Erschienen in: Cell Calcium ; 73 (2018). - S. 25-39
https://dx.doi.org/10.1016/j.ceca.2018.04.001

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

Helmut Plattner[a,*], Alexei Verkhratsky[b,c,d]

[a] *Department of Biology, University of Konstanz, Room M1105, P.O. Box M625, 78457 Konstanz, Germany*
[b] *Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK*
[c] *IKERBASQUE, Basque Foundation for Science, 48011, Bilbao, Spain*
[d] *Department of Neurosciences, University of the Basque Country UPV/EHU, 48940, Leioa, Spain*

Keywords:
Brain
Ca^{2+}
Calcium
Ciliate
Evolution
Neurone
Neurosecretory
Protozoa
Signalling

A B S T R A C T

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, Ca^{2+}-sensors, voltage-gated Ca^{2+}-influx channels and their inhibition by the forming Ca^{2+}/calmodulin complex are conserved, albeit with different subcellular channel localisation, from protozoans to man. Similarly, Ca^{2+}-release channels represented by $InsP_3$ receptors and putative precursors of ryanodine receptors, which all emerged in protozoa, serve for focal intracellular Ca^{2+} signalling from ciliates to mammalian neuronal cells, eventually in conjunction with store-operated Ca^{2+}-influx. Restriction of Ca^{2+} signals by high capacity/low affinity Ca^{2+}-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 Ca^{2+} signals relies upon a complement of voltage-gated and other plasmalemmal Ca^{2+} channels and intracellular Ca^{2+} release channels (CRCs; represented by ryanodine receptors, RyRs, and $InsP_3$ receptors, $InsP_3Rs$). (iii) Cytosolic Ca^{2+}-binding proteins (CaBP) localise Ca^{2+} signals, while Ca^{2+}-ATPases/pumps, together with cation exchangers ascertain homeostatic recovery of $[Ca^{2+}]_i$. (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 Ca^{2+}-sensitive fusogenic protein, synaptotagmin, mediates excoytotic transmitter release and membrane fusions. (vii) Ca^{2+}-dependent cascades provide for internalisation and recycling of the membranes of emptied vesicle. Thus interneuronal communications, as well as integrative processes that occur in pre- and post-

* Corresponding author.
E-mail address: helmut.plattner@uni-konstanz.de (H. Plattner).

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 organellar 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 filastereans, are closest to the evolutionary roots of metazoans (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 protozoans, notably ciliates, and in part also from the myxamoeba *Dictyostlium*, 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 relocalisation 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 saltatory 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

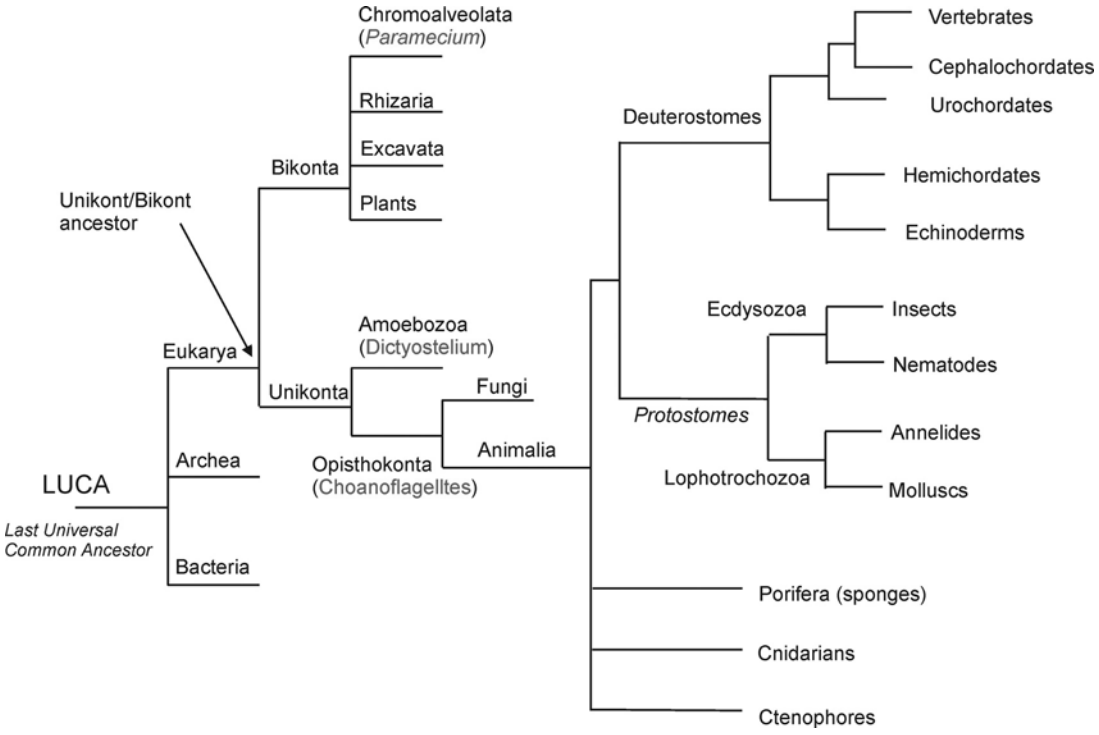

Fig. 1. The tree of life.

Table 1
Selection of genes manually annotated in *P. tetraurelia* by our laboratory (for details, see text and refs. [9,25]) and other groups, as cited, and in *T. thermophila*. The table is founded on coding DNA sequences and on experimentally verified proteins 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/tetrahymena_thermophila.

Type of protein	References
Ca^{2+}-release channels (CRC): *P. tetraurelia*	
inositol 1,4,5 trisphosphate receptor ($InsP_3R$)	[169]
ryanodine receptor-like proteins (RyR-LP)	[102]
Ca^{2+}-ATPases/pumps: *P. tetraurelia*	
plasmamembrane Ca^{2+}-ATPase, PMCA	[223]
sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPAse (SERCA)	[113,114]
Ca^{2+}-bindings proteins: *P. tetraurelia*	
calmodulin and calmodulin-LP	[224,225]
centrin	[186]
copines	[119,120]
SNAREs and SNARE-binding proteins: *P. tetraurelia*	
Synaptobrevin	[28,29]
vesicle-associated membrane protein-associated protein(VAMP-AP)	[28,60]
syntaxin	[30]
Sec1/Munc18 genes (syntaxin-binding proteins); DNA sequence only	[32]
α-SNAP; DNA sequence only	[60]
N-ethylmaleimide-sensitive factor, NSF (SNARE chaperone)	[58]
GTPases	
All Rab types: *T. thermophila*	[27]
Rab 7	[226]
GAPs, GEFs, GIF: *P. tetraurelia* (identification only)	[79]
Vesicle budding	
Clathrin and adaptor proteins: *T. thermophila*	[156]
Adaptor proteins (Aps): *P. tetraurelia* (identification only)	[156]
Dynamin	[156]
H^+-ATPase (V-ATPase)	
A-, C-, D-, E-subunit: *P. tetraurelia*	[31,78,227]
B-subunit: *P.multimicronucleatum*	[228]
A-, B-, C-, D-, E-, F-, H-subunit: *P. tetraurelia*	[31,78,227]
Protein kinases and phosphatases: *P. tetraurelia*	
cGMP-dependent protein kinase	[229]
cAMP-dependent protein kinase	[230,231]
casein kinases	[232–234]
CDPK (Ca^{2+}-dependent protein kinase)	[39]
calcineurin	[149]
Varia*: different species*	
actin isoforms and actin-related proteins (arp): *P. tetraurelia*	[158,159,235]
protein disulfide isomerase: *P. tetraurelia* (gene sequencing only)	[79]
phospholipase C: *T. thermophila*	[166,167]
phospholipase C: *P. tetraurelia*	[167,168]

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 overviewed 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 mucocysts of *Tetrahymena* follow a sortilin-based sorting pathway [10], similar mechanism being in operation in the peripheral and central nervous system of mammals [35].

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, microtubles originate from the cell periphery [25]. Conversely, in neurones 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 [26,38]. (ii) Only neurotransmitter vesicles undergo recycling. (iii) Neither actin nor any other cortical filaments interfere with trichocyst docking, in contrast to neuronal/neuroendocrine cells. (iv) Ciliates do not express true “CaM-kinase” (activated by a complex of Ca^{2+} and calmodulin [CaM]); instead they possess “Ca^{2+}-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].

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 Ca^{2+} 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), (nor)adrenalin is bound, together with ATP, Ca^{2+} and chromogranin in a ternary storage complex [7,42], in which Ca^{2+} serves as a stabiliser – in contrast to trichocyst contents where Ca^{2+} 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), Ca^{2+} entry spots must be closely co-localised with target structures, such as exocytosis sites, to achieve maximal efficiency of signal transfer [43]. The trichocysts as well as the neurotransmitter vesicles aim at discrete locations of the cell membrane. In *Paramecium*, trichocyst exocytosis sites are located in regular, epigenetically determined intervals between ciliary basal bodies [13,26]; Figs. 4 and 5A). Local Ca^{2+} influx sites for regulating exocytosis and ciliary activity are clearly separated. Voltage-gated Ca^{2+}-channels are restricted to ciliary membranes [44] (Fig. 4) where their activation causes a reversal of the direction of ciliary beat [45]. Considering the sequence homology between mammalian and ciliate voltage-gated Ca^{2+}-channels [46,47] this exemplifies re-functionalisation by re-localisation during evolution. In contrast, Ca^{2+} signalling for trichocyst exocytosis takes place independently from these channels [11,13,26,48], as described in more detail in Section 2.3. In *Paramecium*, enhancement of the Ca^{2+} signal is achieved by simultaneous activation of the RyR-type CRCs localised in the membranes of the cortical Ca^{2+}-stores, which flank trichocyst docking sites and influx from the outside medium (Fig. 4).

How the arrangement and function of Ca^{2+}-influx channels in neuroendocrine cells and in neurones are compared? In the adrenal medulla, voltage-gated Ca^{2+}-channels are randomly scattered with no relation to chromaffin vesicle docking sites [49] (5B). In neurones such sites are concentrated at pre-synaptic terminals (Fig. 6). These are endowed with voltage-gated Ca^{2+}-channels [50,51] strictly arranged in rows, being coordinated with docking sites for transmitter vesicles [52].

To achieve a fast exocytotic response in presynaptic nerve terminals,

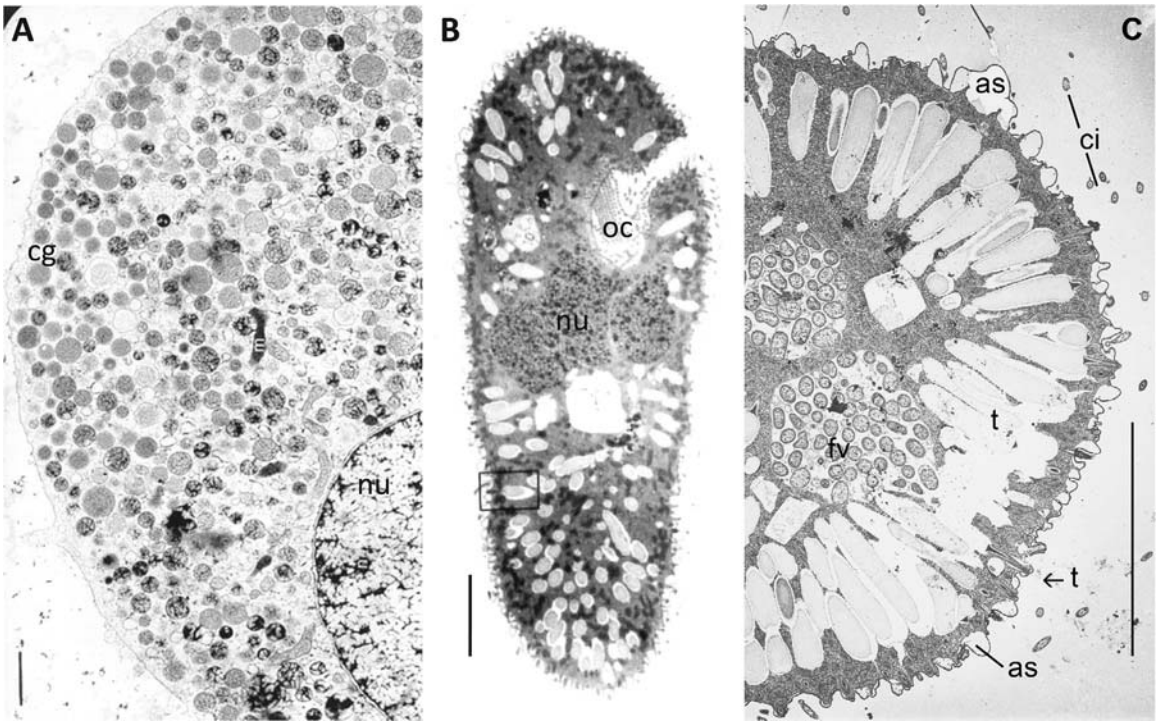

Fig. 2. Cortex (A) of a chromaffin cell from the bovine adrenal medulla and (B, C) of *Paramecium* cells in survey and in detail view. The cell in (A) is densely filled with chromaffin granules (cg) of which only very few approach the cell membrane; nu = nucleus. In (B, C) numerous trichocysts (t) are enriched in the cell cortex where, in appropriate section planes, they are seen attached to the cell membrane (square in B, arrow in C); as = alveolar sacs, ci = cilia, fv = food vacuole, nu = nucleus, oc = oral cavity. In (C) trichocysts present themselves as spindle-shaped dense core-secretory organelles, with a narrow tip and a longer and broader "body" part. On the cell surface, trichocysts alternate with cilia, whereas the rest of the cell membrane is underpinned with alveolar sacs (as). (A) from Plattner et al. [86]. (B and C) Unpublished micrographs. Scale bars: 1 µm (A), 10 µm (B), 5 µm (C).

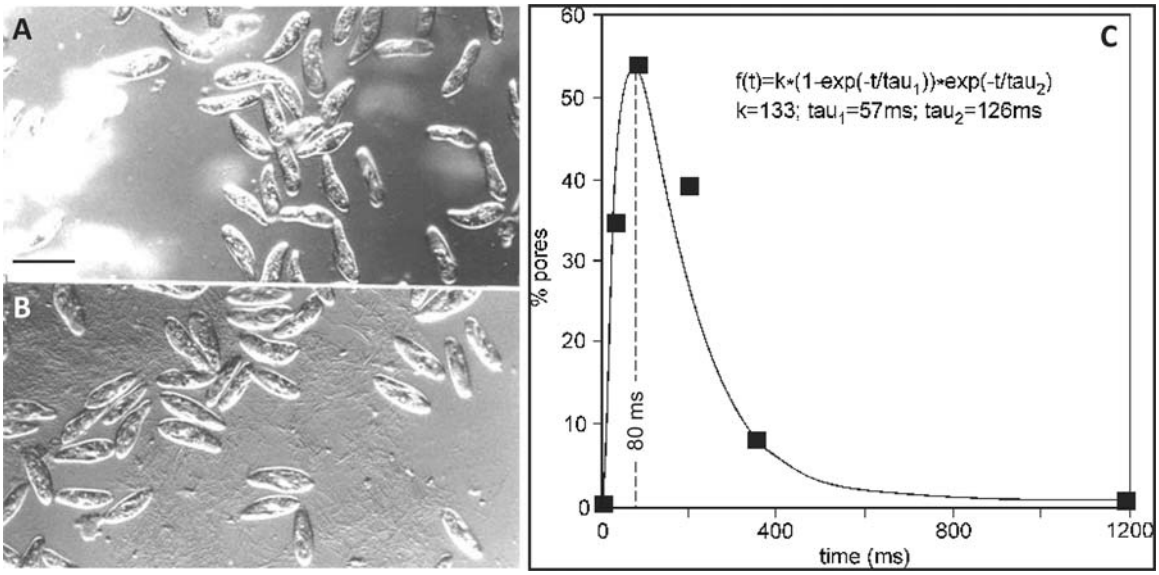

Fig. 3. *Paramecium* cells (A) before) and (B) immediately after stimulation of trichocyst exocytosis. Trichocysts expand to several times their original length in situ, when they come into contact with extracellular Ca^{2+} through the exocytotic opening, once formed. (C) Time course of synchronous trichocyst exocytosis and exocytosis-coupled endocytosis, both within < 0.5 s. Also note calculated apparent time constants for the two processes. (A, B) are from Plattner et al. [236], (C) is from Plattner et al. [237]. Bar in (A) is 100 µm.

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.

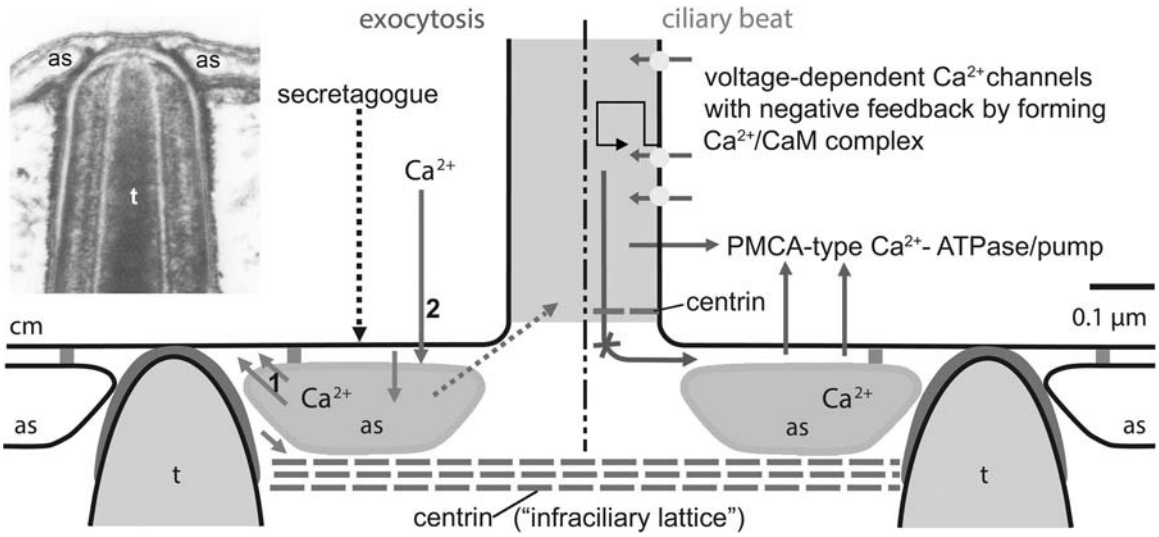

Fig. 4. Relationship of cilia, docked trichocysts (t) and alveolar sacs (as) in the *Paramecium* cortex. The figure schematises the alternating position of the organelles and underpinning of the cell cortex by the high capacity/low affinity CaBP, centrin, which also occurs in association with basal bodies. Note that, during exocytosis stimulation, the first step of Ca^{2+} dynamics is release from alveolar sacs (1), densely followed by Ca^{2+}-influx (2). This SOCE-type mechanism of Ca^{2+} dynamics causes a Ca^{2+} spillover over docking sites, thus triggering exocytosis. Note that exocytosis does not include Ca^{2+}-influx via voltage-dependent Ca^{2+}-channels, as these are restricted to ciliary membranes (right, yellow dots with red arrows for Ca^{2+} flux). Note, however, that some spillover of Ca^{2+} from the exocytotic system into cilia takes place (dotted blue arrow), thus causing ciliary beat reversal. Also note that, as side-effect of ciliary reversal, Ca^{2+}-influx into cilia is abolished by formation of a Ca^{2+}/calmodulin complex – a process also occurring in neurons. *Insert*: Electron micrograph of a trichocyst docked at the cell membrane, with nearby alveolar sacs (as). Also seen are punctual connections between alveolar sacs and the cell membrane as well as occurrence of connecting material between trichocyst tip membrane and cell membrane, as well as a lateral "collar" of diffuse material around the trichocyst tip. The insert is from Plattner [238]. Scale bar: ~0.1 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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 *Pt*Syb5 [29]. Of note, due to difficult 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 SNARE-chaperone N-ethylmaleimide sensitive factor, NSF, is required for the assembly of trichocyst release sites [58,59]. This is remarkable insofar as NSF is generally considered as a chaperone serving for disentangling SNARE complexes after membrane fusion has occurred [55,56]. 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

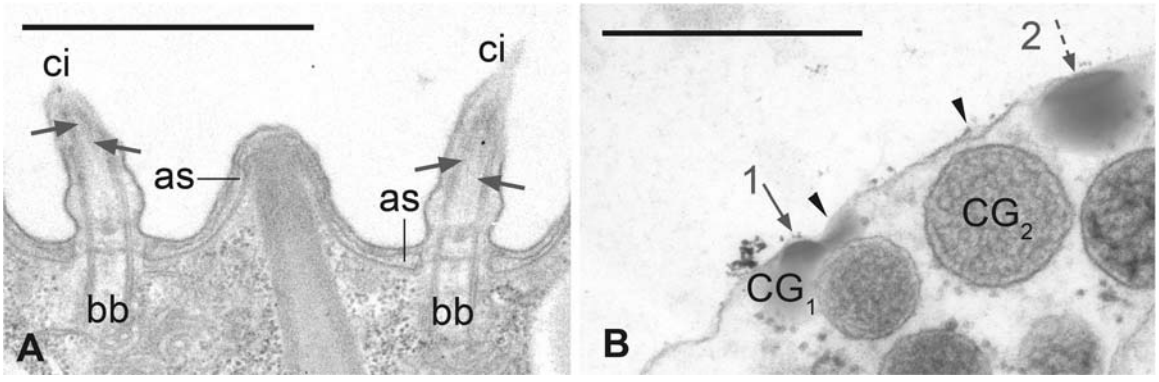

Fig. 5. Surface of as *Paramecium* (A) and of a chromaffin cell (B) isolated from the bovine adrenal medulla. In (A) Ca^{2+}-influx via voltage-dependent channels occurs only in cilia (arrows). as = alveolar sacs, bb = basal bodies of cilia (ci). (B) Two chromaffin granules (CG_1, CG_2) are docked at the cell membrane at two sites (arrowheads). Red arrows 1 and 2 indicate theoretical situations where a voltage-dependent Ca^{2+}-channel would be within sufficiently small distance to trigger exocytosis of a granule (CG_1), while CG_2 would be docked too far off a voltage-dependent Ca^{2+}-channel, thus having low chance to be released due to $[Ca^{2+}]_i$ dilution, as indicated in red. This scheme is based on electrophysiological measurements by Becherer et al. [49]. For details, see text. Electron micrographs: (A) unpublished, (B) from Plattner et al. [86]; scale bars 0.5 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

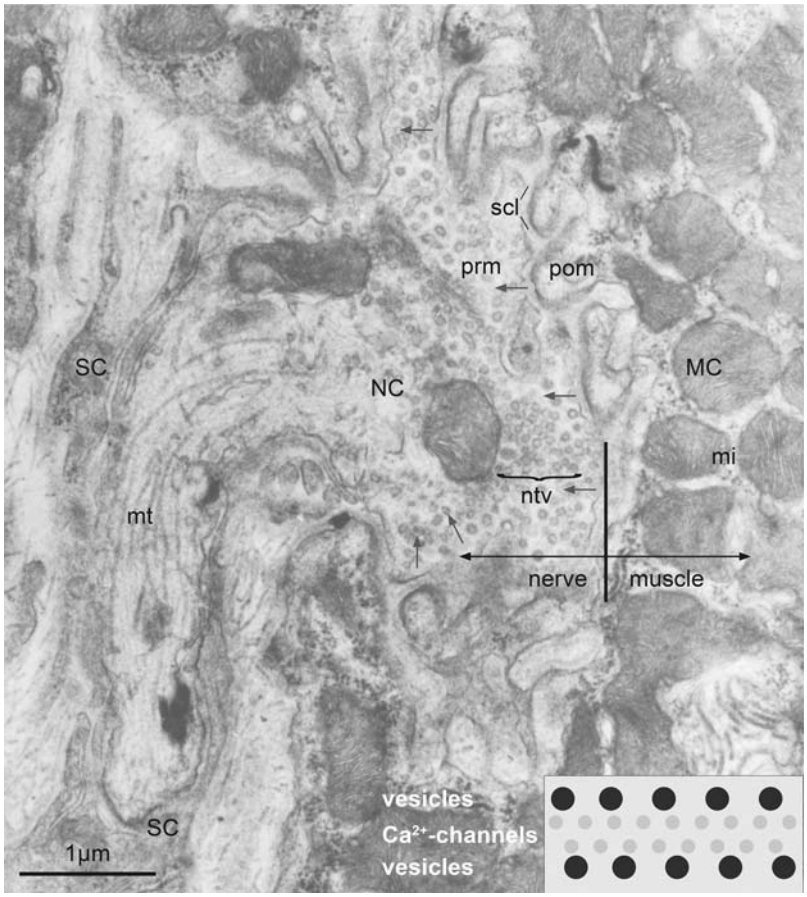

Fig. 6. Neuromuscular junction of the mouse diaphragm. Note a nerve cell (NC) and a muscle cell (MC), with the synaptic cleft (scl) between a presynaptic membrane (prm) and a postsynaptic membrane (pom) with its characteristic folds. Orange dots symbolise voltage-dependent Ca^{2+}-channels in the presynaptic membrane, together with inward Ca^{2+} flux (red arrows). mi = mitochondria, mt = microtubules, ntv = region enriched with neurotransmitter vesicles, SC = Schwann cell. From Plattner [239]; scale bar = 1 µm. *Insert*: arrangement of transmitter vesicles (blue) relative to voltage-dependent Ca^{2+}-influx channels (orange dots) along an "active zone" according to freeze-fracture analyses (see Propst and Ko [52]) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

metazoan precursors it reached ~30 [62]. This emphasises the old heritage of the docking and fusion machinery and its early expansion in ciliates, most likely to provide for their elaborate trafficking system.

For transmitter release in mammalian nervous system, an adequate assembly of Q- and R-SNAREs is required to form mature exocytosis sites [55,56]. SNAREs and auxiliary protein SNAP-25, together with the GTPase Rab3A and H^+-ATPase, are contained in/on vesicle membranes [63]. Another synaptic component, complexin, acts primarily as a break after SNARE assembly and is required for maintaining the exocytosis machinery ready for fusion [64]. This inhibitory function is due to interaction with the Ca^{2+}-signal transducing fusogenic protein synaptotagmin [65]. Complexin has not yet been identified in ciliates, although it was found and functionally scrutinised in choanoflagellates, the protozoan taxon closest to metazoans [66]. This suggests the presence of SNAREs and of an equivalent of synaptotagmin, although the latter has not yet been experimentally verified. Munc-type proteins are additional auxiliary proteins which are found in nerve terminals as well as in choanoflagellates [67] and in *P. tetraurelia* [32], (Table 1).

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* $\geq$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 αP^{32}-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 complex, is inserted into membranes, with the catalytic V1-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 [63] and adrenal medullary chromaffin 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 protozoans.

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 *Paramecium* [79], the assembly of exocytosis systems evidently requires a very similar set of proteins in unicellular and mammalian systems. Flooding of exocytosis sites with Ca^{2+} upon stimulation, however, is achieved in rather different ways, as discussed below.

2.3. Ca^{2+} signals in synaptic terminals and in neurosecretory cells vs. ciliates

In synaptic terminals in the peripheral and in the central nervous

system, local Ca^{2+} 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 synapsin by a Ca^{2+}/CaM activated CaM kinase [82]. These multiple roles of Ca^{2+} have been documented in many neuronal systems [83].

In axonal terminals Ca^{2+} is provided, upon depolarisation, by influx through voltage-gated Ca^{2+}-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 Ca^{2+}-channels, which, however, are not coordinated with exocytosis sites [49]. Here, $[Ca^{2+}]_i$ recorded after caged Ca^{2+} photolysis, after a rapid increase, decays with a $\tau = 20$ s [84] while 98–99 % of the Ca^{2+} entering cells upon stimulation is quickly bound to an endogenous buffer [85]. About 450 dense core "chromaffin granules" (vesicles) are attached to the cell membrane [86]. However, only ~250 vesicles can be immediately released upon depolarization [87]. This implies either that vesicle docking sites are not yet all fully assembled ("matured"), or that they are too remote from randomly scattered voltage-gated Ca^{2+} channels. Both phenomena have been experimentally verified [49]. It seems [49] that a Ca^{2+} channel may not be more distant than ~300 nm from a docked vesicle, i.e. almost one average vesicle diameter (~350 nm), as shown in Fig. 5B. The same study also revealed that increasing extracellular $[Ca^{2+}]$ primes exocytosis sites for release competence, just as in *Paramecium* [11–13].

In summary, only nerve terminals, but not neuroendocrine cells, have a strict coordination of Ca^{2+} signals to exocytosis sites; as discussed below, ciliates have evolved a microanatomical solution to the same problem by side-directed flushing with Ca^{2+} within the subplasmalemmal space.

Altogether the cytosolic Ca^{2+} dynamics is much slower in chromaffin cells than in presynaptic terminals, and the Ca^{2+}-sensing synaptotagmins are also different [88]. In chromaffin cells increase in $[Ca^{2+}]_i$ to 100 µM results in an exocytotic burst [89], whereas 10–25 µM rise in $[Ca^{2+}]_i$ is required for transmitter release at presynaptic active zones [90].

In *Paramecium*, subplasmalemmal $[Ca^{2+}]_i$ increase has been recorded not only by fluorochromes [91,92] but also by whole cell-patch electrophysiology by monitoring activation of different Ca^{2+}/CaM-dependent cation channels [93]. Gene sequences of voltage-gated Ca^{2+}-channels have been detected in choanoflagellates [94], where they may rather function in the flagellum, similarly to the green flagellate, *Chlamydomonas* [95]. Generally, $[Ca^{2+}]_i$ transients are very much determined by immobile Ca^{2+} buffers (CaBPs), not only in mammalian neuronal cells [96,97] but also in *Paramecium* cells [98].

In contrast to chromaffin granules, the major portion of the *Paramecium* trichocysts can be immediately released upon stimulation [11,91,99]. For trichocyst exocytosis, intracellular Ca^{2+}-concentration has to rise locally and transiently at exocytosis sites [91,99]. At the same time, injection of Ca^{2+} does not drive trichocyst exocytosis highlighting the importance of the local source [91]. According to stimulation experiments after injection of mobile Ca^{2+} buffers, local values of $[Ca^{2+}]_i$ ~5–7 µM are required for exocytosis. This is even lower than the range determined for neuroendocrine and neuronal systems [89,90]. Only extended synaptotagmins (eSyntag) are known from *Paramecium* (R. Kissmehl and H. Plattner, unpublished observations), whereas these are more rare than regular types, i.e. those with only two C2-domains, in mammalian cells [88,100].

Trichocyst exocytosis is triggered primarily by Ca^{2+} release from cortical Ca^{2+} stores, the alveolar sacs; it is consecutively augmented, without any obvious delay, by Ca^{2+}-influx [101]. Functional connection between the intracellular stores and plasmalemmal channels remains unknown. However, RyR agonists, caffeine and 4-chloro-*m*-cresol, stimulate trichocyst exocytosis, as they easily permeate the cell membrane and, thus, can activate RyR-like proteins (RyR-LPs) in the outer region of the alveolar sacs membrane facing the cell membrane (Fig. 4). The role for RyR-LPs has been corroborated by significant reduction of Ca^{2+} signals and exocytosis after RyR-LPs post-transcriptional silencing [12,102]. *Paramecium* RyR-LPs are fully operative as Ca^{2+}-release channels, although they lack a large part of the amino-terminal region which, in mammals, exerts a regulatory function on channel activity [103]. Probably caffeine can activate *Paramecium*'s RyR-LPs because binding sites are in a domain adjacent to transmembrane 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 Ca^{2+} release from cortical stores (alveolar sacs) and a store-operated Ca^{2+}-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 Ca^{2+}-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 lower eukaryotes may be endowed with these SOCE-mediating proteins [110]. Pilot data mining in the *Paramecium*DB (*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 Sr^{2+} for Ca^{2+} during quenched flow revealed release of Ca^{2+} from alveolar sacs, superimposed by influx of Sr^{2+} [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 Ca^{2+}-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 Ca^{2+}-ATPase (SERCA-) type Ca^{2+}-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 Ca^{2+} dynamics and signalling in *P. tetraurelia* has been published previously [11,92].

In *Paramecium*, energy-dispersive x-ray microanalysis at electron microscope resolution shows that, upon depolarisation which activates ciliary reversal, no spill-over of Ca^{2+} beyond the ciliary basis occurs [48]. This is in part due to Ca^{2+} binding to basal body-associated CaBPs, such as centrin, as documented by centrin knock out [98]. Of note, in the central nervous system neurones, centrin indirectly contributes to the Ca^{2+} signalling through Ca^{2+}-dependent binding to the Eag1-type K^+-channel which is inactivated in consequence [115]. Another mechanism is the inactivation of voltage-gated Ca^{2+}-channels by Ca^{2+}/CaM complex [116] – an effect documented also for neurones [117,118]. Mobile and immobile Ca^{2+} 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 centrin represents an immobile buffer [10,92,98]. A family of Ca^{2+}-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 Ca^{2+} entry through voltage-gated and other Ca^{2+} transporters [121,122]. In addition to the plasma membrane Ca^{2+}-ATPase (PMCA), Ca^{2+}-exchangers contribute to Ca^{2+} extrusion [123]. Gene sequences encoding voltage-gated Ca^{2+}

channels and a potential Na^+/Ca^{2+} exchanger protein have been recognized by genome data mining in *P. tetraurelia* and *T. thermophila*, respectively [47,124] and in the genome of choanoflagellates [94]. Particularly well characterized at the electrophysiological level are Na^+- and K^+-channels from *Paramecium* [125–127].

When neuronal and ciliated protozoan systems are compared, the common 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 plasmalemmal Ca^{2+} channels in strict (axonal terminals) or random (neurosecretory 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 (ciliates).

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

Many proteins and protein-based mechanisms are shared between protozoa, notably ciliates, 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 synaptic plasticity. In some spines, e.g. of pyramidal cells of the hippocampus and in Purkinje cells of the cerebellum, the smooth endoplasmic reticulum forms a special structure, the “spine apparatus” [128,129] where the endoplasmic reticulum is folded into interconnected 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 ciliates, like in metazoans, the plasma membrane Ca^{2+}-ATPase, 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 which are also found in the *Paramecium* are highlighted in 7A . In the spine plasma membrane excitatory neurotransmitters (glutamate, ATP, acetylcholine) activate ionotropic and metabotropic GluRs, which shape cytoplasmic Ca^{2+} signals. Activation of some AMPA and mainly NMDA receptors as well as opening of voltage-gated Ca^{2+} channels provides for plasmalemmal Ca^{2+} influx, whereas activation of G-protein coupled receptors (GPCR), via phospholipase C (PLC) activation, forms $InsP_3$ and, thus, activates $InsP_3$R-type CRCs in the endoplasmic reticulum or in its derivative, the spine apparatus [130]. This results in Ca^{2+} release into the spine cytosol, which can be further amplified by RyRs [131]. The resulting Ca^{2+} signals regulate vesicle trafficking and SNARE/GTPase dependent exocytosis that contributes to ongoing molecular and structural turnover in the spine. To outline similarities with protozoans, apart from voltage-gated Ca^{2+}-channels, occurrence of GluRs, PLC, $InsP_3$Rs, SERCA- and PMCA-types Ca^{2+}-pumps, and GPCRs are important features [130]. In contrast, Fig. 7B shows examples of salient dissimilarities.

Glutamate acts as an extracellular signalling molecule in protozoa. An ionotropic GluR is present already in bacteria [132]. In *Paramecium*, exogenous glutamate mediates a chemotactic response via cAMP (cyclic adenosine monophosphate) formation and protein kinase A (PKA) activation by instigating ciliary beat within 30 ms, i.e. within one stroke [133]. The nature of underlying receptor remains, however, unknown. In the *Paramecium* genomic database partial sequences of a *N*-methyl D-aspartate receptor (NMDAR) have been identified [134], for which further analysis is required. In ciliates, activation of a putative GluR aims at cilia, i.e. targets different from neurones, although binding constants are similar [135]. At this time, subtyping as ionotropic and metabotropic GluRs is not possible. Endoplasmic reticulum contributes to modulating Ca^{2+} signals in spines. The ER in spines contains SERCA pumps and $InsP_3$Rs [128]; the existence of RyRs in the spine has long been controversial, but more recently their occurrence and activation in spines appears established [131]. Spines also contain various CaBPs [136]. By immuno-gold cytochemistry the presence of calsyntenin has been demonstrated in the spine apparatus (N. Kasielke and H. Plattner, unpublished results; materials provided by P. Sonderegger, University of Zurich). Calsyntenin is a transmembrane CaBP with a highly acidic cytoplasmic domain which can act as a high capacity/low affinity CaBP [137] for rapid removal of Ca^{2+} after activation. This can be considered a functional equivalent of centrin, a non-membrane-bound protein serving for a comparable purpose in the cortex of *Paramecium* [11,13,98].

The Ca^{2+} signal is rapidly reduced within the small spine volume – an important aspect, considering the toxic effect of Ca^{2+} [16]. Formation of spines as well as their modulation during long-term potentiation 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 ciliates, 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 neurons. A CaM kinase *sensu stricto* occurs in the monokont, *Dictyostelium* [41].

Maintenance of synaptic plasticity requires vesicle delivery and retrieval 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 [144] and in ciliates. This also holds for a reggie/flotillin/stomatin-type microdomain-scaffolding protein which also occurs already in ciliates [145].

Synaptic plasticity and memory also depend on protein phosphatase 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 dephosphorylation by CaN [147,148] which also contributes to synaptic plasticity [146]. The hetero-dimeric CaN molecule is present in protozoa, 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, $\gamma-$aminobutyric acid (GABA), is present in pyramidal and cerebellar Purkinje cell spines [131,153] and also in many other inhibitory synapses [154]. Surprisingly, according to proteomics analysis, *Dictyostelium* encodes a GABA receptor [155], its function being not known at this time.

Several proteins contributing to vesicle budding in neurones and ciliates show remarkable similarity. In addition to experimentally verified 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 adaptor proteins (AP1, AP2, AP3) and coatomer (COP) subunits [157]. Furthermore, a collection of actin isoforms is part of the proteome inventory of ciliates [158]. Based on experimental work with *P. tetraurelia*, these actins exert a variety of functions, including phagocytosis regulation [158,159], although effects 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 ciliates does not necessarily imply their functional activity [163]. However, trimeric G-proteins and GPCRs with defined signal transfer

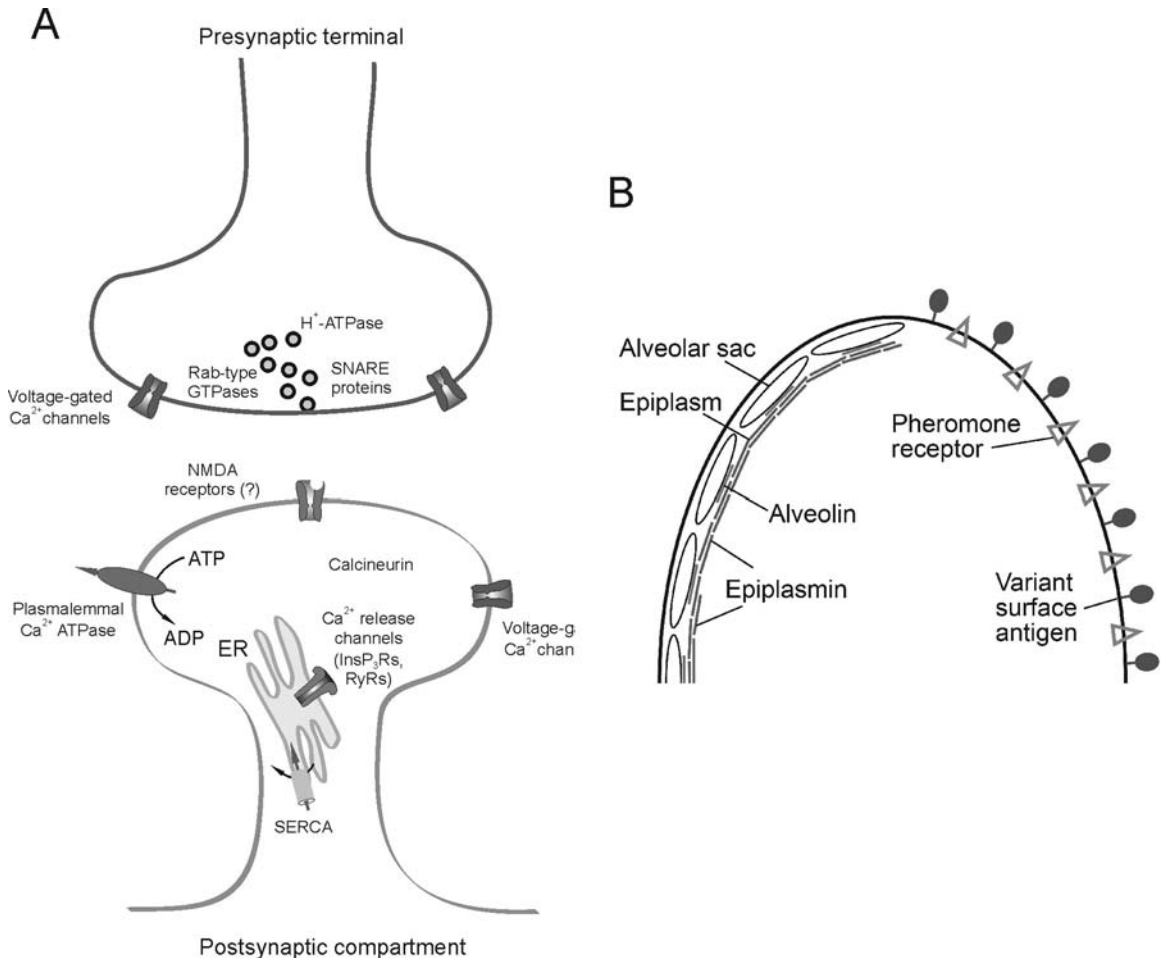

Fig. 7. Conservation of major signalling pathways from protozoa to mammalian nervous system.
(A) Typical synapse comprising presynaptic terminal and spine-associated potsynaptic compartment. Both presynaptic terminal and spine contain a collection of essential proteins existing in protozoa. This includes SNAREs, GTPases, H^+-ATPase/pump, voltage-gated Ca^{2+}-channels, the plasmalemmal Ca^{2+}-ATPase/pump (PMCA), GPCRs, phospholipase C (PLC), $InsP_3R$- and RyR-LP-type Ca^{2+}-release channels, SERCA-type Ca^{2+}-ATPase/pump and GluRs (for details, see text). The scheme is inspired by Rochefort and Konnerth [130] with some modifications. Regulation of neuronal contacts is controlled by numerous pathways that evolved in multicellular organisms; these pathways involve complex intercellular communications. Current discussions about the dynamics of spines in the context of engram formation assumes a much higher number of spines per neurone than previously thought, the number of spines changes in paralleled to changes in transcription of specific proteins, de-/phosphorylation processes and complemented by epigenetic changes [244].
(B) A scheme of a *Paramecium* cell showing examples of proteins not occurring in neurones. This includes proteins of the epiplasm, such as epiplasmin and alveolin associated with the inner membrane domain of cortical Ca^{2+}-stores (alveolar sacs), glycosyl phophatidylinositol-anchored variant surface antigens as well as pheromone/gamone receptors serving in some ciliates, such as *Euplotes* (but not in *Paramecium*) for sexual cell pairing. The molecules are further specified in Refs. [13,18]; specifically for epiplasmin see ref. [240], for alveolin [241], for variant surface antigens [242], and for pheromone receptors see ref. [243]. Note that (A) and (B) are drawn at different scale, a spine being ~1 μm and a *Paramecium* cell ~100 μm long and ~40 μm in diameter. For more details see Table 1.

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 $InsP_3Rs$ [169], although this subpopulation of $InsP_3Rs$ has not been analysed in any detail. $InsP_3Rs$ are wide-spread in protozoa, as discussed previously [13]. According to genomic and proteomic data mining, $InsP_3Rs$ 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 filastereans [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/ultra-fast 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 $\tau_{exocytosis}$ = 57 ms and $\tau_{endocytosis}$ = 126 ms, respectively, under physiological extracellular Ca^{2+} concentration, $[Ca^{2+}]_e$ (Fig. 3B). A rise in $[Ca^{2+}]_e$ 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

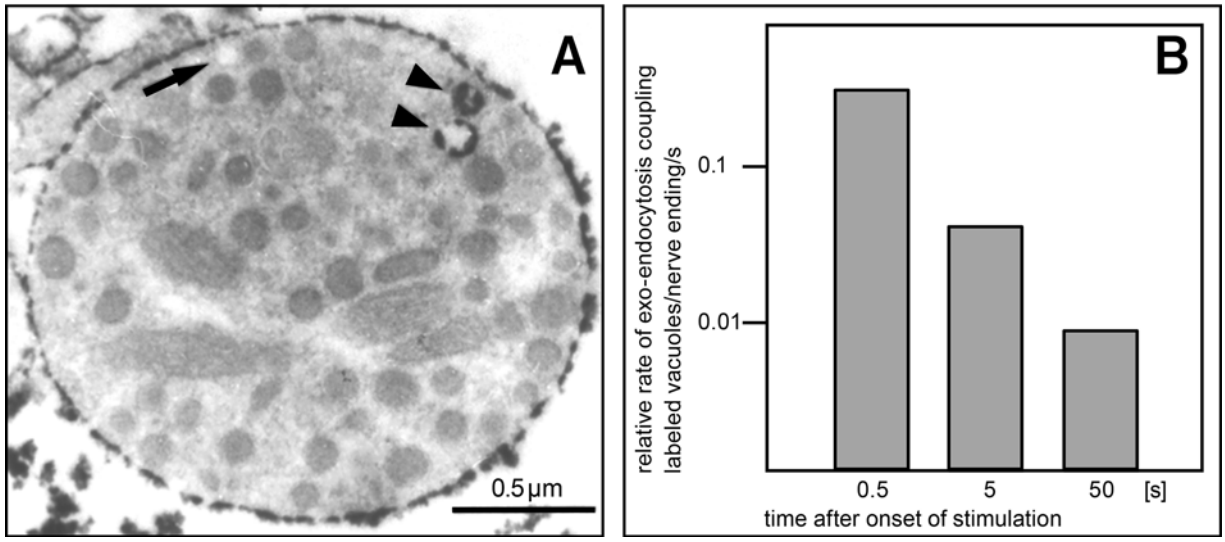

Fig. 8. Rapid exocytosis-endocytosis coupling in a nerve terminal of a peptidergic cell isolated from the posterior pituitary gland of rat. Samples were subjected to chemical depolarisation in presence of the electron microscope marker horseradish peroxidase and rapidly frozen in different time intervals. The peroxidase visualised as electron dense reaction product occurs at the cell surface and in endosomes formed during stimulation (arrowheads), but not in vacuoles formed before (arrow). Highest rate of exocytosis-endocytosis coupling is recognised already within 1 s, with additional events following over longer times. From Knoll et al. [175]; scale bar = 0.5 µm.

in melanotrophic pituitary cells using patch-clamp analysis [176]. Retrieval of chromaffin vesicle "ghosts" proceeds with $\tau = 62$ ms [89], and coupling in different neuronal systems depends on $[Ca^{2+}]_e$, with a similar concentration-dependence and time range [83]. In neurones, exocytosis-endocytosis coupling is enforced by Ca^{2+}/CaN [177]. Though CaN is present in *Paramecium* at distinct sites of constitutive exo-/endocytosis, i.e. "parasomal sacs" [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 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.

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 absence of effects expected from mammalian cells [181], this receptor appears as a "molecular fossil" without precedence. The opposite example is the sensitivity of different cation channels in *Paramecium* which are sensitive to Ca^{2+}/CaM [126,182]: just like in neurones, they are sensitive to anti-CaM drugs, [93,182]. It would be fascinating to see whether binding sites for the Ca^{2+}/calmodulin complex and for the inhibitory drugs are the same in so widely distant organismal groups, protists and mammalians.

An aspect to be further scrutinised in *Paramecium* is the hypothetic occurrence of a Ca^{2+}/polyvalent cation-sensing receptor. This is concluded from the effects of extracellular polyamines, which trigger Ca^{2+} signals and exocytosis [13,26]). Cloning would be required to characterise not only a *bona fide* Ca^{2+}/polyvalent cation-sensing receptor, but also any coupled trimeric G-proteins in ciliates. Such a receptor resembles metabotropic GluRs in neurones [183], or it is assumed to be functionally linked to GluRs in nerve terminals [184,185].

Another matter to be analysed is the importance of the predominant immobile Ca^{2+} buffer, centrin, in the outermost layer of the *Paramecium* 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 $[Ca^{2+}]_i$ recovery upon exocytosis stimulation by about tenfold [98]. As mentioned, calsyntenin 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 are 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 [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 ~1400 Myr has been suggested [193]. A limit of ~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 longin-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 Ca^{2+}-signalling [15]. Some proteins may be kept for identical functions, other changed localisation and/or function [18], a typical example being voltage-gated Ca^{2+}-channels. Some functions, like Ca^{2+} buffering, have been taken over by different CaBPs and the arrangement of Ca^{2+} stores differs, as do some of the specific plasmalemmal channels. Involvement of CaM in channel activity, of the Ca^{2+}-ATPase/pump, PMCA, and of some plasmalemmal 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 Ca^{2+} 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 protozoa?

Such proteins include, for example ion channels and receptors, which already occur in protozoa [127] including ciliates [125]. Voltage-gated cation 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 ~10^{15} 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 Platyhelminthes (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 Ca^{2+}-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 Ca^{2+} 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 splice forms go in parallel with increasingly elaborate cell-to-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.

Acknowledgements

The work of H. P. referred to herein has been supported by the German Research Council (Deutsche Forschungsgemeinschaft, DFG). He also wants to thank the many coworkers who contributed over the years, as cited, to the analysis of trafficking and signalling in *Paramecium* cells. H. P. also thanks Prof. Claudia Stuermer, Chair of Developmental Neurobiology, University of Konstanz, for valuable discussions on different aspects of neurobiology addressed here; any errors, however, are the author's own responsibility.

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