-entry (SOCE) [101]. This also occurs in mammalian cells, including cells of neuronal or neuroendocrine origin [105]. In differentiated neurones a SOCE-type mechanism also exists, although it is less characterised for various reasons [106–108]. Sequences indicating presence of the key molecules mediating SOCE, a Ca2+-sensor anchored in the cortical store membranes, STIM, together with the plasmalemmal channel or channel-associated protein, ORAI, are found in choanoflagellates [94,109], though functional relevance is yet to be investigated. However, not all eukaryotes may be endowed with these SOCE-mediating proteins [110]. Pilot data mining in the Para- meciumbD (P. tetraurelia database) detected neither STIM nor ORAI, although more scrutiny may find some equivalents.

In Paramecium, occurrence of SOCE is supported by fluorescence quenching experiments [111] as well as by quenched-flow stimulation/rapid freezing combined with electron microscopic energy-dispersive x-ray microanalysis. Substituting Sr2+ for Ca2+ during quenched flow revealed release of Ca2+ from alveolar sacs, superimposed by influx of Sr2+ [101]. As proteins responsible for the SOCE remain unknown, mechanisms alternative to STIM/ORAI complex may involve direct binding of cisternae of the endoplasmic reticulum to the cell membrane, similarly to the eSyntag-type Ca2+-sensors identified in neuroblastoma [112]. Physical links to the cell membrane are clearly visible (Fig. 4 insert). Paramecium’s alveolar sacs are derivatives of the endoplasmic reticulum, as visualised by the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA-) type Ca2+-pump coupled to green fluorescent protein [113,114]. These experiments, however, show no continuity between endoplasmic reticulum and alveolar sacs; rather vesicular transport must be assumed. A summary of Ca2+ dynamics and signalling in P. tetraurelia has been published previously [111,92].

In Paramecium, energy-dispersive x-ray microanalysis at electron microscope resolution shows that, upon depolarisation which activates ciliary reversal, no spill-over of Ca2+ beyond the ciliary basis occurs [48]. This is in part due to Ca2+ binding to basal body-associated CaBPs, such as cenrin, as documented by cenrin knock out [98]. Of note, in the central nervous system neurones, cenrin indirectly contributes to the Ca2+ signalling through Ca2+-dependent binding to the Eg1-typical K+ channel which is inactivated in consequence [115]. Another mechanism is the inactivation of voltage-gated Ca2+-channels by Ca2+/CaM complex [116] – an effect documented also for neurones [117,118]. Mobile and immobile Ca2+ buffers with different binding properties are available in neuronal and non-neuronal cells [96]. In chromaffin cells, different CaBPs types serve as buffers [97]. As outlined in more detail below, in Paramecium the cenrin represents an immobile buffer [109,92,98]. A family of Ca2+-dependent phospholipid-binding proteins known as copines appear in Paramecium [119] as well as in neurones [120]. Copines are assumed to serve for membrane binding, but so far any specific functions remain open.

Presynaptic nerve endings are activated by Ca2+ entry through voltage-gated and other Ca2+ transporters [121,122]. In addition to the plasma membrane Ca2+-ATPase (PMCA), Ca2+-exchangers contribute to Ca2+ extrusion [123]. Gene sequences encoding voltage-gated Ca2+
channels and a potential Na\(^+\)/Ca\(^{2+}\) exchanger protein have been re-
recognised by genome data mining in *P. tetraurelia* and *T. thermophila*, re-
spectively [47,124] and in the genome of *choanoflagellates* [94]. Particu-
larly well characterized at the electrophysiological level are Na\(^+\) and K\(^-\)-channels from *Paramecium* [125–127].

When neuronal and ciliated protozoan systems are compared, the com-
mon denominator is a Ca\(^{2+}\) signal restricted in time and space to
narrow strategic sites below the cell membrane. This is generated in
different ways, either by co-localisation of exocytosis sites with plas-
ma-membranal Ca\(^{2+}\) channels in strict (axonal terminals) or random (neu-
rosecretory cells) coordination, or by site-directed Ca\(^{2+}\) flow due to a
SOCE mechanism within the narrow space between cell membrane and
cortical stores surrounding exocytosis sites (ciliaates).

3. Signalling in spines of the central neurones–parallels with
protozoans

Many proteins and protein-based mechanisms are shared between
protozoa, notably ciliaates, and neurones. The post-synaptic element in
central neurones is often represented by dendritic spines. These are
micron-sized bulges that contribute to neuronal plasticity and learning.
Activation of postsynaptic receptors by neurotransmitters instigates
series of coordinated events that underlie information transfer and syn-
aptic plasticity. In some spines, e.g. of pyramidal cells of the hippo-
campus and in Purkinje cells of the cerebellum, the smooth en-
doplasmic reticulum forms a special structure, the “spine apparatus”
[128,129] where the endoplasmic reticulum is folded into inter-
connected stacks of flat sacs. Similarly to the spine apparatus [130],
alveolar sacs are derivatives of the endoplasmic reticulum inasmuch as
they are formed by vesicle transport from the latter compartment
[114]. Both structures, although separate, are endowed with SERCA
pumps. In ciliaates, like in metazoans, the plasma membrane Ca\(^{2+}\)-AT-
ase, PMCA, is clearly different from the SERCA pump [113].

To estimate how much of protozoan proteins altogether may be
encountered in spines we should outline some of their most salient
features. Molecular components essential for synaptic function and
occurrence of partial sequences of trimeric G-protein subunits and of GPCRs in
protozoa, including transmitter vesicle dynamics.

The Ca\(^{2+}\) signal is rapidly reduced within the small spine volume –
an important aspect, considering the toxic effect of Ca\(^{2+}\) [16]. For-
mation of spines as well as their modulation during long-term po-
tentiation requires activation of CaM kinase [138,139]. Salient features of
postsynaptic plasticity, including Na\(^+\) and K\(^-\)-channel activation
via phosphorylation by CaM kinase and vesicle trafficking, have been
widely recognised [36,37,128,130]. CaM kinase also regulates NMDAR
activity [130]. As mentioned, in ciliaates, this kinase is substituted for by
CDPKs [39] – a bikont characteristic [12] which, however, functionally
may be considered an equivalent of the CaM kinase molecule in neu-
rons. A CaM kinase sensu stricto occurs in the monokont, *Dictyostelium*
[41].

Maintenance of synaptic plasticity requires vesicle delivery and re-
trieval to the membrane of the spine. Microtubules emanate from the
main body of the dendrite, i.e. they run in a minus → plus direction (i.e.
from their stable end in the shaft to their reversibly de-/repolymerising
end in the spine) [140]. Vesicle trafficking is mediated by SNAREs and
GTPases [36,37,141], in addition to the activation of both C2 domains
of synaptotagmin [142], and reggie/flotillin proteins combined with
specific GTPases [143]. Some of these components have been identified
in choanoflagellates [14–144] and in ciliaates. This also holds for a reggie/
flotillin/stomatium-type microdomain-scaffolding protein which also
occurs already in ciliaates [145].

Synaptic plasticity and memory also depend on protein phaseate
2B (PP2B), generally known as calcineurin (CaN), which regulates the
activity of several target proteins, including GLuRs [146]. In neurones,
exocytosis-coupled endocytosis is regulated through dynamin dephos-
phorylation by CaN [147,148] which also contributes to synaptic
plasticity [146]. The hetero-dimeric CaN molecule is present in pro-
toza, including *Paramecium* [149], with evidence for regulation not
only of exocytosis, but also of clathrin-mediated endocytosis [150]. CaN
also belongs to the protein inventory of *Dictyostelium* [151,152].

Another CNS transmitter, γ-aminobutyric acid (GABA), is present
in pyramidal and cerebellar Purkinje cell spines [151,153] and also in
many other inhibitory synapses [154]. Surprisingly, according to pro-
teomics analysis, *Dictyostelium* encodes a GABA receptor [155],
its function being not known at this time.

Several proteins contributing to vesicle budding in neurones and
ciliaates show remarkable similarity. In addition to experimentally ver-
ified GTPases [27], dynamin and clathrin, as well as sequences of
several other key proteins have been detected in *T. thermophila*
[10,156]. This includes several adaptors proteins (AP1, AP2, AP3) and
cotomer (COP) subunits [157]. Furthermore, a collection of actin
isomers is part of the proteome inventory of ciliaates [158]. Based on
experimental work with *P. tetraurelia*, these acts exert a variety of
functions, including phagocytosis regulation [159,159], although ef-
ffects on secretory activity have not been found. This is interesting
considering the multiple role of actin in neuronal functions [160],
including transmitter vesicle dynamics.

There are no firm data for functional GPCRs in *Paramecium* [13],
although there are some hints to their existence [161,162]. Occurrence of
partial sequences of trimeric G-protein subunits and of GPCRs in
ciliaates does not necessarily imply their functional activity [163].
However, trimeric G-proteins and GPCRs with defined signal transfer
function occur in the monokont myxamoeba, *Dictyostelium discoideum* [164,165]. This suggests an old genuine signalling function available already before metazoan evolution. Isoenzymes of phospholipase C (PLC), a target protein of GPCRs, have been cloned and expressed in ciliates, such as *Tetrahymena* and *Paramecium* [166–168] where, in addition, PLCs have been localised.

Another similarity between neuronal spines and *Paramecium* cells is associated with CRCs. *Paramecium*’s alveolar sacs contain not only of RyR-LPs, but also InsP3Rs [169], although this subpopulation of InsP3Rs has not been analysed in any detail. InsP3Rs are widespread in protozoa, as discussed previously [13]. According to genomic and proteomic data mining, InsP3Rs are detectable also in choanoflagellates [170], as are RyRs or RyR-LPs [109]. In addition, sequences indicative of both CRC types have also been identified in related flastereans [171].

To summarise, numerous molecular cascades are structurally and functionally conserved from protozoan to mammalian cells of the central nervous system.

**4. Exocytosis-endocytosis coupling**

Using synchronous stimulation of exocytosis, quenched-flow-ultrafast freezing technology, followed by low temperature processing (freeze-substitution) and evaluation of electron micrographs, fast exocytosis-endocytosis coupling has been first scrutinised in *Paramecium* [172]. *Paramecium* is considered the fastest known dense core-secretory organelle system [173]. In a cell population (not in individual cells) undergoing synchronous exocytosis, trichocyst release requires 80 ms and further 250 ms are required for membrane retrieval, with apparent \( \tau_{\text{exocytosis}} = 57 \text{ ms} \) and \( \tau_{\text{endocytosis}} = 126 \text{ ms} \), respectively, under physiological extracellular Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_e\) (Fig. 3B). A rise in \([\text{Ca}^{2+}]_e\) also drives exocytosis-endocytosis coupling in *P. tetraurelia* [174], while new trichocysts are steadily docked, essentially in a Ca\(^{2+}\)-independent manner, depending on *de novo* organelle biosynthesis.

Coupling in the sub-second to second time range has also been observed in peptidergic nerve terminals (Fig. 8), also using quenched flow, but combined with electron microscope labelling [175], as well as
in melanotrophic pituitary cells using patch-clamp analysis [176]. Retrieval of chromaffin vesicle “ghosts” proceeds with $\tau = 62 \text{ ms}$ [89], and coupling in different neuronal systems depends on $[\text{Ca}^{2+}]_c$, with a similar concentration-dependence and time range [83]. In neurons, exocytosis-endocytosis coupling is enforced by Ca$^{2+}$/CaN [177]. Though CaN is present in *Paramaecium* at distinct sites of constitutive exo-/endocytosis, i.e. “parasomal saes” [13,150], this has not been proven as yet for trichocyst exocytosis-endocytosis coupling.

When the rates of exocytosis-endocytosis coupling in peptidergic nerve terminals and in *P. tetraurelia* are compared (Figs. 3B and 8B) much faster kinetics is seen in the latter. In the different types of nerve terminals, coupling time is rather variable [160,178], but altogether it is comparable to most of the systems described here. In conclusion, the dynamics of exocytosis-endocytosis coupling is in the (sub-)second range in so widely different systems as neuronal and protozoan cells.

5. Other mechanisms shared by neurones and protozoa: some salient aspects asking for more scrutiny

Genomic analysis occasionally reveals unexpected details, such as a latrophilin receptor (LP) in *Dictyostelium* [179]. The black widow spider α-latrotoxin removes Ca$^{2+}$-dependence of exocytosis [180]. Considering the otherwise largely idiosyncratic pharmacology of ciliates, which is often different from mammalian cells, i.e. frequent kinetics are seen in the latter. The different types of neurones coupling time is rather variable [160,178], but altogether it is comparable to most of the systems described here. In conclusion, the dynamics of exocytosis-endocytosis coupling is in the (sub-)second range in so widely different systems as neuronal and protozoan cells.

Another matter to be analysed is the importance of the predominant immobile Ca$^{2+}$ buffer, centrin, in the outermost layer of the *Paramaecium* cell [98]. Here it forms the predominant component of the “infraciliary network” [186], with centrin as a main component. Its elimination by molecular biology methodology delays $[\text{Ca}^{2+}]_i$ recovery upon exocytosis stimulation by about tenfold [98]. As mentioned, cal-syntenin may be a functional equivalent contained in the spine apparatus.

6. Time scale and basic changes during molecular evolution

Evolution leaves behind molecular traces or “molecular fossils” [187]. Among them we find genes/proteins pertinent to many fundamental cellular functions. How does the finding of specific proteins in both, protozoa and neurones, compare with the age of the taxa under consideration and what is the basic ancient heritage? Molecular data for a time scale of the early evolution of eukaryotes is still controversial. The estimated age of the earliest eukaryotes is indicated as 1500 Myr (million years) [188], but shorter, or longer times have also been proposed, e.g. $< 1300$ Myr [189]. There are different estimates of the evolutionary age of the taxa under consideration. Fossil records of ciliates are estimated to be between 660 and 740 Myr old [190]. On a molecular basis, the age of ciliate clades have been considered to be between 800 and 850 Myr [191,192]. However, based on extremely low mutation rate, for *P. caudatum*, recently $\sim 1400$ Myr has been suggested [193]. A limit of $\sim 600$ Myr is assumed for the extinction of choanoflagellate progenitors [194,195]. As generally surmised, choanoflagellates have aggregated to early metazoans, such as sponges [9,196] which, however, are devoid of a nervous system. Remarkably, widely different time ranges are assumed also for formation of multicellular animals, i.e. between 650 [196] and 1500 Myr [188]. Despite these wide divergences, it could be safely conjectured that precursors of both, monokonts and bikonts, may have transmitted genes/proteins from a common gene pool for basic molecular functions.

In this context the probably monophyletic origin of all eukaryotes [197] suggests a common molecular legacy from both, mono- and bikonts. In retrospect, this justifies our focus on ciliates, as currently the largest amount of data is available from this group [10,13]. With this regard, functional and topological data for choanoflagellates and their relatives, the most likely remote ancestors of metazoans, still strongly
lag behind genomic data mining in these organisms. Once experimental work has sufficiently progressed, important new insights also for the evolution of neuronal systems will be delivered.

In conclusion, the genes for many basic eukaryotic functions were possibly shared between early unikont and bikont ancestors. This seems to be corroborated by the considerable similarity of numerous gene products, although with some exceptions. Examples are CDPks and Lokin-type synaptobrevins in ciliates [12,18]. A large number of genes can be assumed to be pan-eukaryotic also because bacterial genes contribute ~37% to mammalian gene sequences [198], whereas a common pool of 44% of protein encoding genes are expressed in all major mammalian tissues [199]. Important cytoskeletal elements are found already in bacteria [200], as are some posttranslational protein modifications [201].

All this supports the assumption that basic functional capabilities have been essentially conserved from the protozoan to highest metazoan level [10,21,62,72]. The underlying molecules have been designated “eukaryotic signature proteins” [202] and their list is extending, e.g., by proteins engaged in vesicle trafficking and Ca2+-signalling [15]. Some proteins may be kept for identical functions, other changed localisation and/or function [18], a typical example being voltage-gated Ca2+ channels. Some functions, like Ca2+ buffering, have been taken over by different CaBPs and the arrangement of Ca2+-stores differs, as do some of the specific plasmalemmal channels. Involvement of CaM in channel activity, of the Ca2+-ATPase/pump, PMCA, and of some plasmalemal cation channels in neuronal assemblies is also shared by unicellular systems. This heritage also includes adaptor proteins, clathrin, COPs and calcineurin for vesicle budding and recycling via endocytosis. In addition we see basic parts of the trafficking machinery such as SNAREs and CaBPs of different sensitivity, including Ca2+-sensor proteins. Of course, many novelties had to be “invented” during evolution, particularly with respect to increasing structural and functional complexity, including formation of increasingly complex neuronal networks [203].

Choanoflagellates contain molecules important for neuronal function including precursors of cadherins [204] and of postsynaptic density scaffolding proteins [205,206], tyrosine phosphorylation and receptor tyrosine kinases [207] etc. Some experimental work with choanoflagellates shows, e.g., the interaction of syntaxin 1 with the auxiliary protein, Munc 18 [67]. In choanoflagellates, such functional scrutiny still has to be performed for proteins relevant for cell-cell adhesion, skeletal regulators, signalling and signal transduction [208].

7. Perspectives

Increase of neuronal complexity during evolution has many facets. Can we identify key proteins relevant for highest intellectual and emotional functions already in protozoans?

Such proteins include, for example ion channels and receptors, which already occur in protozoa [127] including ciliates [125]. Voltage-gated channel channels allow for quick responses, including behavioural activities in ciliates and in green flagellates [95]). Genomic data mining has revealed these channels also in monokont choanoflagellates [94]. This, together with additional molecular details discussed previously [10,13,17], and in this article, suggests the emergence of increasingly complex cells based on both, bikont (leading to plants which otherwise “invented” strictly different cation channels) and monokont (leading to vertebrates) heritage in agreement with a recent suggestion of a common ancestry of mono- and bikonts [197]).

Furthermore, emergence of multicellularity called for cell adhesion molecules and increased information exchange between cells. Precursors (homologs) of cell adhesion molecules have been detected in choanoflagellates, which may also experience intracellular communication [204–208]. Similar adhesion molecules and intercellular communication are also known from the myxamoeba, Dictyostelium [41,209].

Cell-to-cell contacts were prerequisite to the advances of neuronal systems by increasing cell numbers and anatomical complexity, with ~7500 synapses in the nematode Caenorhabditis elegans and probably ~1015 or more synapses in humans. This enabled enormous increase in information processing in neuronal systems during metazoan evolution. Though the number of protein encoding genes in eukaryotes increases only moderately during evolution, e.g. when protozoans and humans are compared, as discussed previously [18]), there is sufficient innovation in the protein inventory, particularly for intercellular contacts and signalling. Evolution of multicellularity included multiple independent origins, with both, conservation and innovation [210] as well as collapse and expansion of cell adhesion molecule gene clusters; this is the case in the nematode C. elegans, which already contains different types of cell adhesion molecules with a number of paralogs [211]. Remarkably primitive superfamily of immunoglobulin cell adhesion molecules (IgCAMs) emerges already in neurones of the primitive taxon of Platyhelmintes (planariae) [212]. At the end of metazoan evolution, neurones dispose of a spectrum of specific cell adhesion molecules contributing to structural changes and functional plasticity, such as long-term potentiation or depression [213]. Regulation of neuronal contacts also includes protein de-/phosphorylation processes and GPCR signalling, as pointed out above for several protozoan lines.

Also the number of splice variants increases considerably during evolution [214,215]. Since this also applies to neurones [216,217], splice variants, e.g. of SNAREs, CRCs or Ca2+-pumps and particularly of cell adhesion molecules [218], can contribute substantially to increasing complexity. In contrast, splicing is rare in protozoans and almost absent in Paramecium, as summarized recently [18].

Generally, an increase of the number of transcription factors during evolution is prerequisite to individual differentiation processes [219,220]. Expansion of the neocortex is accompanied with an emergence of “human-specific gene signatures”, including transcription factors [221]. Some of these transcription factors are regulated by Ca2+ or by calcineurin [222] – an old heritage from monokont- and bikont-type protozoa.

In conclusion, important components of neuronal signalling machinery have been found in the genomes of choanoflagellates, myxamoebae and ciliates. Many other components, specifically those relevant for basic cell functions, and pertinent to the evolution of nervous system emerge from protozoa. Beyond this basic legacy, a slight increase in genes during metazoan evolution and a significant increase of alternative splicing go in parallel with increasingly elaborate cell-cell interactions, signalling and differential gene expression patterns, enabled by an increasing repertoire of transcription factors etc., can account for increasing neural complexity.

Conflict of interest

The author declares that there is no conflict of interest.

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References


I.M. Sehring, C. Reiner, H. Plattner, The actin subfamily
S.J. Lee, Y. Escobedo-Lozoya, E.M. Szatmari, R. Yasuda, Activation of CaMKII in
A.D. Nusblat, L.J. Bright, A.P. Turkewitz, Conservation and innovation in
B. Coukell, Y. Li, J. Moniakis, A. Cameron, The Ca2+/calcineurin-regulated cup
X.S. Wu, Z. Zhang, W.D. Zhao, D. Wang, F. Luo, L.G. Wu, Calcineurin is universally
K. Baumgärtel, I.M. Mansuy, Neural functions of calcineurin in synaptic plasticity
N.C. Elde, G. Morgan, M. Winey, L. Sperling, A.P. Turkewitz, Elucidation of cla-
P. Burkhardt, The origin and evolution of synaptic proteins
G.Q. Chen, C. Cui, M.L. Mayer, E. Gouaux, Functional characterization of a po-
V. Bodrikov, A. Pauschert, G. Kochlamazashvili, C.A. Stuermer, Reggie-1 and re-
E.B. Merriam, M. Millette, D.C. Lumbard, W. Saengsawang, T. Fothergill, X. Hu,
G. Glöckner, Phylogeny-wide analysis of social amoeba genomes highlights an-
U. John, M. Schleicher, L. Eichinger, M. Platzer, A.A. Noegel, P. Schaap,
A.M. Hofer, Another dimension to calcium signaling: a look at extracellular cal-
P. Hunter, Molecular fossils probe life
C.A. Elzie, J. Colby, M.A. Sammons, C. Janetopoulos, Dynamic characterization of NMDA-
G. Glöckner, Phylogeny-wide analysis of social amoeba genomes highlights an-
H. Plattner, C. Schütt, J. Hentschel, Facilitation of membrane fusion during exocy-