Secretory vesicle protein homologues in choanoflagellates

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

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Faculty of Mathematics and Natural Sciences

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List of abbreviations

ATP	Adenosine triphosphate
A. queenslandica	*Amphimedon queenslandica*
B. dendrobatidis	*Batrachochytrium dendrobatidis*
BLAST	Basic local alignment search tool
bp	Base pairs
Ca^{2+}	Calcium ions
Cas9-RFP	CRISPR associated protein 9-Red fluorescent protein
cDNA	Complementary Deoxyribonucleic acid
co-IP	Co-immunoprecipitation
COPI/II	Coatomer protein complex I/II
C. owczarzaki	*Capsaspora owczarzaki*
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CSP	Co-chaperone cysteine string protein
D. melanogaster	*Drosophila melanogaster*
Doc2	Double C2 domain protein
D. rerio	*Danio rerio*
E. coli	*Escherichia coli*
ELKS	Protein rich in the amino acids E, L, K, and S
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
GRAVY	Grand average of hydropathicity
H+-ATPase	Hydrogen-exporting adenosine triphosphatase
h	Hour(s)
IP	Immunoprecipitation
IPT/TIG	Immunoglobulin, plexins, transcription factors-like/transcription factor Immunoglobulin
kDa	Kilodalton
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
MAL2	Myelin and lymphocyte protein 2
M. brevicollis	*Monsiga brevicollis*
MCTP1	Multiple C2 and transmembrane domain-containing protein 1
min	Minute(s)
M. leidyi	*Mnemiopsis leidyi*
Munc18	Mammalian uncoordinated-18
NCBI	National Center for Biotechnology Information
NSF	*N*-ethylmaleimide-sensitive fusion protein
N. vectensis	*Nematostella vectensis*
OD	Optical density
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
pI	Isoelectric point
PTSG	Proterospongia sp. ATCC 50818
Rab	Rat sarcoma virus-associated-binding proteins
rBoNT/D-LC	Recombinant botulinum Neurotoxin Type D Light Chain
rec	Recombinant
RIM	Rab3-interacting molecule
RIM-BPs	RIM-binding proteins
rpm	Revolutions per minute
$rpl36a^{P56Q}$	Ribosomal protein gene rpl36a with a conversion of the 56^{th} proline to glutamine
RNA	Ribonucleic acid

SCAMP	Secretory carrier-associated membrane protein
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC1	Secretory mutant 1
SM proteins	Sec1/Munc-18
SMP	Synaptotagmin-like, Mitochondrial and lipid-binding Protein
SNAP	Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein
SNAP-25	Synaptosome-associated protein-25 kDa
SNARE(s)	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor(s)
R. oryzae	*Rhizopus oryzae*
S. cerevisiae	*Saccharomyces cerevisiae*
*Sp*Cas9	*Streptococcus pyogenes* CRISPR associated protein 9
S. purpuratus	*Strongylocentrotus purpuratus*
S. rosetta/Sros	*Salpingoeca rosetta*
SV2	Synaptic vesicle protein 2
SVOP	SV2-related protein
Syb	Synaptobrevin
T. adhaerens	*Trichoplax adhaerens*
tracrRNA	Trans-activating CRISPR RNA
t-SNARE	Target-membrane SNARE
v-SNARE	Vesicle-membrane SNARE
VAChT	Vesicular acetylcholine transporter
VAMP	Vesicle-associated membrane protein
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter
VMAT	Vesicular monoamine transporter
Ypt	Yeast protein transport
ZnT3	Zinc transporter 3

Abstract in English

Regulated vesicle exocytosis, as in the case of highly specialised synaptic vesicle secretion, is controlled by a set of evolutionarily conserved proteins. Many of these proteins predate animals. The aim of this thesis was to study the origin and evolution of presynaptic protein complexes that are important for transmitter release. The main model organism used for this purpose was the choanoflagellate species *Salpingoeca rosetta* (*S. rosetta*). Choanoflagellates are not only the closest unicellular relatives of animals, but they also possess numerous synaptic protein homologues. The use of a wide range of techniques, such as homology-based protein searches, 3D vesicle reconstructions, immunostaining, genome editing, subcellular fractionation, immunoprecipitation, and mass spectrometry analysis, allowed direct comparisons with synapses and synaptic proteins. These revealed that choanoflagellates possess diverse, polarised vesicular landscapes and encode many of the investigated core neurosecretory vesicle proteins in their genomes. One of these neurosecretory vesicle core proteins is a homologue of secretory synaptobrevin, which is localised at both vesicle-rich poles of *S. rosetta* according to immunostainings. Similar to synapses, synaptobrevin in *S. rosetta* is most likely located in the membrane of vesicles transported along the cytoskeleton, as suggested by immunostaining and subcellular fractionation results. Co-immunoprecipitation (co-IP) with an antibody against *S. rosetta* synaptobrevin uncovered several potential interaction partners of synaptobrevin in *S. rosetta*, some of which were also found in proteomic studies on synaptic vesicles. Moreover, CRISPR/Cas9-mediated knockouts of synaptobrevin in *S. rosetta* suggest that synaptobrevin, similar to other organisms, is likely an essential protein for choanoflagellates. Like animal synaptobrevin 2, *S. rosetta* synaptobrevin has amino acids at key positions suspected to be important for substrate recognition by botulinum neurotoxin D (BoNT/D). In contrast to animal synaptobrevin 2, however, recombinant *S. rosetta* synaptobrevin is not cleaved by BoNT/D. The observed similarities between the vesicle secretion machinery in choanoflagellates and animal synapses provide evidence for a common origin of regulated vesicle secretion, which may be older than originally thought.

Abstract in Norwegian

Regulert vesikeleksocytose, som i tilfellet med høyt spesialisert synaptisk vesikelsekresjon, styres av et sett evolusjonært konserverte proteiner. Mange av disse proteinene er eldre enn dyrene. Målet med denne avhandlingen var å studere opprinnelsen til og evolusjonen av pre-synaptiske proteinkomplekser som er viktige for transmitterfrigjøring. Hovedmodellorganismen jeg brukte til dette formålet, var choanoflagellaten *Salpingoeca rosetta* (*S. rosetta*). Choanoflagellater er ikke bare de nærmeste encellede slektningene til dyr, men de har også mange synaptiske proteinhomologer. Bruken av et bredt spekter av teknikker, som homologibaserte proteinsøk, 3D-vesikelrekonstruksjoner, immunfarging, genomredigering, subcellulær fraksjonering, immunopresipitering og massespektrometrisk analyse, muliggjorde direkte sammenligninger med synapser og synaptiske proteiner. Disse avslørte at choanoflagellater har mangfoldige, polariserte vesikellandskap og koder for mange av de undersøkte kjerneproteinene i nevrosekretoriske vesikler i genomene sine. Et av disse neurosekretoriske vesikelkjerneproteinene er en homolog til sekretorisk synaptobrevin, som ifølge immunfargingene er lokalisert ved begge vesikelrike poler i *S. rosetta*. I likhet med synapser er synaptobrevin i *S. rosetta* sannsynligvis lokalisert i membranen til vesikler som transporteres langs cytoskjelettet, noe immunfarging og subcellulær fraksjonering tyder på. Co-immunopresipitering (co-IP) med et antistoff mot synaptobrevin i *S. rosetta* avslørte flere potensielle interaksjonspartnere for synaptobrevin i *S. rosetta*, hvorav noen også ble funnet i proteomiske studier av synaptiske vesikler. Dessuten tyder CRISPR/Cas9-mediert knockout av synaptobrevin i *S. rosetta* på at synaptobrevin, i likhet med andre organismer, sannsynligvis er et essensielt protein for choanoflagellater. I likhet med synaptobrevin 2 fra dyr har synaptobrevin fra *S. rosetta* aminosyrer i nøkkelposisjoner som mistenkes å være viktige for substratgjenkjenning av botulinum nevrotoksin D (BoNT/D). I motsetning til dyrenes synaptobrevin 2 spaltes imidlertid ikke rekombinant synaptobrevin fra *S. rosetta* av BoNT/D. De observerte likhetene mellom vesikelsekresjonsmaskineriet hos choanoflagellater og dyresynapser tyder på at den regulerte vesikelsekresjonen har en felles opprinnelse som kan være eldre enn først antatt.

List of Publications

Paper I:

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Paper II:

Göhde, R. and Burkhardt, P.

"Characteristics of secretory synaptobrevin homologues in the closest unicellular relative of animals". (in preparation)

1. Introduction

1.1. Secretory pathway

Eukaryotic cells are separated by the endomembrane system into distinct subcellular compartments. This separation of diverse functional environments (e.g., nucleus, endoplasmic reticulum (ER), Golgi network, lysosome) provides a high degree of cellular specialisation (reviewed in Lee et al., 2004; Søreng et al., 2018). Parts of the endomembrane system were first described in the late 18th century by Camillo Golgi who used silver staining to visualise the Golgi network under a light microscope (Golgi, 1898). Later, after the application of electron microscopy in combination with cell fractionation, the essential interactions between the different cellular compartments in form of membrane trafficking were discovered (Jamieson & Palade, 1967; Palade, 1975).

The secretory pathway is one of the major membrane trafficking pathways. It is responsible for protein biogenesis, modification, sorting, secretion, and lipid transport (Palade, 1975). Studies of isolated secretion mutants in yeast provided profound knowledge for the understanding of the secretory pathway (Novick et al., 1980; Novick & Schekman, 1979), which was only later shown to be highly conserved in eukaryotes (Brennwald et al., 1994; d'Enfert et al., 1992; Griff et al., 1992; Protopopov et al., 1993; Wilson et al., 1989). Nearly all extracellular and membrane-bound proteins in a eukaryotic cell are transported via the secretory pathway to their destination (Palade, 1975). This pathway can be distinguished between constitutive and regulated secretion. Constitutive secretion is believed to continually deliver newly synthesised proteins and lipids to the cell membrane in all cell types, whereas specific secretory cells, such as neurons, store secretory vesicles in pools. Upon a trigger signal, secretory vesicles can be released in a highly regulated manner from these pools (Gumbiner & Kelly, 1982; Tartakoff et al., 1977). Cargos of both pathways are assumed to follow the same route until they are sorted in the *trans*-Golgi network (Griffiths & Simons, 1986; Orci et al., 1987).

The secretory pathway begins at the ER membrane, where ribosomes dock onto protein pores to release nascent polypeptides (Blobel & Dobberstein, 1975; Caro & Palade, 1964). Here proteins are translocated or integrated into the ER to be post-translationally modified, folded, assembled, and transported to the Golgi network. In the Golgi network proteins are further processed while travelling between the different cisternae (*cis*-, *medial*-, *trans*-compartments of the Golgi network) (Baeuerle & Huttner, 1987; Balch et al., 1984a; Dunphy et al., 1985; Griffiths et al., 1983). Only after leaving the *trans*-Golgi network, proteins destined for the plasma membrane, lysosomes or regulated secretion follow different paths (Griffiths & Simons, 1986; Orci et al., 1987).

Directional transport to specific locations, like the plasma membrane, is carried out by motor proteins moving along the cytoskeleton (including microtubules and actin filaments) (Bi et al., 1997; Govindan et al., 1995; Hall & Hedgecock, 1991; Johnston et al., 1991; Kuznetsov et al., 1992; reviewed in Nirschl et al., 2017; Novick & Botstein, 1985). To travel between the different compartments, proteins are packed in transport vesicles (Balch et al., 1984b; Caro & Palade, 1964; Jamieson & Palade, 1967; Orci et al., 1986) (**Figure 1.1**). These vesicles bud off from the donor membrane driven by specific coat proteins that allow for cargo sorting and directionality (Balch et al., 1994; Barlowe, 2003; Cosson & Letourneur, 1994; Davis et al., 1986; Nishimura & Balch, 1997). In the case of ER-derived transport, vesicles are encapsulated with the Coatomer protein complex II (COPII) for the anterograde transfer (Barlowe et al., 1994). In many eukaryotes COPII-coated vesicle budding is regionally restricted to specific ER-exit sites, which have been proposed to facilitate coupling with the retrograde recycling (Aridor et al., 1995; Bannykh et al., 1996; Orci et al., 1991). Between ER and Golgi, the protein homeostasis is maintained by retrieving ER escaped proteins in COPI-coated vesicles from the Golgi network (Letourneur et al., 1994; Lewis & Pelham, 1996). Also within the Golgi network, proteins are assumed to be transported in COPI-coated vesicles (Malhotra et al., 1989; Orci et al., 1989). Vesicles transported between the *trans*-Golgi network and the endosome, lysosome or plasma membrane are coated with clathrin and its adaptor proteins (Ford et al., 2021; Friend & Farquhar, 1967; Pearse, 1975; Roth & Porter, 1964; Tooze & Tooze, 1986) (**Figure 1.1**).

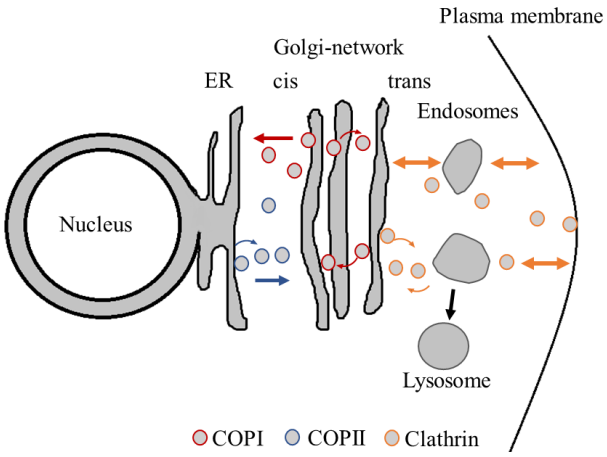


Figure 1.1 Simplified scheme of the secretory pathway and the different transport steps. The different transport steps are indicated by colour-coded arrows and coat proteins (COPI, COPII and Clathrin). Proteins are packed in transport vesicles encapsulated by different coat proteins which drive vesicle budding, mediate cargo sorting and directionality. Transport vesicles which bud off from the ER in direction of the Golgi network are coated with Coatomer protein complex II (COPII, blue). Retrograde transported vesicles between Golgi and ER, as well as vesicles transported between the different cisternae of the Golgi-network, are assumed to be coated with COPI (red). Clathrin and its adaptor proteins (orange) coat vesicles transported between the *trans*-Golgi network, the endosome, lysosome, or plasma membrane. The figure was created according to the scheme described by Bonifacino and Glick (2004) (also reviewed by Ford et al., 2021).

Although there are variations between vesicle trafficking regulated by the different coat proteins, they all follow the same pattern involving cargo sorting, coat assembly, budding, uncoating, recycling of coat proteins, tethering, docking and fusion with the target membrane. Prior to fusion, tethering of vesicles to the target membrane is believed to be the first contact to the acceptor compartment. Together with Rab (Ras-associated-binding) proteins, tethering factors participate in the regulation of membrane docking and fusion with the target membrane (reviewed in Bonifacino & Glick, 2004; Kirchhausen, 2000; Lee et al., 2004).

The importance of Rab proteins in the regulation of the secretory pathway was first discovered in yeast, where these proteins are known as Yeast protein transport (Ypt) GTPases (Peter et al., 1994; Salminen & Novick, 1987; Segev et al., 1988). Ypt and

Rab GTPases are highly conserved from yeast to mammals and participate in the regulation of many different membrane trafficking steps (Haubruck et al., 1989; Li & Warner, 1996; Tisdale et al., 1992). These steps include, for instance, vesicle formation, uncoating, the transport by specific motor proteins, docking and fusion (reviewed in Horgan & McCaffrey, 2011; Stenmark, 2009).

The final step, the fusion between vesicles and their target membrane, is mediated by a set of proteins called soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) (Söllner et al., 1993a). Together with regulatory factors, SNARE proteins catalyse membrane fusion, which leads to the release of cargo from the transport vesicles (described in more detail in chapter 1.2. SNAREs).

1.2. SNAREs

SNARE proteins are integral membrane proteins which initiate fusion between two opposing membranes (Hu et al., 2003). It was demonstrated by *in vitro* studies using cell-free extracts that three cognate SNARE proteins localised on a target (t-SNAREs) and vesicular membrane (v-SNAREs) form a highly stable ternary complex (Söllner et al., 1993b). In neurons, synaptic vesicles fuse with the presynaptic membrane by the formation of an SDS-resistant complex consisting of the v-SNARE, synaptobrevin (also called VAMP, vesicle-associated membrane protein) and the t-SNAREs, syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa) (**Figure 1.2**) (Hayashi et al., 1994; Söllner et al., 1993b). The identification of these three proteins as targets of the clostridial neurotoxins, botulinum and tetanus toxin, provided early evidence for their central role in synaptic vesicle exocytosis (Blasi et al., 1993a; Blasi et al., 1993b; Link et al., 1992; Niemann et al., 1994; Schiavo et al., 1993a; Schiavo et al., 1993b; Schiavo et al., 1992). Together, these three SNARE proteins form a thermally stable coiled coil structure of a four-helix bundle (Fasshauer et al., 1997a; Fasshauer et al., 1998a; Fasshauer et al., 1997b; Hayashi et al., 1994; Sutton et al., 1998). In case of the synaptic fusion complex, the four-helix bundle consists of two alpha-helices

contributed by SNAP-25 and one alpha-helix contributed by synaptobrevin and syntaxin each, which align in parallel to each other (Fasshauer et al., 1998a; Poirier et al., 1998; Sutton et al., 1998). This arrangement of exocytotic SNAREs is highly conserved from yeast to mammals (Katz et al., 1998; Rossi et al., 1997).

A variety of different SNARE proteins have been discovered. So far, 41 members of the SNARE family have been found in *Homo sapiens*, 62 in *Arabidopsis thaliana* and 26 in *Saccharomyces cerevisiae* (Kloepper et al., 2007). These are localised to different subcellular compartments and display enriched expression profiles for specific cells in animals (Aalto et al., 1993; Advani et al., 1998; Antonin et al., 2000; Bennett et al., 1993; Jahn & Scheller, 2006; Nagahama et al., 1996; Søgaard et al., 1994). Even though this diversity indicates a contribution to mediating membrane fusion specificity, the exact role of SNARE proteins in targeting specificity is still unclear (Fasshauer et al., 1999; Söllner et al., 1993b; Yang et al., 1999). Experiments using combinations of different v-SNAREs and t-SNAREs in liposome reconstitution assays suggest the specificity of SNARE pairing. Other studies argue, SNARE proteins alone are not sufficient to ensure specific membrane fusion (Brandhorst et al., 2006; Furukawa & Mima, 2014; McNew et al., 2000). Furthermore, it is difficult to assess the extent to which SNARE proteins exhibit compartment specificity, as some SNAREs are involved in more than a single fusion step with different SNARE partners, whereas other SNAREs may substitute for each other (Antonin et al., 2000; Liu & Barlowe, 2002; Wang et al., 2004). Recent microinjection experiments have shown that during vesicle budding, selectively sorted SNARE proteins and combinations mediate targeting via recruitment of specific tethering factors independent of SNARE pairing. According to these findings, some SNARE proteins are not only important for membrane fusion, but also actively participate in vesicle targeting (Koike & Jahn, 2019).

The characteristic sequence that allows SNARE proteins to interact with each other is called the “SNARE motif”. This is an evolutionarily conserved stretch of 60-70 amino acids shared by all proteins belonging to the SNARE superfamily. It contains a heptad repeat pattern that can be grouped into 15 layers of hydrophobic residues and one

hydrophile layer with an ionic residue in the centre. After the SNARE complex has formed, the zero ionic layer, which consists of one arginine and three glutamine residues, is shielded from the surrounding solvent by the outer hydrophobic leucine-zippers. Each of these ionic residues originates from one of the four alpha helices that form the SNARE complex (Bock et al., 2001; Fasshauer et al., 1998b; Sutton et al., 1998; Weimbs et al., 1997; Weimbs et al., 1998).

Based on the amino acid in their zero ionic layer, a different classification of SNARE proteins into R-SNAREs (arginine) and Q-SNAREs (glutamine) has been established. This terminology also covers homotypic fusion events, for example, fusion between two vesicles where no assignment in v- and t-SNAREs is possible (Fasshauer et al., 1998b). Q-SNAREs can be further differentiated into Qa, Qb and Qc SNAREs based on their homology to the C-terminus of syntaxin (Qa), the N- (Qb) and C-terminus (Qc) of SNAP-25 (Bock et al., 2001).

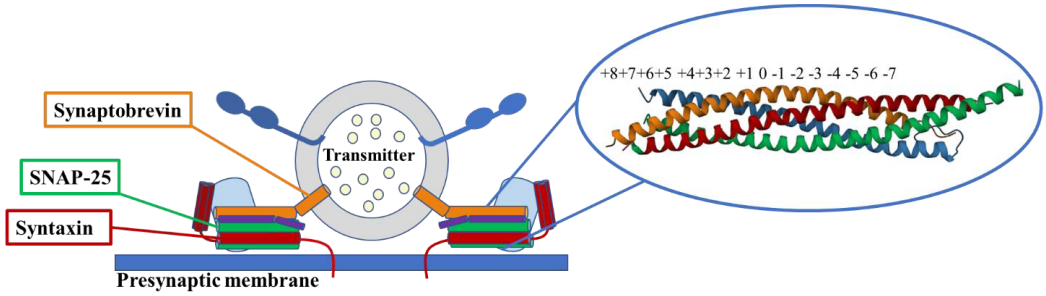


Figure 1.2 Schematic illustration of the assembled trans-SNARE complex. The assembled trans-SNARE complex consists of the t-SNARES/Q-SNARES syntaxin and SNAP-25 and the v-SNARE/R-SNARE synaptobrevin, which dock a synaptic vesicle to the presynaptic membrane. Circled in blue is the crystal structure of the assembled SNARE complex comprising a four-helix bundle with 15 hydrophobic layers and one ionic layer in the centre (0). One helix is contributed by synaptobrevin (orange), one by syntaxin (red) and two by SNAP-25 (green (Qb) and blue (Qc)). The illustration was created according to the model described by Südhof (2013) and the crystal structure (PDB ID: 1SFC from Sutton and Brunger (1998)) was included.

When SNAREs localised on opposing membranes come together, they form a *trans*-SNARE complex which bridges the two membranes and induces membrane fusion

(**Figure 1.2**). In the course of the fusion reaction, this complex is converted into a *cis*-SNARE complex in which all SNARE proteins form a parallel four-helix bundle located in the same membrane (Hanson et al., 1997b; Lin & Scheller, 1997; Weber et al., 1998). Assembly of the *cis*-complex is believed to be accomplished by directional "zippering" initiated at the N-terminus in the direction of the C-terminus (Gao et al., 2012; Hanson et al., 1997a; Hua & Charlton, 1999; Pobbati et al., 2006). The energy required to overcome the repulsive forces between the two membranes to merge them together is expected to be inherent in the energetically favoured assembly of the SNARE complex, which acts as an internal driving force (Fasshauer et al., 1997b; Hanson et al., 1997b; Lin & Scheller, 1997; Weber et al., 1998).

Together with soluble NSF attachment proteins (SNAPs), the *cis*-complex is reversibly disassembled under ATP hydrolysis by the ATPase NSF (*N*-ethylmaleimide-sensitive fusion protein) allowing SNARE proteins to undergo repetitive rounds of fusion (Hayashi et al., 1995; Söllner et al., 1993a; Söllner et al., 1993b; Weber et al., 2000).

Reconstitution assays using recombinantly expressed SNAREs demonstrated that SNARE proteins alone catalyse spontaneous fusion between artificial vesicles. However, the assembly rate of SNARE complexes *in vitro* is slow compared to the rapid regulated fusion events *in vivo* (Fasshauer et al., 2002; Sabatini & Regehr, 1996; Weber et al., 1998). Another *in vitro* study demonstrated that artificially stabilising a syntaxin/SNAP-25 acceptor complex for synaptobrevin accelerates liposome fusion (Pobbati et al., 2006). Other regulatory subunits are therefore needed to enable fast and precise membrane fusion in and between cells (Shen et al., 2007).

1.3. Molecular assembly for fast regulated exocytosis

The SM proteins, Sec1 and Munc18, first discovered in mutants of yeast and the nematode *Caenorhabditis elegans*, are of fundamental importance for *in vivo* membrane fusion (Brenner, 1974; Hata et al., 1993; Novick & Schekman, 1979; Pevsner et al., 1994; Verhage et al., 2000). Together with the priming factor Munc13,

SM proteins directly interact with SNARE proteins to organise and promote proper assembly of the fusion complex (Augustin et al., 1999; Baker et al., 2015; Parisotto et al., 2014; Richmond et al., 1999; Shen et al., 2007; Shu et al., 2020; Varoqueaux et al., 2002; Wang et al., 2019). Neuronal syntaxin1 forms a stable complex with Munc18-1 in a closed conformation that prevents interactions with the other two SNAREs (Burkhardt et al., 2008; Dulubova et al., 1999; Pevsner et al., 1994). In a mechanism that is not yet fully understood, the closed conformation of syntaxin1 is released via interaction with the MUN domain of Munc13-1 (Basu et al., 2005; Ma et al., 2011; Richmond et al., 1999; Richmond et al., 2001; Wang et al., 2017). The subsequent proper assembly of the ternary SNARE complex is believed to be orchestrated by Munc13 and Munc18. According to recent experiments, an intermediate is formed in which Munc18 provides a template for synaptobrevin and syntaxin simultaneously. This template organisation initiates and accelerates SNARE assembly most likely with the assistance of Munc13. In addition, this organisation might protect the complex from premature NSF-SNAP disassembly (Jiao et al., 2018; Lai et al., 2017; Ma et al., 2013; Parisotto et al., 2014; Shu et al., 2020; Stepien et al., 2019; Stepien et al., 2022; Wang et al., 2019). Similar observations of non-neuronal SM proteins, which act as important templates for membrane fusion complexes, indicate the conservation of this mechanism (Baker et al., 2015; Burkhardt et al., 2011; Jiao et al., 2018; Parra-Rivas et al., 2022).

Another important aspect of fast regulated membrane fusion is the priming of vesicles in a release-ready state docked to the target membrane prior to fusion. During this step, SNARE proteins become arrested in a partly assembled state (Hua & Charlton, 1999; Pobbati et al., 2006; Walter et al., 2010). Priming and subsequent efficient fusion of a vesicle likely involves the cooperation of multiple SNARE complexes under tight regulation by several proteins (Radhakrishnan et al., 2021; Shi et al., 2012; Sinha et al., 2011). In addition to Munc18 and Munc13, which are both essential priming factors (Lai et al., 2017; Varoqueaux et al., 2002; Verhage et al., 2000), complexin and synaptotagmin-1 are believed to be important priming regulators for fast Ca^{2+}-dependent neurotransmitter release (Brose et al., 1992; Cai et al., 2008; Fernández-Chacón et al., 2001; Li et al., 2011; Malsam et al., 2020). Although the exact molecular mechanism remains unknown, several studies indicate a cooperation between

synaptotagmin-1 and complexin to lock the *trans*-SNARE complex in an activated state. Upon Ca^{2+}-binding to the calcium sensor synaptotagmin-1, the locked state is released allowing SNAREs to fully assemble and initiate fusion (Brose et al., 1992; Giraudo et al., 2006; Li et al., 2011; Maximov et al., 2009; Rizo, 2022; Tang et al., 2006; Zhou et al., 2017).

To achieve fast Ca^{2+}-triggered exocytosis of synaptic vesicles, Ca^{2+} channels must be positioned in close proximity to primed vesicles (Eggermann et al., 2012; Katz, 1969; Llinas et al., 1992; Stanley, 1993). This is accomplished by the scaffold proteins Rab3-interacting molecule (RIM) and RIM-binding proteins (RIM-BPs) which tether Ca^{2+} channels to the active zone (Kaeser et al., 2011; Wu et al., 2019). Since RIM binds to the synaptic vesicle-localised Rab3 protein, it was suggested that RIMs link synaptic vesicles to Ca^{2+} channels (Kaeser et al., 2011). In addition, RIM is also involved in reversing the autoinhibition of Munc13 by disrupting its homodimeric state and making it accessible for vesicle priming (Deng et al., 2011). It was therefore proposed that RIMs form a priming complex with Rab3 and Munc13 (Dulubova et al., 2005; Kaeser et al., 2011).

In conclusion, precise vesicle fusion is regulated by multiple specialised proteins, such as SNAREs, Rab GTPases, NSF, SNAPs, SM, scaffold and tethering proteins, like Munc13, and in the case of fast Ca^{2+}-triggered release by complexin and synaptotagmin (reviewed in Brunger et al., 2019; Koike & Jahn, 2022; Rizo, 2022; Zhang & Hughson, 2021). Despite all the progress made on vesicle fusion, there is still much to learn (Brose et al., 2019; Rizo, 2022). Many questions remain about the precise molecular details at each step of membrane fusion, the interaction partners involved and the identity of possibly still unknown actors. As the basic set of the membrane fusion machinery seems to be highly conserved in eukaryotes, comparative approaches using unicellular relatives of animals can help to address these questions (Burkhardt et al., 2011; Kloepper et al., 2007; Novick & Schekman, 1979; Parra-Rivas et al., 2022; Protopopov et al., 1993).

1.4. Synaptic transmission and secretory vesicles

Neurons communicate with each other via synapses. These are the small endings used to transmit a signal from one neuron to another. Synapses can either be of electrical or chemical nature (Bennett et al., 1963; Dale & Gaddum, 1930; Furshpan & Potter, 1959; Loewi, 1921). The focus in this thesis is on chemical synapses. In these, neurotransmitters are stored in synaptic vesicles at the presynaptic active zone (de Iraldi & de Robertis, 1963; De Robertis & Bennett, 1955; Del Castillo & Katz, 1955; Palade, 1954; Politoff et al., 1975; Robertson, 1956). Many scaffolding and tethering proteins, like Piccolo, Bassoon, ELKS, RIM, RIM-BP and Munc13 participate in organising synaptic vesicles and Ca^{2+} channels at the release site of a vertebrate synapse (Kaeser et al., 2011; Mukherjee et al., 2010; Wang et al., 2016). Upon arrival of an action potential, the membrane is temporarily depolarised, and voltage-gated Ca^{2+} channels are activated resulting in an influx of Ca^{2+}. The increase of the intracellular Ca^{2+} concentration triggers exocytosis, which leads to the release of neurotransmitters into the synaptic cleft within less than 100 µs (Del Castillo & Katz, 1954; Fatt & Katz, 1952; Heuser et al., 1979; Katz & Miledi, 1967; Liley, 1956; Sabatini & Regehr, 1996). Neurotransmitters diffuse across the synaptic cleft to the opposing postsynaptic membrane where they activate their target neurotransmitter receptors (Craig et al., 1994; Del Castillo & Katz, 1955; Triller et al., 1985). This leads to the conversion of a chemical signal into an electrical signal. By allowing ion flux across the postsynaptic membrane, the membrane potential changes, resulting in an inhibitory or excitatory postsynaptic potential. Depending on the nature of the neurotransmitter receptor, the ion flow is either directly activated by ionotropic receptors or modulated by metabotropic receptors that activate G-proteins and trigger second messenger cascades (Bormann et al., 1987; Bührle & Sonnhof, 1983; Del Castillo & Katz, 1955; Sugiyama et al., 1987; Sun et al., 2001). In vertebrates, received signals from dendrites are integrated at the axon initial segment, where they decrease or increase the probability of the generation of an action potential in an all-or-none manner (Adrian, 1914; Coombs et al., 1957; Fuortes et al., 1957; Hill et al., 2008; Palmer & Stuart, 2006). When an action potential is generated, the membrane is depolarised, resulting in

opening of voltage-gated ion channels and propagation of the action potential down the axon (Bender & Trussell, 2009; Hodgkin & Huxley, 1952; Hodgkin & Katz, 1949). As explained earlier, the voltage-gated Ca^{2+} channels open in response to membrane depolarisation as soon as the action potential reaches the synaptic endings, and the Ca^{2+} influx triggers vesicle exocytosis (Del Castillo & Katz, 1954; Fatt & Katz, 1952; Heuser et al., 1979; Katz & Miledi, 1967; Liley, 1956).

Most synapses which contain the neurotransmitter glutamate are excitatory (Curtis et al., 1959a; Takeuchi & Takeuchi, 1963), whereas y-aminobutyric acid (GABA) and glycine containing synapses are often inhibitory (Curtis et al., 1959b; Krnjević & Schwartz, 1967; Werman et al., 1967). However, there are also examples of GABA acting as an excitatory transmitter, presumably by mediating depolarisation at high intracellular chloride concentrations (Choi et al., 2008; Haam et al., 2012). It has been shown, that not only the interplay, but also direct interactions between excitatory and inhibitory systems contribute to another level of complexity in the regulation of neuronal transmission (Wen et al., 2022).

Electron microscopic studies revealed that two morphologically different types of secretory vesicles can be found in neurons, which undergo regulated secretion. These are clear synaptic vesicles (40-60 nm) and large dense core vesicles (70-120 nm). The latter appear granular in electron micrographs due to an electron dense core (De Robertis & Bennett, 1955; Hökfelt et al., 1977; Larsson, 1977; Lundberg & Hökfelt, 1983; Palade, 1954; Pelletier et al., 1984; Pelletier et al., 1981; Pickel et al., 1979; Richardson, 1962; Robertson, 1956). The protein composition of both vesicle types has been investigated by mass spectrometry (Bark et al., 2012; Bradberry et al., 2022; Grønborg et al., 2010; Morciano et al., 2005; Takamori et al., 2006; Taoufiq et al., 2020; Wegrzyn et al., 2007; Wegrzyn et al., 2010). Large dense core vesicles are loaded at the *trans*-Golgi network from which they bud off as immature large dense core vesicles (Hummer et al., 2017; Orci et al., 1987; Tooze & Huttner, 1990; Wu et al., 2004). Their cargo are neuropeptides, amines, neurotrophic factors or other modulatory substances essential for a variety of functions that modulate synaptic transmission (de Iraldi & de Robertis, 1963; de Wit et al., 2006; Larsson, 1977; Lochner

et al., 2006; Michael et al., 1997; Pelletier et al., 1984; Pelletier et al., 1981; Wu et al., 2004). Large dense core vesicles are distributed throughout the neuron and have different release sites, whereas synaptic vesicles are stored and released at presynaptic active zones (de Wit et al., 2006; Lochner et al., 2006; Matsuda et al., 2009; Moro et al., 2021; Pelletier et al., 1984; Persoon et al., 2018; van de Bospoort et al., 2012). Compared to synaptic vesicles, large dense core vesicles exhibit distinct characteristics in biogenesis, dynamic transport, and release organisation. They are highly mobile and are continuously transported along the cytoskeleton (Bharat et al., 2017; de Wit et al., 2006; Matsuda et al., 2009; Wong et al., 2012). Moreover, in mammalian neurons, large dense core vesicles do not appear to be pre-docked at release sites but are instead located in the periphery of synaptic vesicles pools. This may explain the slow-release properties and requirement for strong, prolonged stimulation for the release of large dense core vesicles (Hartmann et al., 2001; Persoon et al., 2018; van de Bospoort et al., 2012). However, the molecular details of the individual steps in the life cycle of large dense core vesicles are still elusive and are only beginning to be understood (Bharat et al., 2017; de Wit et al., 2006; Hoogstraaten et al., 2020; Hummer et al., 2017; Moro et al., 2021; van de Bospoort et al., 2012; Wong et al., 2012). Future research on large dense core vesicles in neurons may reveal unexpected complexity in different types of neurons (Merighi, 2018).

In contrast to large dense core vesicles, synaptic vesicles are not only retrieved from the *trans*-Golgi network but can also be recycled locally at the synapse (Ceccarelli et al., 1973; Heuser & Reese, 1973; Prior & Clague, 1997). The cargo of synaptic vesicles is loaded by neurotransmitter transporters at the presynapse. Synaptic vesicles store small classical neurotransmitters, such as acetylcholine, GABA, glutamate, or glycine. (Burger et al., 1989; Hell et al., 1990; Kish et al., 1989; Maycox et al., 1988; Politoff et al., 1975; Takamori et al., 2000). Apart from minor deviations, the overall protein composition of synaptic vesicles appears to be largely similar, even if they carry different cargos (Grønborg et al., 2010). However, diverse experiments suggest that synaptic vesicles can be assigned to distinct pools at the presynapse, which seem to involve different molecular components (Chanaday & Kavalali, 2018; Crawford & Kavalali, 2015; Evstratova et al., 2014; Raingo et al., 2012). These pools of synaptic

vesicles are functionally categorised based on their release characteristics in a readily releasable pool, a reserve pool, and a resting pool (Südhof, 2000). Synaptic vesicles of different pools might participate in the different release modes during neurotransmission. Here, a distinction is made between fast synchronous release in response to high Ca^{2+} concentrations and delayed asynchronous release in response to lower Ca^{2+} concentrations, as well as action potential-independent spontaneous release (Barrett & Stevens, 1972; Chanaday & Kavalali, 2018; Crawford & Kavalali, 2015; Evstratova et al., 2014; Fatt & Katz, 1952; Goda & Stevens, 1994; Raingo et al., 2012). It has been suggested that the readily releasable pool and reserve pool form together the activity-dependent recycling pool. The resting pool, on the other hand, has been proposed to be activity-independent and to store vesicles that are spontaneously released (Chanaday & Kavalali, 2018; Fredj & Burrone, 2009; Südhof, 2000).

Synaptic vesicles and large dense core vesicles also share common features. For example, both types of vesicles depend on a fusion machinery consisting of neuronal SNAREs and their release is triggered by Ca^{2+} influx (Hartmann et al., 2001; Hoogstraaten et al., 2020; Matsuda et al., 2009; Persoon et al., 2018; Shimojo et al., 2015; van de Bospoort et al., 2012). Western blot analyses of isolated large dense core vesicles indicated that they contain several classical synaptic vesicle proteins (synaptic vesicle protein 2 (SV2), Rab3, synaptophysin, (Berg et al., 2000)). Some of these and other proteins common to both vesicle types were also identified by mass spectrometry analysis (Bark et al., 2012; Takamori et al., 2006; Wegrzyn et al., 2010). Furthermore, the loading of both synaptic vesicles and monoamine-containing large dense core vesicles is dependent on neurotransmitter transporters and an electrochemical proton gradient generated by a vacuolar H^+-ATPase (Birinci et al., 2020; Edwards, 2007; Eriksen et al., 2016; Hell et al., 1990; Hummer et al., 2017; Kish et al., 1989; Matsuda et al., 2009; Maycox et al., 1988).

1.5. The evolution of the nervous system

One of the central questions in the evolution of animals is how the nervous system and its diverse components originated (Marlow & Arendt, 2014). This question is closely linked to two other important issues concerning (1) the definition of a neuron and (2) the debate about the basal animal phylogeny (Bucher & Anderson, 2015; Marlow & Arendt, 2014). As for the difficulty of defining a neuron (1), no pan-neuronal genetic markers have yet been identified that apply to all neurons across all species. It is rather the case that some characteristics are not present in all neurons or are not exclusive to neuronal cells. For example, osteocytes and glia cells have long processes resembling dendrites, but some interneurons do not have clearly defined axons and dendrites (Bucher & Anderson, 2015). Moreover, many genes important for neuronal transmission are also expressed in cells outside of the nervous system (Bucher & Anderson, 2015; Moroz, 2009; Moroz & Kohn, 2015). Classical neurotransmitters and their receptors, for instance, play important roles in non-neuronal cells (Genever et al., 1999; Lv & Liu, 2017; Xiang et al., 2007). In addition, many key features of the nervous system can be found outside of the animal kingdom. For example, voltage-gated ion channels are present in choanoflagellates, bacteria and viruses (Liebeskind et al., 2011; Moran & Zakon, 2014; Plugge et al., 2000; Ren et al., 2001). Components involved in cellular polarisation are highly conserved from yeast to animals (Sarto-Jackson & Tomaska, 2016). Electrophysiological recordings showed that the unicellular protist *Odontella sinensis* can generate fast sodium-based action potentials similar to cardiac and skeletal muscle cells of animals (Taylor, 2009).

Regarding the animal phylogeny (2), there are three animal lineages that have clearly recognisable neurons—bilaterians, cnidarians (sea anemones, corals, jellyfish, and hydroids) and ctenophores (comb jellies). Since neither muscle cells nor *bona fide* neurons appear to be present in poriferans (sponges) and placozoans (e.g., *Trichoplax adhaerens*, *Hoilungia hongkongensis,* and *Polyplacotoma mediterranea*), and due to their morphological simplicity, these lineages were considered to be among the earliest-branching animals (de Ceccatty, 1974a, 1974b; Philippe et al., 2009; Pick et al., 2010; Schierwater, 2005; Schierwater et al., 2009;

Schulze, 1892; Srivastava et al., 2008). In the poriferan *Amphimedon queenslandica*, many synaptic genes are not co-regulated like in animals with clearly defined neurons. Assuming that poriferans are the sister group to all animals, this could mean that pre-existing proteins were used to form a synapse after coordination and expansion of interaction networks of protosynaptic genes (Conaco et al., 2012; Sakarya et al., 2007; Srivastava et al., 2010; Wong et al., 2019).

Several studies have challenged this view and suggested ctenophores to be the first animal group that branched off the animal tree of life. This could imply that either the last common ancestor of all animals already possessed a nervous system that was lost in poriferans and placozoans, or that the nervous systems of ctenophores and other animals (cnidarians and bilaterians) evolved independently (Dunn et al., 2008; Moroz et al., 2014; Ryan & Chiodin, 2015; Ryan et al., 2013; Shen et al., 2017). Although many components important for synaptic signal transmission are present in ctenophores, other proteins involved in neuronal fate, patterning, synaptic functions (e.g., neurogenin, synaptotagmin, neuroligin) as well as some canonical neurotransmitters (e.g., serotonin, acetylcholine, dopamine, noradrenaline, histamine) seem absent (Moroz et al., 2014; Ryan et al., 2013; Sachkova et al., 2021). However, many aspects of the nervous system of ctenophores and their neurotransmission are still unknown, making it difficult to draw conclusions about functional conservation (Burkhardt et al., 2023; Burkhardt & Sprecher, 2017; Moroz & Kohn, 2015; Sachkova et al., 2021). Phylogenomic analyses indicating that poriferans should keep the basal position, on the other hand, do not exclude multiple origins of the nervous system, but they also suggest that the nervous system could have evolved only once and was lost in placozoans (Pisani et al., 2015; Simion et al., 2017). In summary, depending on the scenario, different assumptions can be made about the evolution of the nervous system. Four of these scenarios are shown in **Figure 1.3** (reviewed by Jékely et al., 2015).

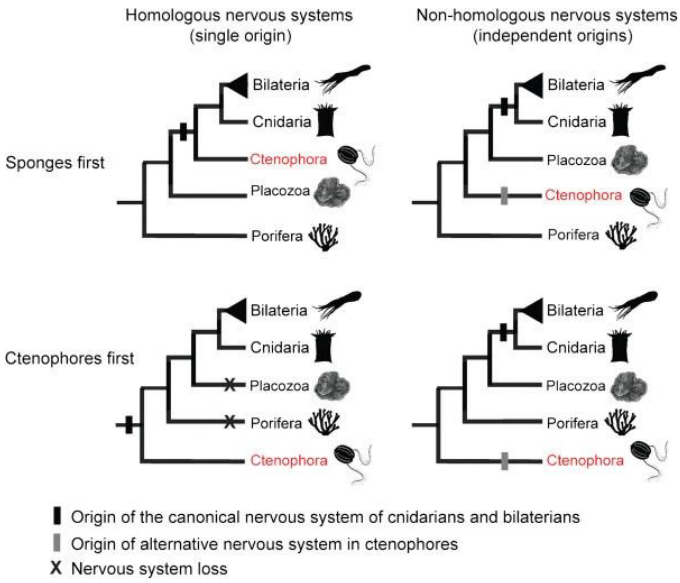


Figure 1.3 Different scenarios for the evolution of the nervous system. The Illustration summarises four different scenarios on the origin of the nervous system. Depending on the phylogeny of animals and the homology of animal nervous systems, it can be assumed that the nervous system evolved only once (two scenarios on the left) or multiple times independently of each other (two scenarios on the right). If the nervous system evolved once, it could either have evolved in the last common ancestor of ctenophores, cnidarians and bilaterians (sponges first), or it was lost in placozoans and sponges (ctenophores first). In the case that the nervous systems are not homologues and evolved independently multiple times, either ctenophores or sponges could be the earliest-branching animals (reviewed by Jékely et al., 2015). Illustration from Jékely et al. (2015).

There are various theories about the origin and evolution of the nervous system and neurons (reviewed in Arendt, 2021; Mackie, 1990; Moroz, 2009). Already in 1872, the German biologist Nicolaus Kleinenberg made first assumptions about the emergence of neurons. Kleinenberg suggested that neurons and muscle cells originated from a cell similar to the epithelial muscle cells of Hydra. Before this cell specialised into distinct contractile and excitable cells, it would have formed the basis for muscle and neuronal cells (Kleinenberg, 1872). In contrast, other theories assumed an independent evolution of sensory and receptor cells from (ciliated) epithelial cells (Hertwig & Hertwig, 1879; Jékely, 2011; Parker, 1919).

However, a different perspective emerged from theories that focused on tissue levels assuming cellular specialisations evolved after nerve nets (Pantin, 1956). One of these hypotheses was that the nervous system arose from a primordial epithelium capable of conducting electrical events and contraction (Mackie, 1970). This was based on the finding that hydrozoans have electrically excitable epithelia which receive sensory information and signal to effector cells, even though they do not exhibit specificity and directionality (Mackie & Passano, 1968). Several other examples of conductive tissues in plants, algae, and animals were considered to support the theory that a primordial conductive tissue may have formed the starting point for the evolution of localised, polarised information transfer resulting in specialisations, such as muscles and neurons. In addition, consideration was given to whether electrical transmission might be more ancient than chemical transmission (Mackie, 1970). Today, it is unclear whether electrical synapses were used by the last common ancestor of animals, since innexins and connexins, important proteins for the formations of gap junctions, are missing in poriferans and placozoans and have not been found outside of animals (Cai, 2008; Welzel & Schuster, 2022).

Other theories suggest that neurons and the nervous system evolved from secretory cells or a secretory network (Colgren & Burkhardt, 2022; Grundfest, 1959; Haldane, 1954; Horridge, 1968; Jékely, 2021; Lentz, 1968). One of these hypotheses is that neurons may have evolved from environment-and microbe-sensing cells, which share a common origin with immune cells. These cells interacted with its microbiome and released antimicrobial neuropeptides in response to microbial cues (Klimovich & Bosch, 2018). Another theory in favour of the secretory network was formulated by Gáspár Jékely in the ‘chemical brain hypothesis’ (Jékely, 2021). He proposed that neurosecretory cells arose by specialisation from a ciliated tissue. Under these conditions, the release of neuropeptides presumably served to synchronise sensory, motile ciliated cells. According to the ‘chemical brain hypothesis’, the evolution of a neurosecretory network connected by synapses was important to overcome the limitations caused by increasing body size and inefficient diffusion of neuropeptides. Another strategy to cope with the difficulties posed by the constraint diffusion and large body size had been the emergence of a circulatory system and neurohemal organs

(Jékely, 2021). The signalling machinery used for neuropeptide-based intercellular communication may have originally been involved in ciliary communication of ancestral protists and has been adopted for signalling in animals (Jékely, 2021).

As early as in 1954, Haldane suggested that chemical communication in neurons and protists involves similar mechanisms and hence evolved from a unicellular ancestor (Haldane, 1954). This theory was further elaborated by the hypothesis that membrane repair mechanisms in the last common ancestor of eukaryotes could have set the foundation for combining contraction and secretion in response to Ca^{2+} influx and depolarisation (Brunet & Arendt, 2016).

To date, it is still uncertain whether the nervous system evolved from a contractile-conductive or sensory-secretory basis, or whether it has several different origins (Arendt, 2021). In the future, further comparative analyses in animals and organisms outside of the animal kingdom will contribute to a deeper understanding of the evolutionary history of the nervous system (Arendt, 2020; Burkhardt & Sprecher, 2017; Colgren & Burkhardt, 2022).

1.6. Choanoflagellates

Choanoflagellates are unicellular eukaryotes that live in various aquatic environments all over the globe (Auer & Arndt, 2001; Buck & Garrison, 1988; Leadbeater, 2015; Leakey et al., 2002; Thomsen et al., 1997; Thomsen & Larsen, 1992). They have a characteristic cell morphology which resembles choanocytes, the feeding cells of sponges (Clark, 1866; Kent, 1880; Laundon et al., 2019; Maldonado, 2004; Nichols et al., 2009). The cell body of choanoflagellates is spherical to ovoid with an apical flagellum surrounded by a collar of actin-filled microvilli which they use to trap bacteria and detritus for phagocytosis (Boenigk & Arndt, 2000; Karpov & Leadbeater, 1998; Pettitt et al., 2002). Morphological and phylogenetic analyses identified choanoflagellates as the closest unicellular relatives of animals (**Figure 1.4**). It was therefore assumed that the last common ancestor of animals and choanoflagellates

resembled a choanoflagellate cell (Carr et al., 2008; Clark, 1871; King & Carroll, 2001; Lang et al., 2002; Ruiz-Trillo et al., 2008; Steenkamp et al., 2005). Together with animals, filastereans and ichthyosporeans, choanoflagellates form the clade of holozoans (Carr et al., 2008; Lang et al., 2002).

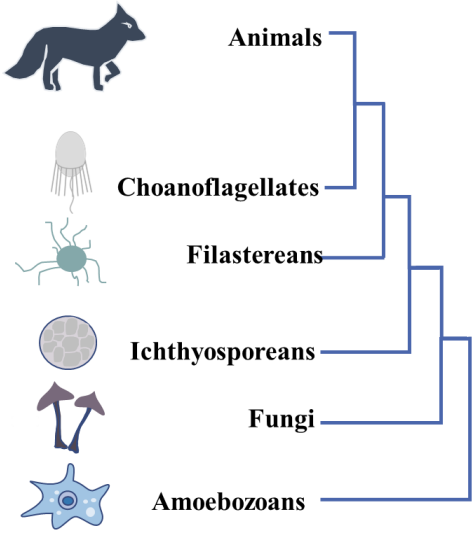


Figure 1.4 Phylogenetic relationship between opisthokonts and amoebozoans. The opisthokonts include holozoans (animals, choanoflagellates, filastereans, and ichthyosporeans) and fungi. Phylogenetic analyses position choanoflagellates as the closest unicellular relatives of animals (Carr et al., 2008; King & Carroll, 2001; Lang et al., 2002; Ruiz-Trillo et al., 2008; Steenkamp et al., 2005). The illustration shows a simplified scheme, created according to the phylogenomic analysis of mitochondrial proteins by Ruiz-Trillo et al. (2008).

Choanoflagellates display complex behaviours in response to diverse environmental stimuli, such as photo-, chemo- and mechanosensation (Brunet et al., 2021; Brunet et al., 2019; Kirkegaard et al., 2016; Miño et al., 2017; Reyes-Rivera et al., 2022; Ros-Rocher & Brunet, 2023; Woznica et al., 2017; Woznica et al., 2021). Studies of choanoflagellates are key for understanding the origin and evolution of animals (Brunet & King, 2017; Goldstein & King, 2016; Hoffmeyer & Burkhardt, 2016; López-Escardó et al., 2019). For example, comparative analyses of the ciliary proteomes of choanoflagellates and different animals provided information on the ancestry of ciliary signalling proteins. In the same study, they identified previously uncharacterised ciliary

proteins conserved from choanoflagellates to mammals, offering insights into the aetiology of ciliopathies (Sigg et al., 2017). As choanoflagellates are a new model organism, many techniques still need to be established. Nevertheless, fully sequenced genomes and transcriptomes are available for several choanoflagellates species, and transgenic experiments are possible in the choanoflagellate species *Salpingoeca rosetta* (*S. rosetta*) and *Monsiga brevicollis* (*M. brevicollis*) (Booth & King, 2020, 2022; Booth et al., 2018; Brunet et al., 2019; Fairclough et al., 2013; King et al., 2008; López-Escardó et al., 2019; Richter et al., 2018; Wetzel et al., 2018; Woznica et al., 2021).

In the present study, *S. rosetta* was used as a model to investigate the biology of synaptic protein homologues. One of the great advantages of working with *S. rosetta* is that it can easily be cultured in the laboratory and has a short generation time (Dayel et al., 2011; Fairclough et al., 2010). Moreover, *S. rosetta* has a complex life history which includes differentiation into diverse life stages (**Figure 1.5**). Among these are sexual, asexual, different unicellular, and multicellular life history stages (Dayel et al., 2011; Fairclough et al., 2010; Levin & King, 2013). The different life stages can be induced by environmental cues (Alegado et al., 2012; Dayel et al., 2011; Fairclough et al., 2010; Levin & King, 2013; Woznica et al., 2016; Woznica et al., 2017). For example, the chondroitinase EroS (extracellular regulator of sex), secreted by the bacterium *Vibrio fischeri,* induces swarming and mating in *S. rosetta* (Woznica et al., 2017). Another example is the so-called “rosette” colony, one of the multicellular stages of *S. rosetta*. Rosette colonies are formed by serial cell division in response to a bacterial lipid produced by *Algoriphagus machipongonensis* (Alegado et al., 2012; Alegado et al., 2013; Dayel et al., 2011; Fairclough et al., 2010; Woznica et al., 2016).

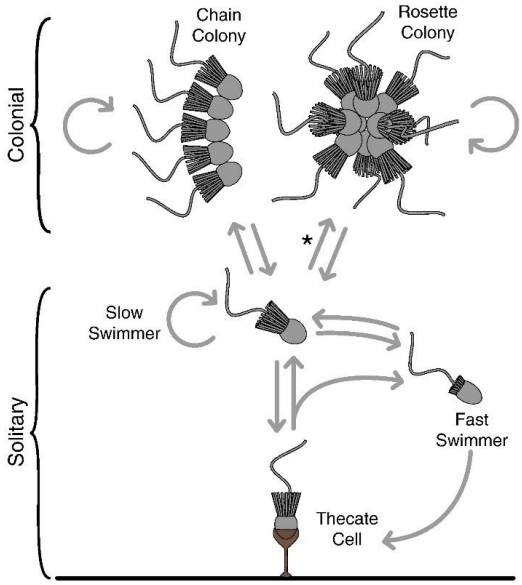


Figure 1.5 Schematic drawing of the life history of *S. rosetta*. *S. rosetta* can differentiate into different forms of solitary and multicellular stages. Arrows show the observed transitions. Slow swimmer cells can form chain colonies, where cells are attached by an extracellular matrix and intercellular bridges. Upon addition of *Algoriphagus machipongonensis* (*), slow swimmers can form rosette colonies by cell division. In this stage, cells have filopodia, are connected by intercellular bridges, and share an extracellular matrix. Slow swimmers are also assumed to be able to differentiate into fast swimmers and produce more slow swimmers by cell division. By settlement on surfaces, fast swimmers can become thecate cells that can become swimmers again by theca abandonment. Thecate cells produce an extracellular matrix and can give rise to a swimming and a thecate cell through cell division. The model and illustration were obtained from Dayel et al. (2011).

Within a colony, cells share an extracellular matrix and are connected by fine intercellular bridges. These intercellular bridges might mediate signalling via diffusion of small molecules from one cell to another (Dayel et al., 2011). Structural analyses of colonies have shown that cells within a rosette colony display cell disparity. This indicates the presence of distinct cell states or even cell types within a *S. rosetta* colony (Laundon et al., 2019; Naumann & Burkhardt, 2019).

1.7. Studies on choanoflagellates to reconstruct the ancestry of synapses

Neurons that possess chemical synapses are specialised secretory cells, that allow for ultrafast synaptic vesicle secretion in a highly coordinated and regulated manner (Rizo, 2022; Sabatini & Regehr, 1996; see also chapters 1.3 and 1.4). To study the evolution of the nervous system and chemical signal transmission, it is necessary to investigate the onset of regulated secretion. To date, fossil records provided only limited information on the earliest appearance of a nervous system in animals (Budd, 2015; Burkhardt & Jékely, 2021; Liu et al., 2014).

Several studies in yeast supported the hypothesis that the mechanism of synaptic vesicle exocytosis has evolved from the constitutive secretory pathway of single celled eukaryotes (Aalto et al., 1993; Brennwald et al., 1994; Novick & Schekman, 1979; Protopopov et al., 1993; Rossi et al., 1997). As the closest unicellular relatives of animals that do not possess a nervous system, choanoflagellates represent an important model to study the onset and transition from a unicellular organism to a complex nervous system-controlled animal (Carr et al., 2008; King & Carroll, 2001; Lang et al., 2002; Ruiz-Trillo et al., 2008). Choanoflagellate genomes encode an astonishingly high number of diverse synaptic protein homologues. These include presynaptic protein homologues involved in vesicle exocytosis (secretory SNAREs, Munc18, Munc13) (Burkhardt et al., 2011; Fairclough et al., 2013), cell adhesion and signalling proteins (cadherins, Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), Hedgehog, septins and tyrosine kinases) (Fairclough et al., 2013; King & Carroll, 2001; King et al., 2008; Snell et al., 2006), voltage-gated sodium and calcium channels (Cai, 2008; Liebeskind et al., 2011; Moran & Zakon, 2014), postsynaptic scaffolding proteins (Shank, Homer, Discs large) (Alié & Manuel, 2010; Anderson et al., 2016; Burkhardt et al., 2014; Moroz & Kohn, 2015; Sakarya et al., 2007) and ionotropic glutamate receptors (Moroz et al., 2021; Tikhonenkov et al., 2020). In particular, many of the proteins that are highly abundant on synaptic vesicles and assumed to be key for regulating synaptic secretion appear to be conserved in choanoflagellates (Buckley & Kelly, 1985; Burkhardt et al., 2014; Ciruelas et al., 2019; Göhde et al., 2021; Jahn &

Boyken, 2016; Liao et al., 2007; Rizo, 2022; Sugita et al., 1999; Taoufiq et al., 2020; Zhang & Castle, 2011; Zhang et al., 2015). There are, for instance, homologues of synaptic vesicle protein 2 (SV2), synaptogyrin, secretory carrier-associated membrane proteins (SCAMPs) and SV2-related protein (SVOP) in the genomes of *S. rosetta* and *M. brevicollis* (Burkhardt et al., 2014; Göhde et al., 2021). Complexin, one of the key proteins regulating synaptic vesicle exocytosis, is also present in the genome of *M. brevicollis* (Burkhardt et al., 2014). So far, no homologue of the calcium sensor synaptotagmin could be found (Göhde et al., 2021). However, other calcium sensors could be responsible for a regulated secretion in choanoflagellates. In neurons, Doc2 is assumed to be another calcium sensor that may be involved in asynchronous and/or spontaneous release of neurotransmitters (Courtney et al., 2018; Díez-Arazola et al., 2020; Yao et al., 2011). Using basic local alignment analysis with human Doc2 as query, I identified other potential calcium sensors in the predicted proteomes of *S. rosetta* and *M. brevicollis* (**Table 1**). Another important finding in *S. rosetta* was the presence of the neuropeptide precursor sequences phoenixin and nesfatin, which are likely secretory proteins and might regulate feeding (Yañez-Guerra et al., 2022).

Comparative studies of the secretion apparatus of neurons and *M. brevicollis* demonstrated that the key proteins Munc18 and syntaxin1 not only structurally resemble the vertebrate complex, but also interact in a very similar manner (Burkhardt et al., 2011). In *M. brevicollis* these proteins seem to be localised at the apical pole, where also numerous vesicles can be found (Burkhardt et al., 2011; Göhde et al., 2021). Immunostainings in *S. rosetta* indicated the presence of synaptobrevin, another secretory key protein, at both poles of the cell (Göhde et al., 2021). In combination with diverse vesicular landscapes observed in *S. rosetta* and *M. brevicollis*, these results suggest that the secretory function of the neurosecretory apparatus may be conserved in choanoflagellates (Burkhardt et al., 2011; Göhde et al., 2021; Laundon et al., 2019). It remains to be seen whether also its regulation or role in intercellular communication is conserved in choanoflagellates and has a proto-synaptic function.

In summary, many of the building blocks of the nervous system have a premetazoan origin and thus predate neurons, suggesting that the nervous system evolved in a

stepwise assembly from pre-existing components (Arendt, 2020; Burkhardt et al., 2014; Burkhardt & Jékely, 2021; Burkhardt et al., 2011; Göhde et al., 2021; Jékely, 2021; Moroz & Kohn, 2015; Varoqueaux & Fasshauer, 2017; Yañez-Guerra et al., 2022).

So far, proteomic studies on secretory vesicle proteins have mainly been conducted in animals (Bradberry et al., 2022; Grønborg et al., 2010; Morciano et al., 2005; Takamori et al., 2006; Taoufiq et al., 2020). The functions of synaptic protein homologues in non-animal organisms are still largely unclear (Burkhardt et al., 2014; Colgren & Burkhardt, 2022; Göhde et al., 2021). However, the proteomic and functional studies that have been carried out on unicellular eukaryotes have provided fascinating insights into the ancestry of animal proteins (Bhattacharyya et al., 2016; Brennwald et al., 1994; Henriksen et al., 2012; Parra-Rivas et al., 2022; Protopopov et al., 1993; Sebé-Pedrós et al., 2016; Sigg et al., 2017).

One example of a functional study that helped to characterise properties and regulatory interactions of syntaxin was an interspecies complementation experiment. Here syntaxin was knocked out in *Caenorhabditis elegans* and rescued by expressing a chimeric syntaxin containing a Habc domain from *M. brevicollis*. The rescue by the choanoflagellate chimera suggested a conserved function that predates synapses and animals (Parra-Rivas et al., 2022).

The inclusion of the closest unicellular relatives of animals in investigations of the evolution of the nervous system and synapses—the smallest units for neuronal transmission—promise a different perspective that can bring clarity to many fundamental questions (Burkhardt & Sprecher, 2017). Choanoflagellates are a unique model for studying the evolution of regulated secretion under direct environmental influence (Burkhardt, 2015; Dayel et al., 2011; Hoffmeyer & Burkhardt, 2016). Due to its multicellularity and established techniques, *S. rosetta* is an ideal model that offers a great opportunity to investigate whether the last common ancestor of animals and choanoflagellates might have already used a similar apparatus for (regulated) secretion or intercellular communication as the synapses of animals (Booth & King, 2020, 2022;

Booth et al., 2018; Dayel et al., 2011; Fairclough et al., 2013; Fairclough et al., 2010; Wetzel et al., 2018). Comparative studies including animals and their closest relatives allow us to explore how vesicle secretion became highly specialised and linked to intercellular signal transduction, as in the case of neurotransmission. In addition, investigating homologues of synaptic vesicle proteins in close animal relatives may help to identify previously unknown molecular functions and potential interaction partners of synaptic proteins (Burkhardt et al., 2014; Burkhardt et al., 2011; Colgren & Burkhardt, 2022; Parra-Rivas et al., 2022; Yañez-Guerra et al., 2022).

2. Aim of the thesis

The aim of this thesis was to investigate the evolutionary origin of synaptic proteins, especially those which are important for vesicle secretion. The main model organism for this thesis was the choanoflagellate species *Salpingoeca rosetta* (*S. rosetta*), in whose genome we find a great variety of highly conserved synaptic protein homologues. Studying protein homologues in choanoflagellates, the closest unicellular relative of animals, promises to uncover whether key synaptic vesicle proteins may have been part of an ancient secretion complex that evolved before the emergence of the first nervous systems.

The general focus of this thesis, exploring the evolutionary history of regulated, synaptic vesicle release, was investigated in the context of two studies. The first of these studies (Paper I) aimed to show that most of the core neurosecretory vesicle proteins are conserved in the genomes of unicellular organisms and thus predate animals. Another important component of this study were investigations of intracellular vesicles in two choanoflagellate species (*M. brevicollis* and *S. rosetta*) to allow a comparison between highly specialised secretory cells such as neurons and choanoflagellates.

The second study (Paper II) intended to further assess and characterise homologues of exocytotic synaptobrevin in *S. rosetta*. Exocytotic synaptobrevin is one of the key proteins for vesicle secretion in eukaryotes and can be used as a marker for secretory vesicles. So far, not much is known about the exact function and localisation of synaptobrevin in choanoflagellates. For direct comparisons between synaptobrevin from *S. rosetta* and secretory synaptobrevin of animals, I used a combination of comparative studies, targeted genome editing and various biochemical techniques, like co-immunoprecipitation, subcellular fractionation, enzyme assays, SDS-PAGE, Western blot, and mass spectrometry analysis. The purpose of this was to study synaptobrevin of *S. rosetta* from many different angles and to learn more about its pre-metazoan role.

3. Summary of the results

3.1. Choanoflagellates and the ancestry of neurosecretory vesicles (Paper I)

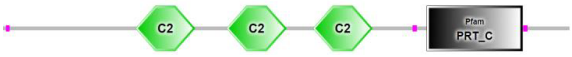
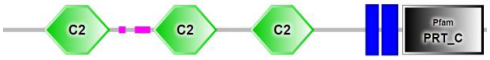
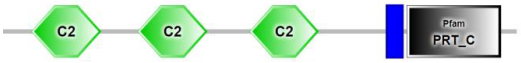
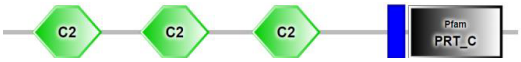
Comparative analysis of neurosecretory vesicle proteins

Neurosecretory vesicles are storage organelles that contain, for example, neuropeptides or neurotransmitters, which are released in a highly regulated manner. They represent an important part of the chemical signal transmission of the nervous system (see chapter 1.4; also reviewed by Hannah et al., 1999). Although there are many different theories about the evolution of the nervous system, its origin is still unclear (reviewed in Arendt, 2021; Mackie, 1990; Moroz, 2009). In paper I, we investigated the evolutionary history of neurosecretory vesicles using a comparative analysis of 28 core proteins of the neurosecretory vesicle proteome in 13 different eukaryotic species. Among these were animals with clearly recognisable neurons (zebrafish (*Danio rerio*), sea urchin (*Strongylocentrotus purpuratus*), fruit fly (*Drosophila melanogaster*), sea anemone (*Nematostella vectensis*), ctenophore (*Mnemiopsis leidyi*)), animals without recognisable neurons (placozoan (*Trichoplax adhaerens*), sponge (*Amphimedon queenslandica*)), the closest unicellular relatives of animals (two choanoflagellate species (*S. rosetta*, *M. brevicollis*) and a filasterean (*Capsaspora owczarzaki*)), and three different fungi species (*Batrachochytrium dendrobatidis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*) (**figure 1**). We searched for homologues of neurosecretory vesicle core proteins belonging to the following categories: ATPases; transporters and transporter-like proteins; proteins with four transmembrane domains; synapsins; synaptotagmins; SNAREs; SNARE co-chaperones; SNARE binding partners and Rab proteins (**figure 1**). Of the proteins studied, about 39% appear to be present exclusively in animals. These include synapsin, the synaptic-associated zinc transporter ZnT3, synaptotagmin1, chaperone cysteine string protein (CSP), myelin and lymphocyte protein 2 (MAL2), and synuclein. In contrast, most of the neurosecretory vesicle proteins studied also appear to be present in unicellular opisthokonts (~61%) (**figure 1**). All examined species seemed to possess homologues of secretory SNAREs, Rab7, V-and P-ATPases. In *S. rosetta* and *M. brevicollis* we also found homologues of

synaptogyrin, SCAMP1/2, synaptic vesicle protein 2 (SV2) and SV2-related protein (SVOP). In summary, in paper I we have shown that most of the synaptic vesicle core proteins analysed seem to occur in unicellular opisthokonts and thus predate animals.

In addition to these results, I searched for the presence of Doc2 in the predicted proteomes of *S. rosetta* and *M. brevicollis* (additional results, **Table 1**). Doc2 is another calcium sensor in neurons and potentially responsible for asynchronous or spontaneous transmitter release in neurons (Courtney et al., 2018; Díez-Arazola et al., 2020; Yao et al., 2011). The results summarised in **Table 1** show *S. rosetta* and *M. brevicollis* proteins with a sequence similarity of more than 25% to human Doc2. Reciprocal searches against the human proteome using the identified choanoflagellate protein sequences as queries indicated similarity to multiple C2 and transmembrane domain-containing protein 1 (MCTP1). The protein domain architecture, including the phosphoribosyl transferase-like domain of the *S. rosetta* protein PTSG_11158, also displays similarity to that of MCTP1. It can therefore be assumed that the genome of *S. rosetta* possibly encodes for an MCTP protein homologue.

Table 1 Protein domain compositions of Doc2A from *Homo sapiens* and Doc2A-like proteins from *M. brevicollis* and *S. rosetta* that exhibit sequence similarity. The Doc2A protein sequence from *Homo sapiens* was used as query for searches against the predicted proteomes of *S. rosetta* and *M. brevicollis* using the basic local alignment sequence similarity search tool (BLASTp) at the National Center for Biotechnology Information (NCBI) with the default BLAST parameters (Altschul et al., 1997). The identified choanoflagellate proteins were used as queries for reciprocal blasts against the *Homo sapiens* proteome. To further investigate the conservation of proteins, protein domains were analysed using Pfam (Punta et al., 2012) and SMART (Letunic & Bork, 2017; Schultz et al., 1998). As predicted by SMART and Pfam, the green hexagons represent C2 domains, small pink boxes are low complexity regions, blue boxes are transmembrane regions, and black boxes are domains found at the C-terminus of phosphoribosyl transferases and phosphoribosyl transferase-like proteins.

	Protein	Organism	Accession number	E-value	% identity
Query:	**Doc2A protein**	*Homo sapiens*	AAH41769.2		
Domain architecture					
	A9VA04_MONBE	*M. brevicollis*	XP_001749528.1	8e-15	27.88%
Domain architecture					
	PTSG_07483	*S. rosetta*	XP_004990989.1	5e-13	25.35%
Domain architecture					
	PTSG_11158	*S. rosetta*	XP_004987806.1	6e-13	29.04%
Domain architecture					
Query:	**A9VA04_MONBE**	*M. brevicollis*	XP_001749528.1		
	multiple C2 and transmembrane domain-containing protein 1 isoform 14	*Homo sapiens*	NP_001380474.1	1e-100	31.39%
Domain architecture					
Query:	**PTSG_07483**	*S. rosetta*	XP_004990989.1		
	multiple C2 and transmembrane domain-containing protein 1 isoform 13	*Homo sapiens*	NP_001380473.1	1e-95	31.79%
Domain architecture					
Query:	**PTSG_11158**	*S. rosetta*	XP_004987806.1		
	multiple C2 and transmembrane domain-containing protein 1 isoform 11	*Homo sapiens*	NP_001380471.1	7e-76	29.70%
Domain architecture					

Synaptobrevin in *S. rosetta*

Of the neurosecretory vesicle core protein homologues in *S. rosetta*, we examined the SNARE protein synaptobrevin more closely and compared it to human synaptobrevin 1 and 2 (**figure 2 a, b**). The amino acid sequence of *S. rosetta* synaptobrevin displays a sequence identity of 38% with human synaptobrevin 1 and 36% with human synaptobrevin 2 (**figure 2 b**). Since synaptobrevin and its homologue snc1/2 are localised on membranes of secretory vesicles in animals and yeast, we used it as a putative marker for secretory vesicles (Baumert et al., 1989; Protopopov et al., 1993). Immunostainings of *S. rosetta* single and colonial cells with an antibody directed against the cytosolic domain of *S. rosetta* synaptobrevin indicated the presence of synaptobrevin at the apical and basal pole of the cells (**figure 2 c–f**). Here, the anti-synaptobrevin signal seemed to overlap with signals of the anti-tubulin antibody (**figure 2 e, f**). In addition, we saw an overlap between cytoskeletal filaments (tubulin) and apical vesicles in transmission electron microscopy sections (**figure 2 g, h**). However, we did not observe any synaptobrevin antibody staining at putative plasma membrane contact sites of colonial *S. rosetta* cells (**figure 2 e´´**).

Vesicular landscapes in choanoflagellates

We investigated the quantity and diversity of intracellular vesicles in a *M. brevicollis* and *S. rosetta* cell by 3D reconstructions of the vesicular landscapes from serial ultrathin sections obtained by transmission electron microscopy (**figure 3**). Both species displayed diverse, polarised vesicular landscapes. Similar to the study by Laundon et al. (2019), we observed different vesicle populations with distinct morphologies. We assigned these vesicles to five types according to their size, location, and electron densities.

In the *M. brevicollis* cell, we detected 163 vesicles (**figure 3 a**, **b-f**, **b´-f´;** electronic supplementary material of Paper I, supplementary video 1). These include (1) electron-dense Golgi-associated vesicles (N = 79; mean diameter 54 nm) near the Golgi apparatus at the apical side of the cell, and between endoplasmic reticulum (ER) and

Golgi apparatus (**figure 3 b, b´**). Although vesicles between ER and Golgi cisternae display more heterogenous electron densities and often seem to be slightly more electron-lucent, they have the same size as vesicles near the Golgi apparatus at the apical side of the cell (**figure 3 b´**). Throughout the *M. brevicollis* cell and especially in the basal region there are (2) small electron-lucent vesicles (N = 51; mean diameter 72 nm; **figure 3 c**, **c´**). These are slightly larger, but otherwise resemble the electron-lucent vesicles between the Golgi apparatus and ER. (3) Apical vesicles (N = 6; mean diameter 116 nm) show a higher electron density and are present in low numbers at the apical pole of the cell (**figure 3 d**, **d´**). (4) Large extremely electron-lucent vesicles (N = 15; mean diameter 129 nm; **figure 3 e**, **e´**) are dispersed throughout the whole *M. brevicollis* cell. In the basal area of the cell soma are (5) large electron-dense vesicles (N = 12; mean diameter 129 nm; **figure 3 f**, **f´**).

In the *S. rosetta* cell, we found 314 vesicles (**figure 3 a**, **g-k**, **g´-k´**; electronic supplementary material of Paper I, supplementary video 2). Here we also observed (1) electron-dense Golgi-associated vesicles (N = 206; mean diameter 55 nm) near the Golgi apparatus at the apical side of the cell (**figure 3 g, g´**). In contrast to *M. brevicollis*, we detected (2) small electron-lucent vesicles (N = 31; mean diameter 79 nm) mainly in the apical region in *S. rosetta* (**figure 3 h**, **h´**). These resemble the Golgi-associated vesicles observed between the Golgi apparatus and the ER in the *M. brevicollis* cell (**figure 3 b**, **b´**) and the small vesicles of *M. brevicollis* (**figure 3 c, c´**) due to their homogenous electron density. Near the apical complex of the *S. rosetta* cell are (3) larger apical vesicles (N = 39; mean diameter 175 nm) (**figure 3 i**, **i´**). In comparison with the apical vesicles of *M. brevicollis*, those of *S. rosetta* often have a more ovoid shape and are more electron-lucent. (4) Large extremely electron-lucent vesicles (N = 28; mean diameter 209 nm) can be found in the whole cell soma of the *S. rosetta* cell (**figure 3 j**, **j´**). The few (5) medium-sized, electron-lucent vesicles (N = 10; mean diameter 129 nm) are scattered throughout the *S. rosetta* cell (**figure 3 k**, **k´**).

We found similarities and differences between the vesicle types of the two choanoflagellate species, which also differed in number (**figure 3 l**). Four of the vesicle

types were common to both species (Golgi-associated vesicles, small electron-lucent vesicles, apical vesicles and large extremely, electron-lucent vesicles), whereas large electron-dense vesicles and medium-sized, electron-lucent vesicles appear to be present in only one species each. The Golgi-associated vesicles of both species have a similar mean diameter but are 2.6 times more abundant in *S. rosetta*. Small vesicles also display a similar mean diameter but are slightly larger in *S. rosetta*. Moreover, they are 1.6 times more abundant in *M. brevicollis* and differ in their localisation (**figure 3 c**, **h**). Apical vesicles are 6.5 times more abundant in *S. rosetta* and their mean diameter is 1.5 times larger in *S. rosetta*. In addition, their shape is spherical in *M. brevicollis*, whereas they are spherical to ovoid in *S. rosetta*. Large extremely electron-lucent vesicles are 1.9 times more abundant in *S. rosetta* and their mean diameter is 1.6 times larger in *S. rosetta*. The large electron-dense vesicles of *M. brevicollis* appear to be a different type of vesicles from the medium-sized, electron-lucent vesicles of *S. rosetta*, as they show no similarities (**figure 3 f**, **k**).

Since a single cell of each species was examined in paper I, it should be noted that there may be cell-to-cell variations within the same species (Laundon et al., 2019). Therefore, conclusions based on comparisons between the vesicles of the two species should be considered very carefully. Nevertheless, our results revealed that both species exhibit diverse, polarised vesicular landscapes in which vesicles can be assigned to different types.

3.2. Characteristics of secretory synaptobrevin homologues in the closest unicellular relative of animals (Paper II)

The aim of paper II was to analyse and describe secretory synaptobrevin homologues of *S. rosetta*. In neurons, synaptobrevin1/2 is localised on the membrane of synaptic vesicles and is one of the key proteins for synaptic vesicle exocytosis. There it forms a complex with the two presynaptic membrane-bound SNARE proteins SNAP-25 and syntaxin, which is crucial for transmitter release (Baumert et al., 1989; Bennett et al.,

1992; Oyler et al., 1989; Schiavo et al., 1992; Söllner et al., 1993b; 1.2. SNAREs). Through a homology-based search against the *S. rosetta* database at NCBI, we found two homologues of *Homo sapiens* synaptobrevin1/2 in *S. rosetta*. These are potentially involved in exocytosis according to searches against the SNARE Database (Kloepper et al., 2007). One of these synaptobrevins was the one we have already started to examine in paper I. In paper II, we named this synaptobrevin homologue synaptobrevin 1. We have named the other synaptobrevin we found in the genome of *S. rosetta*, synaptobrevin 2 (**Figure 1 A**). The expression of synaptobrevin 2 was verified by PCR-based amplification and sequencing of cDNA from single and rosette colony-forming cells of *S. rosetta* (**Figure 1 B, Figure S1**). *S. rosetta* therefore possibly possesses two exocytotic v-SNAREs.

Functional analysis of synaptobrevin in *S. rosetta*

We have attempted to analyse the function of the two synaptobrevins in *S. rosetta* by CRISPR/CAS9-mediated knockouts. For this purpose, we designed different guide RNAs to target Cas9 to the DNA region encoding for the cytosolic part of synaptobrevin 1 and 2. The associated DNA repair templates were designed with homology arms flanking the cleavage site 50 nucleotides each upstream and downstream. Downstream of the cleavage site, a stop cassette for premature termination of synaptobrevin was included in the repair templates (**Figure 2 A**). Repair templates and pre-assembled ribonucleoprotein complexes consisting of Cas9 pre-loaded with a single guide RNA were delivered into *S. rosetta* cells by nucleofection. The nucleofection step, with a genome editing frequency of ~1%, is believed to be the biggest limiting factor for the genome editing efficiency (Booth & King, 2020; Booth et al., 2018).

To increase the likelihood of enriching for edited cells, we used two different selection techniques. First, we co-transfected the *S. rosetta* cells with another ribonucleoprotein complex and a repair template mediating cycloheximide resistance (rpl36a^{P56Q}) (Booth & King, 2020). This allowed us to select cells for cycloheximide resistance for four

days prior to clonal isolation. For the positive and negative control, cells were transfected only with rpl36a^{P56Q}, with and without Cas9. During selection, we observed cell growth in the positive control (cells transfected with rpl36a^{P56Q}+Cas9), the two synaptobrevin 1-targeting knockouts+rpl36a^{P56Q} and the synaptobrevin 2-targeting knockout+rpl36a^{P56Q}. In contrast, no cell growth was observed in the negative control (cells transfected with rpl36a^{P56Q} without Cas9). These results indicated the successful delivery and incorporation of the cycloheximide resistance-mediating mutation. After selection, DNA from cycloheximide-resistant *S. rosetta* cells was isolated and screened for the integration of the stop cassette by PCR-based amplification and treatment with the restriction enzyme PacI. Since the stop cassette contains a PacI recognition site, which is normally absent in the genes encoding synaptobrevin 1 and 2, a successfully edited gene would be digested by PacI in two fragments. This is demonstrated by a shift on an agarose gel compared to undigested or non-edited DNA. Of all samples screened for the presence of the stop cassette in the DNA encoding for synaptobrevin 1 and 2, none showed digestion products of PacI (**Figure 2 C, D**). This means that despite the observed cell growth during cycloheximide selection, which indicates successful delivery and editing of rpl36a^{P56Q}, no successful synaptobrevin 1 knockout could be observed.

To avoid losing cells carrying the edited gene due to the duration of the selection process, we used an alternative, faster selection technique (**Figure 2 B**). For this, cells were transfected with a ribonucleoprotein complex containing the enzyme Cas9-RFP. This allowed clonal isolation of the transfected cells by fluorescence-activated cell sorting (FACS) already one day after transfection. After clonal isolation, we could observe proliferating cells in all samples. These included cells transfected with the two synaptobrevin 1-targeting knockouts and the synaptobrevin 2-targeting knockout. However, the presence of edited synaptobrevin 1 or synaptobrevin 2 could not be detected by PacI digestion (**Figure 2 E-G**). Thus, the presence of successful synaptobrevin 1 and 2 knockouts could not be confirmed after a faster selection procedure.

Since we assumed the knockouts could potentially be lethal, we tried to rescue one of the synaptobrevin 1 knockouts. For this, *S. rosetta* cells were first transfected with the synaptobrevin 1-targeting knockout. After 4 h of recovery, cells were co-transfected with a plasmid encoding for synaptobrevin 1. Following clonal isolation by FACS sorting and five days in which the cells were allowed to grow, we isolated the DNA of the co-transfected cells. Although, we detected the synaptobrevin 1 plasmid (**Figure 2 I**), we could not find an edited synaptobrevin 1 (**Figure 2 H**). Accordingly, no synaptobrevin 1 knockout could be observed after co-transfection with a synaptobrevin 1 plasmid.

Comparative cleavage assay of human and *S. rosetta* synaptobrevin

To further characterise synaptobrevin 1 of *S. rosetta* and compare it to human synaptobrevin 2, we performed a comparative cleavage assay. For this assay, we used botulinum neurotoxin D light chain (BoNT/D-LC), a highly specific protease that cleaves human synaptobrevin 2, 3 (cellubrevin) and to a minor extent synaptobrevin 1 (Yamamoto et al., 2012). The protein sequence of *S. rosetta* synaptobrevin 1 contains five amino acids at key positions that are assumed to be important for substrate recognition by BoNT/D-LC (**Figure 3 A**). These are methionine at position 25 (46*), valine 21 (42*), arginine 26 (47*), lysine 31 (52*) and arginine 35 (56*), all of which are also present in human synaptobrevin 2 at positions marked with asterisks (Arndt et al., 2006; Yamasaki et al., 1994). We incubated equal concentrations and volumes of recombinant *S. rosetta* synaptobrevin 1 without the transmembrane domain (rec. *Sros* Syb1 [1-75]) or recombinant human synaptobrevin 2 (without most of the transmembrane domain, rec. human Syb [1-96]) with two different concentrations of recombinant BoNT/D-LC (rBoNT/D-LC). The negative controls contained recombinant proteins mixed with assay buffer. The samples were analysed by SDS-PAGE and Coomassie staining (**Figure 3 B**). Recombinant *S. rosetta* synaptobrevin 1 [1-75] has a molecular weight of 9.27 kDa and human synaptobrevin 2 [1-96] of 10.5 kDa. Both displayed slightly higher bands than their predicted molecular weights (**Figure 3 B**). Similar differences between predicted molecular weight and migration

distance on SDS gels have been reported in other studies for synaptobrevin (Baumert et al., 1989; Zeng et al., 1998). These could be due to the low Grand average of hydropathicity (GRAVY) values of recombinant *S. rosetta* synaptobrevin 1 (-1.463) and recombinant human synaptobrevin 2 (-0.649). Their low GRAVY values may lead to lower binding of sodium dodecyl sulphate and thus lower electrophoretic mobility of the proteins (Scheller et al., 2021; Shirai et al., 2008). Recombinant *S. rosetta* synaptobrevin 1 shows two bands on SDS gels (**Figure 3 B**; **Figure S2**). The lower band is probably the result of protein degradation by non-specific thrombin cleavage. According to our result, both concentrations of rBoNT/D-LC led to cleavage of recombinant human synaptobrevin 2. In comparison, no cleavage could be observed for recombinant *S. rosetta* synaptobrevin 1 [1-75] by rBoNT/D-LC (**Figure 3 B**).

Potential interaction partners of *S. rosetta* synaptobrevin 1

Potential interaction partners of synaptobrevin 1 in *S. rosetta* were analysed by co-immunoprecipitation (co-IP) and label-free mass spectrometry analysis (**Figure 4 A**). We performed two independent co-IP experiments with the synaptobrevin 1 antibody already used for immunostainings in paper I (**figure 2 c-f**). In both experiments, we used a co-IP with another antibody (anti-shank) as negative control. Of the two experiments, all eluates, supernatants, last wash samples, and the *S. rosetta* cell lysate were examined for the presence of synaptobrevin 1 by SDS-PAGE and Western blot analysis (**Figure 4 B and C**). We detected a synaptobrevin 1 signal in the *S. rosetta* cell lysate, all supernatants, and the eluate samples of the synaptobrevin 1 co-IPs (using purified and non-purified antibody). Compared to the other samples of the first co-IP experiment, the signal of the eluate of the synaptobrevin 1 co-IP seemed to be the strongest (**Figure 4 B**). Since less eluate was loaded in comparison to the other samples, this result indicates a successful enrichment of synaptobrevin 1 by co-IP.

For the first co-IP experiment, we used non-affinity purified synaptobrevin 1 antibody. Using mass spectrometry analysis, we identified 171 proteins that were exclusively present in the eluate of the synaptobrevin 1 co-IP (**Table S2**). This list of identified

proteins was compared to proteomic analysis of isolated synaptic vesicles (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020). Besides synaptobrevin, four cytoskeletal proteins, two signalling molecules, seven metabolic enzymes, two chaperones, the small GTPase-related protein Rab9, annexin, the V-ATPase subunit H, a subunit of the oligosaccharyl transferase complex, von Willebrand factor A (VWFA) domain-containing protein, a mitochondrial, three proteasomal and 18 ribosomal proteins were present in the synaptobrevin 1 co-IP and in synaptic vesicle isolates (**Table S2**) (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020). Three of these proteins (synaptobrevin, V-ATPase subunit H and tubulin beta chain) were among the most abundant protein species in the synaptic vesicle isolates studied by Taoufiq et al. (2020). Two other proteins that were co-immunoprecipitated with synaptobrevin 1 are folate gamma-glutamyl hydrolase and transmembrane protein 35A. The folate gamma-glutamyl hydrolase is a peptidase known to be secreted and present in lysosomes or vacuoles (Bakthavatsalam & Gomer, 2010; Barrueco et al., 1992; O'Connor et al., 1991; Orsomando et al., 2005; Yao et al., 1996). Transmembrane protein 35A (or novel acetylcholine receptor chaperone (NACHO)) is localised in the ER of neurons, where it is involved in the assembly of nicotinic acetylcholine receptors (Gu et al., 2016; Matta et al., 2017). In summary, using non-affinity purified synaptobrevin 1 antibody, synaptobrevin 1 was enriched together with 170 other proteins from *S. rosetta* cell lysate in the first synaptobrevin 1 co-IP.

To validate the specificity of the 171 co-precipitated proteins in the first synaptobrevin 1 co-IP, the experiment was repeated using non-affinity and affinity-purified synaptobrevin 1 antibody in parallel. In addition, another negative control containing only magnetic beads without antibodies ("beads only") was included and an optimised ratio of beads to *S. rosetta* lysate with higher protein concentrations was used. However, for the co-IP with affinity-purified synaptobrevin 1 antibody, smaller amounts of antibody had to be used, as the purification process caused a large loss of antibody protein concentration. According to the Western blot results of the second, optimised co-IP experiment, synaptobrevin 1 was successfully enriched in the eluate samples of both synaptobrevin 1 co-IPs (performed with affinity- and non-affinity purified antibody, **Figure 4 C**).

We identified 88 proteins exclusively present in the two synaptobrevin 1 co-IPs of the second, optimised experiment by mass spectrometry analysis (**Table S3**). Of these, 87 proteins were enriched in the co-IP with the non-affinity purified synaptobrevin 1 antibody. Five proteins were enriched in the co-IP performed with affinity-purified synaptobrevin 1 antibody. Compared to the co-IP with non-affinity purified synaptobrevin 1 antibody performed in parallel, this is 17.4 times fewer proteins. Four of these proteins (synaptobrevin 1, two metabolic enzymes, and an RNA helicase) were also present in the co-IP with non-affinity purified synaptobrevin 1 antibody performed in parallel and in synaptic vesicle isolates (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020). Accordingly, synaptobrevin 1 was also successfully enriched in the second, optimised co-IP experiment with affinity- and non-affinity-purified synaptobrevin 1 antibody. Here, substantially fewer proteins were co-enriched using the affinity-purified antibody.

Several of the proteins identified in the second, optimised co-IP experiment with non-affinity purified synaptobrevin 1 antibody were also found in proteomic studies on isolated synaptic vesicles. Among these were the SNARE regulating ATPase NSF, gamma-soluble NSF attachment protein (γ-SNAP), dynamin, the voltage-dependent anion-selective channel protein 2, a cytoskeleton protein, four metabolic enzymes, the signalling protein Major vault protein and the mitochondrial dynamin-like GTPase OPA1 (**Table S3**) (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020).

Other proteins that were identified in the second, optimised experiment included the translocon-associated protein subunit delta and a Notch-like protein. Although the former protein was assigned as translocon-associated protein subunit delta, according to our homology-based search against the *Homo sapiens* database at NCBI, it could also be a 40-kDa Huntingtin-associated protein homologue (**Table S4**). A complex of 40-kDa Huntingtin-associated protein and Huntingtin is believed to be involved in the regulation of early endosome motility through interaction with Rab5 (Pal et al., 2006). The protein Notch is part of an evolutionarily conserved family that, among many other important cellular processes, is also involved in intercellular communication (Gazave et al., 2009; Kopan & Ilagan, 2009; Vlachakis et al., 2020). The canonical protein

domain architecture of Notch consists of one to several epidermal growth factor repeats, a Notch domain, transmembrane and Ankyrin domains in the order listed here (Gazave et al., 2009; Kidd et al., 1986; Richter et al., 2018; Wharton et al., 1985). Since the Notch-like protein identified here lacks the Notch and Ankyrin domains but contains repeats of the epidermal growth factor and two transmembrane regions that are also common in other proteins, it cannot be regarded as a clear Notch homologue (Gazave et al., 2009; Kidd et al., 1986; Richter et al., 2018; Wharton et al., 1985).

Of the proteins enriched in the first and second, optimised experiment, eleven proteins were present in both datasets (**Table S2**, **Table S3**, indicated with ●). In addition to synaptobrevin 1, these also included two IPT/TIG domain-containing proteins, dual oxidase maturation factor 1, CYRIA/CYRIB Rac1 binding domain-containing protein, uncharacterised protein PTSG_00332, four metabolic enzymes and AKT protein kinase. Of these, synaptobrevin and the metabolic enzyme inosine-5'-monophosphate dehydrogenase were also found in synaptic vesicle isolates (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020).

IPT/TIG domains (immunoglobulin, plexins, transcription factors-like/transcription factor immunoglobulin) are present, for example, in the animal exocyst complex component 2 (also called sec5) (Aravind & Koonin, 1999; Kee et al., 1997). The exocyst is a highly conserved complex involved in vesicle trafficking (Koumandou et al., 2007; Mei & Guo, 2018). When we used human exocyst complex component 2 as a query for a homology-based search against the *S. rosetta* database on NCBI, we discovered that it exhibits a 30.85% protein sequence identity with one of the co-precipitated IPT/TIG domain-containing proteins (PTSG_12887) (E-value=5e-04, **Table S4**). However, we did not detect a sec5 domain in the IPT/TIG domain-containing protein of *S. rosetta*.

The intensity of synaptobrevin 1 was one of the strongest signals measured in both co-IP experiments (**Table S2**, **Table S3**). Although comparisons between independently measured mass spectrometry samples without a standard must be considered very carefully, we assume from the strong signal that the enrichment of

synaptobrevin 1 was successful. It should be noted that all identified proteins in both co-IP experiments were annotated using the UniProt database. Future analyses of the domain architecture of each potential interaction partner will either confirm the previous annotations or possibly suggest alternative assignments.

Localisation of synaptobrevin 1 in *S. rosetta*

In paper I, we showed that the anti-synaptobrevin 1 antibody detects an antigen at both poles of *S. rosetta* cells, where we also observed numerous vesicles (Paper I: **figure 2 c–f**). However, it is still unclear whether synaptobrevin 1 is localised on secretory vesicles in *S. rosetta*, similar to animals or yeast (Baumert et al., 1989; Protopopov et al., 1993). Therefore, we examined the localisation of synaptobrevin 1 using a subcellular fractionation protocol and the synaptobrevin 1 antibody. For this, we mechanically homogenised *S. rosetta* cells and separated subcellular fractions by differential centrifugation (**Figure 5 A**). All obtained fractions were analysed for the presence of synaptobrevin 1 by SDS-PAGE and Western blot analysis with the synaptobrevin 1 antibody (**Figure 5 B**). The homogenate (H) and pelleted non-homogenised *S. rosetta* cells (control pellet = PC and control = C) showed a synaptobrevin 1 signal. As expected, no signal was observed in the two supernatants that were supposed to be free of *S. rosetta* cells, such as the bacterial supernatant (BS) obtained from pooling the *S. rosetta* cells and the supernatant of the non-homogenised control cells (SC). The homogenate was centrifugated at low speed to pellet nuclei, cellular debris, and unbroken cells (pellet 1 = P1) (Begovic, unpublished; Liu & Fagotto, 2011; Michelsen & Von Hagen, 2009; Walworth & Novick, 1987; Zinser et al., 1991). The viscosity of the sample and the smeared signal of pellet 1 (P1) was an indicator for the presence of large amounts of nucleic acids (Kurien & Scofield, 2012). Pellet 2 (P2), obtained by centrifugation of supernatant 1 (S1) at increased speed, was also viscous and gave a smeared signal. This could be due to mitochondrial DNA, since mitochondria were supposed to be pelleted by second centrifugation step. Both supernatants (S1 and S2) showed a clear synaptobrevin 1 signal, indicating a successful homogenisation of *S. rosetta* cells. Moreover, the supernatants exhibited

fewer non-specific bands, with S2 showing even fewer non-specific bands than S1. Based on previous studies, we assumed that the supernatant S2, obtained by the second centrifugation step at increased speed, still contained the membrane and cytosolic fractions. In contrast, the nuclear (P1) and the mitochondrial fractions (S1, P2) should be largely absent in S2 (Begovic, unpublished; Liu & Fagotto, 2011; Michelsen & Von Hagen, 2009; Walworth & Novick, 1987; Zinser et al., 1991). The result therefore indicated that synaptobrevin 1 is present in the membrane and cytosolic fraction.

4. Discussion

4.1 The premetazoan origin of neurosecretory vesicle protein homologues

In paper I, we used a comparative cross-species analysis to investigate the presence or absence of neurosecretory vesicle core proteins in different eukaryotic organisms. In line with previous studies, our findings revealed that many of the neurosecretory vesicle core proteins appear to be conserved as homologues in unicellular eukaryotes (Abrams & Sossin, 2019; Burkhardt et al., 2014; Burkhardt et al., 2011; Moroz & Kohn, 2015) (**figure 1**). This indicates that many of the neurosecretory vesicle core proteins may have evolved before the occurrence of the first animals and thus the first neurons. Some of the proteins we found in the predicted proteomes of the choanoflagellate species *S. rosetta* and *M. brevicollis* are involved in the regulated exocytosis of neurosecretory vesicles. Among these are a complete set of putative secretory SNAREs (synaptobrevin, syntaxin and SNAP-25), secretory carrier-associated membrane proteins (SCAMPs), synaptic vesicle protein 2 (SV2), synaptogyrin, and SV2-related protein (SVOP) (Buckley & Kelly, 1985; Ciruelas et al., 2019; Liao et al., 2007; Sugita et al., 1999; Zhang & Castle, 2011; Zhang et al., 2015). As previously shown by Burkhardt et al. (2014; 2011), complexin and Munc18, two other key proteins for regulated vesicle exocytosis, are also conserved in choanoflagellates. The presence of all these proteins in the closest unicellular relatives of animals suggests that they may have been involved in regulated secretion before the first nervous systems evolved.

So far, we could not find a homologue of the calcium sensor synaptotagmin in organisms other than animals. This result is consistent with other studies and suggests that synaptotagmin may have originated in animals (Barber et al., 2009; Burkhardt et al., 2014; Craxton, 2007, 2010). However, this needs to be confirmed by future studies on other close relatives of animals, as it could also have been lost in the course of evolution (Richter et al., 2018).

Other proteins similar to synaptotagmin have been identified in plants and yeast (Barber et al., 2009; Craxton, 2007, 2010; Creutz et al., 2004; Manford et al., 2012; Saheki & De Camilli, 2017). Although these proteins are also called synaptotagmins in plants, they are more similar to mammalian extended-synaptotagmins or tricalbins in yeast. They all contain an additional domain called Synaptotagmin-like, Mitochondrial and lipid-binding Protein (SMP) domain and function as ER to plasma membrane tethers (Craxton, 2010; Giordano et al., 2013; Manford et al., 2012; Pérez-Sancho et al., 2015; Ruiz-Lopez et al., 2021; Toulmay & Prinz, 2012).

Here, I show proteins of *S. rosetta* and *M. brevicollis* which display over 25% sequence similarity to Doc2 (**Table 1**). Doc2 is another calcium sensor in neurons that could be responsible for spontaneous or asynchronous transmitter release (Courtney et al., 2018; Díez-Arazola et al., 2020; Yao et al., 2011). In contrast to the choanoflagellate proteins shown here, vertebrate Doc2 has only two C2 domains (Craxton, 2010; Orita et al., 1995). Multiple C2 domains are, for example, present in the extended-synaptotagmins (or tricalbins in yeast) (Creutz et al., 2004; Min et al., 2007). These, however, possess the aforementioned characteristic SMP domain, which is not present in the choanoflagellate proteins shown here (**Table 1**; Lee & Hong, 2006). Reciprocal BLAST searches using the identified *S. rosetta* protein sequences as queries against the human proteome revealed a sequence identity of ~30% for multiple C2 and transmembrane domain-containing protein 1 (MCTP1). In fact, the protein domain architecture was also more reminiscent of MCTP1 than of Doc2 (**Table 1**). MCTPs are evolutionarily conserved. Studies have shown that MCTPs are mainly present in multicellular eukaryotes, like animals and plants, whereas they seem to be absent in the unicellular organisms studied, with the exception of choanoflagellates (Barber et al., 2009; Liu et al., 2018; Shin et al., 2005; Téllez-Arreola et al., 2022; Zhao et al., 2022). Although the functions of MCTPs are not entirely clear, recent studies suggest that MCTPs are involved in neurotransmitter release, perhaps as endoplasmic reticulum-localised calcium sensors (Genç et al., 2017; Téllez-Arreola et al., 2020). It remains to be seen whether the choanoflagellate proteins identified here are calcium sensors or whether they are involved in other tasks.

4.2 The evolution of regulated secretion and directed vesicle transport at cell-cell contact sites

Regulated secretion, the basis for neurotransmitter release, appears to occur not only in animals, but can also be observed in unicellular eukaryotes (Bresslau, 1921; Chin et al., 2004; Munoz et al., 1991; Satir, 1977). So far, however, only limited information is available on the molecular details of regulated secretion in unicellular organisms (Aquilini et al., 2021; Coleman et al., 2018; Kuppannan et al., 2022; Sparvoli et al., 2018). Alveolates, for example, which are distantly related to opisthokonts, exhibit regulated secretion, e.g. for the purpose of host invasion, predation or defence against predators (Buonanno et al., 2014; Ewing & Kocan, 1992; Harumoto & Miyake, 1991; Keeling et al., 2014; Sparvoli & Lebrun, 2021; Steenkamp et al., 2005). Some of the proteins involved in the regulated exocytosis of alveolates are conserved in ciliates, dinoflagellates, and apicomplexans, but appear to be restricted to alveolates (Aquilini et al., 2021; Kuppannan et al., 2022). During invasion, apicomplexan parasites inject rhoptry proteins into the host cell (Boothroyd & Dubremetz, 2008; Håkansson et al., 2001; Nichols et al., 1983). To date, no SNARE proteins have been identified that are involved in the regulated rhoptry exocytosis (Aquilini et al., 2021; Sparvoli & Lebrun, 2021). In general, it is still largely unknown whether and to what extent the molecular mechanisms of regulated exocytosis in animals and the various unicellular organisms share similarities and if they have a common origin (Briguglio et al., 2013; Coleman et al., 2018; Kaur et al., 2017; Kuppannan et al., 2022; Sparvoli & Lebrun, 2021; Sparvoli et al., 2018; Verbsky & Turkewitz, 1998).

In yeast, secretory vesicles do not appear to be accumulated and released by regulated exocytosis, but constitutively (Novick & Schekman, 1979; Walworth & Novick, 1987). However, in choanoflagellates, the closest unicellular relative of animals, it is not clear whether vesicles may also be released by regulated exocytosis. The presence of the previously mentioned proteins, which are known to be involved in the release and regulation of synaptic vesicles, suggests a mechanism of regulated secretion in choanoflagellates (**figure 1**).

In paper I, we 3D reconstructed the vesicular landscapes of two choanoflagellate species *S. rosetta* and *M. brevicollis*. This allowed the identification of distinct vesicle populations (**figure 3**). Based on the size, location, and electron densities, we identified five different types of vesicles in each species. These included Golgi-associated, small electron-lucent, apical, large electron-lucent and large electron-dense vesicles or medium-sized, electron-lucent vesicles. Some of the vesicles were difficult to classify as they displayed intermediate features of different types. This illustrates the high dynamic nature of these organelles and is represented by the large whiskers in **figure 3 l**. Nevertheless, the properties of the vesicle types differ considerably from each other, indicating that our classification is likely correct.

However, further molecular studies could allow the classification of additional vesicle types. This could, for example, enable a subdivision of Golgi-associated vesicles. Apical Golgi-associated vesicles could possibly be *trans*-Golgi vesicles that are transported to different destinations in the cell, while vesicles localised between ER and Golgi could be anterograde and retrograde vesicles (COPII/COPI coated vesicles, see also chapter 1.1).

In both choanoflagellate species, we observed vesicle populations at the apical and basal pole of the cell. The polarised distribution of the vesicular landscapes in both choanoflagellate species is reminiscent of the clustered organisation of synaptic vesicles in presynapses of the nervous system (Richardson, 1962). Based on our immunostaining results in paper I (**figure 2 c-f**), we suspect that the homologue of the synaptic vesicle protein synaptobrevin is also localised at both cell poles of *S. rosetta.* These results are consistent with a previous study on *M. brevicollis* where immunostainings indicated the presence of synaptobrevin at the apical pole of the cell (Burkhardt et al., 2011). Considering the presence of a presumably secretory synaptobrevin at both cell poles, we assume that the apical and basal vesicles are secretory in *S. rosetta*.

Vesicles localised at the apical pole of *S. rosetta* are in close proximity to tubulin filaments (**figure 2 g, h**). Together with the overlapping immunostaining signals of

synaptobrevin and the cytoskeleton protein tubulin in *S. rosetta* (**figure 2 f**), these results suggest a directed transport system in choanoflagellates where vesicles are trafficked along the cytoskeleton.

So far, we do not know what these vesicles contain or what functions they have. Despite this, it is tempting to speculate that vesicles localised at the basal pole of *S. rosetta* may contain rosetteless and/or other extracellular matrix material. The secretion of these components from the basal part of *S. rosetta* cells appears to be crucial for rosette development (Dayel et al., 2011; Larson et al., 2020; Levin et al., 2014; Wetzel et al., 2018). The protein rosetteless was identified by Levin et al. (2014) in a forward genetic screen of *S. rosetta* cells displaying defects in rosette development in the presence of rosette-inducing factors. One of these mutants had a defect in a C-type lectin, which turned out to be essential for the formation of rosette colonies. This C-type lectin was subsequently named "rosetteless". Rosetteless has a predicted secretion signal and is basally secreted into the extracellular matrix of rosette colonies. In the same study they showed that lysates of rosettes, single cells and chain colonies of *S. rosetta* contain similar amounts of rosetteless. Immunostainings of these three stages, on the other hand, indicated different quantities of rosetteless. Rosette colonies showed the strongest signal for rosetteless when analysed by immunostaining, whereas the other two stages showed weak to no signals (Levin et al., 2014). This could mean that rosetteless is only secreted during rosette colony development and is otherwise stored in vesicles in *S. rosetta* cells, where it may not be recognised by the rosetteless antibody. Therefore, rosetteless-containing vesicles are potential candidates for regulated secretion, that are possibly stored during the unicellular state. Apical vesicles, on the other hand, may contain enzymes that are released in direction of the feeding collar for extracellular digestion, similar to the enzymes released by yeast (Berthelot, 1860; Novick & Schekman, 1979; Sanchez et al., 1984). It is also possible that these vesicles are used for intercellular communication (Rosati & Modeo, 2003). Based on our finding that putative sialin-like transporters exist in the genomes of *S. rosetta* and *M. brevicollis*, the apical vesicles could contain sialic acid, aspartate, or glutamate (Miyaji et al., 2008). Future studies on isolated vesicles from choanoflagellates (which are currently in preparation in our laboratory) promise to reveal more about the content

of these vesicles. In addition, further 3D reconstructions of the vesicular landscapes of different cell stages of *S. rosetta*, as performed by Laundon et al. (2019) and in paper I, will show whether the vesicle populations themselves or their amounts change. In the study by Laundon et al. (2019), a higher number of Golgi-associated vesicles was observed in *S. rosetta* single cells compared to colonies. This could indicate an increased secretion of vesicles containing, for example, rosetteless, extracellular matrix material or other components important for rosette development (Dayel et al., 2011; Levin et al., 2014; Wetzel et al., 2018).

As mentioned earlier, rosette colonies of *S. rosetta* are connected by fine intercellular bridges of unknown molecular composition (Dayel et al., 2011). These bridges have electron dense-plates and are suggested to be involved in intercellular communication (Chaigne & Brunet, 2022; Colgren & Burkhardt, 2022; Dayel et al., 2011; Laundon et al., 2019). Since we did not observe synaptobrevin localisation or vesicle accumulations at these cellular connections, an exchange of signalling molecules via intercellular bridges would likely be independent of vesicle exocytosis and thus different from chemical neurotransmission (paper I, Laundon et al., 2019). However, small signalling molecules or ions could still be exchanged (Dayel et al., 2011). Cytoplasmic bridges in plants are used for the transfer of metabolites, but also serve for the transmission of electrical signals (Canales et al., 2018; Mackie, 1970; Meiners et al., 1988). It has been hypothesised that in animals, functionally similar conductions that also transport metabolites originally formed the basis for specialised conduction by muscles, neurons and neurosensory cells (Mackie, 1970; Meiners et al., 1988). Although gap junctions are not connected by a continuous membrane like cytoplasmic bridges, and the key proteins for the formation of gap junctions (innexins/pannexins and connexins) at electrical synapses appear to be an animal innovation, intercellular bridges of choanoflagellates may still share some similarities with electrical signal transmission (Cai, 2008; Colgren & Burkhardt, 2022; Moroz & Kohn, 2015). Moreover, there are possibly other, so far unknown proteins that form gap junctions in animals which do not possess connexins or pannexins (Slivko-Koltchik et al., 2019). Recent studies have shown that filamentous cyanobacteria use septal junctions for cellular communication that are mechanistically similar to gap

junctions (Kieninger & Maldener, 2021; Weiss et al., 2019). In general, it cannot be ruled out that choanoflagellates have functionally similar intercellular channels that may allow intercellular communication.

In summary, in paper I we observed several features in *M. brevicollis* and *S. rosetta* which are also present in neurons, highly specialised secretory cells. These include a large number of synaptic protein homologues, polarised vesicular landscapes, overlapping synaptobrevin and tubulin staining indicating a directed transport system, and plasma membrane contact sites in the case of multicellular *S. rosetta* cells (Bentley & Banker, 2016).

In paper I, we propose a scenario that could explain the similarity between choanoflagellates and neurons. According to our findings, several features and structural components of neurosecretory vesicles appear to be ancient. We therefore suspect that a basic vesicle secretion machinery was already used by the last common ancestor of animals and choanoflagellates for the secretion of signalling molecules or other substances. Although this premetazoan vesicle secretion system may have already been used for intercellular communication, it was not yet positioned at cellular contact sites. Instead, signalling molecules were released into the environment. With the emergence of animals and increasing number of cells within an organism, this machinery was shifted to contact sites between cells. Under these conditions, the localisation of signal transmission would have been an evolutionary novelty and would have provided the basis for specific pre- and postsynaptic signalling. The ancestral directed transport and vesicle secretion system would thus have been co-opted for signal transmission at cellular contact sites. This may have been the foundation for the evolution of the many structurally distinct types of synapses, such as presynaptic triads in ctenophores, or neuromuscular synapses in many other animals (Hernandez-Nicaise, 1968; Heuser & Reese, 1973; Palade, 1954; Robertson, 1956). Further future studies investigating the molecular machinery for intercellular communication in unicellular relatives of animals will test the validity of this scenario.

4.3 Studies of *S. rosetta* synaptobrevin allow comparisons with the secretory synaptobrevins of animals (Paper II)

Synaptobrevin1/2 and its yeast homologue snc1/2 are the exocytotic SNARE proteins that are localised on the membrane of secretory vesicles (Baumert et al., 1989; Protopopov et al., 1993). They are crucial components of the basic vesicle secretion apparatus in eukaryotes (Baumert et al., 1989; Bennett et al., 1992; Protopopov et al., 1993; Schiavo et al., 1992; Söllner et al., 1993b; 1.2. SNAREs). To learn more about the evolutionary history of one of the key proteins for vesicle exocytosis, we investigated the properties of secretory synaptobrevin homologues in choanoflagellates. These included studies of the function, sensitivity to rBoNT/D-LC, identification of potential interaction partners and the localisation of synaptobrevin in *S. rosetta*.

When we tried to knock out putative secretory synaptobrevins in *S. rosetta* using the CRISPR/CAS9 system, we could not detect any cells containing the edited DNA of synaptobrevin 1 and 2 (**Figure 2 C-H**). One explanation for the absence of the edited genes could be the low gene editing efficiency (~1%). The efficiency is believed to be mainly limited by the delivery of the ribonucleoprotein complex and repair templates (Booth & King, 2020). However, the successful co-transfection of the synaptobrevin 1 plasmid (**Figure 2 I**) and rpl36a^{P56Q}, as indicated by cell growth under cycloheximide selection, suggested that the delivery of Cas9 and repair templates was likely not the limiting factor. We used two different selection techniques to increase the chances of enriching cells carrying edited synaptobrevin 1 and 2. The second selection technique aimed to shorten the time between transfection and clonal isolation to reduce the loss of possibly genome-edited cells (**Figure 2 B**). We could not detect any edited synaptobrevin here either (**Figure 2 E-H**). Although we used different guides and targeted two different genes to minimise the chance of failure due to low binding efficiency, the designed sequences may not have been optimal. Since not only the complementarity between the guide RNA and target DNA, but also the presence of the protospacer-adjacent motif (PAM) is essential for recognition by Cas9 (Leenay & Beisel, 2017), we verified the presence of the target sequences in advance by

sequencing amplified DNA with appropriate primers. If the loss of synaptobrevin 1 and 2 causes a growth defect, it is also possible that successfully edited cells are outcompeted by cycloheximide-resistant or wild type cells. Another explanation could be that knocking out synaptobrevin 1 and 2 is lethal for *S. rosetta*, similar to animals or yeast (Liu et al., 2011; Nystuen et al., 2007; Protopopov et al., 1993; Schoch et al., 2001). If synaptobrevin 1 and 2 are essential and their knockouts prevent *S. rosetta* cells from proliferating, this would explain why we could not detect the edited genes in *S. rosetta* cells.

Transient overexpression of synaptobrevin 1 as fluorescent fusion protein also appears to significantly decrease cell growth of *S. rosetta*, suggesting an essential role (personal communication with Jeffrey Colgren). In summary, no functional phenotype of a synaptobrevin 1 or 2 knockout could be studied in detail with this approach. Since our results suggest that putative secretory synaptobrevin 1 and 2 may be essential for *S. rosetta*, its function could possibly be investigated in the future using an inducible knockdown system. In alveolates, yeast and different animal cells, for instance, the function of essential and presumably fitness-conferring genes was assessed using an auxin-inducible degron system, which allows rapid depletion of the targeted protein (Adhikari et al., 2021; Aquilini et al., 2021; Natsume et al., 2016; Nishimura et al., 2009). Alternatively, CRISPR/Cas9-mediated synaptobrevin 1 and 2 knockouts could be performed in haploid *S. rosetta* cells, in which mating is induced immediately afterwards to generate diploid cells to complement the knockout (Levin et al., 2014; Levin & King, 2013; Wetzel et al., 2018; Woznica et al., 2017).

In paper I, we showed that *S. rosetta* synaptobrevin 1 has a protein sequence identity of 38% with human synaptobrevin 1 and 36% identity with human synaptobrevin 2 (Paper I, **figure 2 b**). Although the sequence identity of human synaptobrevin 2 and *S. rosetta* synaptobrevin 1 is lower, they share amino acids at specific positions which likely play an important role in substrate recognition by BoNT/D-LC (Paper II, **Figure 3 A**) (Arndt et al., 2006; Yamasaki et al., 1994). As one of the highly specific clostridial neurotoxins, BoNT/D-LC specifically cleaves synaptobrevin 2 and 3 in vertebrates, whereas synaptobrevin 1 is only cleaved to a lesser extent (Arndt et al.,

2006; Yamamoto et al., 2012; Yamasaki et al., 1994). The lack of rBoNT/D-LC cleavage that we observed in recombinant *S. rosetta* synaptobrevin 1 [1-75] could mean that *S. rosetta* synaptobrevin 1 either cannot be recognised in general or that other factors are also involved in substrate recognition (**Figure 3 B**). For example, the yeast synaptobrevin 1/2 homologues snc1/2 are also not cleaved by clostridial neurotoxins (Niemann et al., 1994). However, it has been shown that that an engineered chimera of human synaptobrevin 2 and yeast snc2 not only functions in yeast, but is also cleaved by botulinum neurotoxin B (Fang et al., 2006). This study allowed the comparative investigation of botulinum cleavage in a eukaryotic cell and of recombinantly expressed human synaptobrevin 2 *in vitro*. There, point mutations showed different effects on the cleavage sensitivity. In particular, the effects of mutations near or within the transmembrane region of the chimera protein on the cleavage efficiency by botulinum neurotoxin B in yeast cells could be observed, indicating the importance of the transmembrane region (Fang et al., 2006). Moreover, cleavage of animal synaptobrevin 2 by clostridial neurotoxins has been shown to be strongly enhanced by the presence of lipid membranes (Caccin et al., 2003). According to these studies, both the use of full-length *S. rosetta* synaptobrevin 1 [1-96] and its incorporation into a lipid membrane could potentially improve substrate recognition of recombinant *S. rosetta* synaptobrevin 1 by rBoNT/D-LC. In the future, cleavage of *S. rosetta* synaptobrevin 1 could be investigated by delivery of rBoNT/D-LC via nucleofection into *S. rosetta* cells. Alternatively, a *S. rosetta* synaptobrevin 1/snc2 chimera could be expressed in yeast cells and treated with BoNT/D-LC, similar to the study by Fang et al. (2006). Different recombinant chimeras of human synaptobrevin 2 and *S. rosetta* synaptobrevin 1 might also be used for an *in vitro* cleavage assay to compare *S. rosetta* synaptobrevin 1 with animal synaptobrevin 2. Moreover, higher concentrations of rBoNT/D-LC should be tested, as rat synaptobrevin 1 requires an almost 3700-fold higher concentration to be cleaved (Yamasaki et al., 1994). In addition, native synaptobrevin 1 from *S. rosetta* cells enriched by immunoprecipitation or recombinant full-length *S. rosetta* synaptobrevin 1 reconstituted in artificial vesicles could be used for a rBoNT/D-LC cleavage assay (Weber et al., 1998). Other botulinum neurotoxins specific for synaptobrevin can also be tested in a comparative cleavage assay.

For example, the botulinum neurotoxin type X light chain (BoNT/X-LC), which also cleaves non-secretory R-SNAREs in rat (synaptobrevin 1, 2, 3, 4, 5 and ykt6), might be used (Zhang et al., 2017).

For the identification of potential interaction partners of synaptobrevin 1 in *S. rosetta*, we performed two independent co-IP experiments that were analysed by mass spectrometry (**Figure 4 A**). In these experiments, we identified 171 and 88 proteins, respectively (**Table S2**, **Table S3**). Of the proteins identified in the first and second co-IP, ~28% and ~22%, respectively, were also found in proteomic studies on isolated synaptic vesicles (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020). Many of the co-immunoprecipitated proteins have been shown to participate in vesicle transport, secretion, cellular signalling, intercellular communication and migration (Aravind & Koonin, 1999; Bakthavatsalam & Gomer, 2010; Fort et al., 2018; Gu et al., 2016; Kee et al., 1997; Kopan & Ilagan, 2009; Matta et al., 2017; O'Connor et al., 1991; Pal et al., 2006; Shang et al., 2018; Vlachakis et al., 2020; Yuki et al., 2019). Among these were, for example, a Notch-like protein, a potential 40-kDa Huntingtin-associated protein homologue, CYRIA/CYRIB Rac1 binding domain-containing protein, transmembrane protein 35A, folate gamma-glutamyl hydrolase and two IPT/TIG domain-containing proteins. The CYRIA/CYRIB Rac1 binding domain-containing protein and IPT/TIG domain-containing proteins were detected in both co-IP experiments.

CYRIA/CYRIB Rac1 binding domains are part of the evolutionarily conserved protein CYRI, which influence cell migration, chemotaxis, epithelial polarisation through direct interaction with the small GTPase Rac1 (Fort et al., 2018; Shang et al., 2018; Yuki et al., 2019).

Since the IPT/TIG domain is also part of sec5, the animal exocyst component 2 (Aravind & Koonin, 1999; Kee et al., 1997), we searched the *S. rosetta* protein database on NCBI for a potential homologue, using human sec5 as query (**Table S4**). Indeed, one of the two IPT/TIG domain-containing proteins enriched by the synaptobrevin 1 co-IPs displays a ~31% sequence similarity to human sec5, even

though it lacks the sec5 domain (**Table S4**). The exocyst complex is a conserved octameric complex important for polarised secretion of secretory vesicles in eukaryotes. Originally identified in yeast, it was later shown to be conserved from plants to mammals (Guo et al., 1999; Hsu et al., 1996; Koumandou et al., 2007; Mei & Guo, 2018; Novick et al., 1980; Novick & Schekman, 1979; Pereira et al., 2023; TerBush et al., 1996). Different subunits of the exocyst complex interact with SNAREs, Rab GTPases, and myosin. These interactions are thought to be important for the recognition, tethering and membrane fusion of secretory vesicles (Ahmed et al., 2018; Dubuke et al., 2015; Jin et al., 2011; Moskalenko et al., 2002; Mott et al., 2003; Munson & Novick, 2006; Shen et al., 2013; Yue et al., 2017). The IPT/TIG domain of sec5 in animals allows interaction with the Ras family GTPase Ral, which is believed to be involved in recruitment of the exocyst complex (Mei & Guo, 2018; Moskalenko et al., 2003; Mott et al., 2003; Sugihara et al., 2002). In yeast and animals, different models for the assembly of the exocyst complex exist. Some of these suggest that sec5, together with other exocyst components, forms a subcomplex that localises to the plasma membrane or binds to secretory vesicles before the complete octameric complex is formed (Ahmed et al., 2018; Boyd et al., 2004; Maib & Murray, 2022; Moskalenko et al., 2003; Pereira et al., 2023). Our co-IP datasets did not include any of the other sec components, including those reported to interact directly with SNARE proteins and shown to precipitate with sec5 (Ahmed et al., 2018; Dubuke et al., 2015; Katoh et al., 2015; Shen et al., 2013; Yue et al., 2017). In the future, it must be tested whether the co-precipitated IPT/TIG domain-containing protein from *S. rosetta* is a sec5 homologue and whether it interacts directly with synaptobrevin 1 in choanoflagellates. If the IPT/TIG domain-containing protein from *S. rosetta* is indeed part of an exocyst complex, this may imply that a sec5 protein from choanoflagellates bears a closer resemblance to the animal sec5 than to yeast due to the absence of the IPT/TIG domain in *Saccharomyces cerevisiae* (Mott et al., 2003).

Among the proteins identified in the synaptobrevin 1 co-IPs were ribosomal, proteasomal and metabolic proteins. Some of these exhibited high intensities and were also found in the proteomes of isolated synaptic vesicles (Bradberry et al., 2022; Takamori et al., 2006; Taoufiq et al., 2020). Of these proteins, not all are likely to be

specific interaction partners of synaptobrevin 1, but instead are highly abundant in the cytosol. It is possible that signals from these abundant proteins suppressed the signals from specific interaction partners with lower abundance (Sun et al., 2005; Taoufiq et al., 2020). Although we assume that, similar to yeast, only a few vesicles are docked to and in contact with membrane-bound SNAREs, we would still have expected to find other SNARE proteins among the interaction partners (Paper I; Bradberry et al., 2022; Brennwald et al., 1994; Laundon et al., 2019; Takamori et al., 2006; Taoufiq et al., 2020). One possibility is that all SNARE complexes were efficiently disassembled. This is indicated by the presence of the ATPase NSF and γ-SNAP, which were enriched in the second co-IP experiment (Table S3; Hayashi et al., 1995; Söllner et al., 1993a; Söllner et al., 1993b). Another possibility is that the synaptobrevin 1 antibody used for the co-IP experiments blocks the interaction with other SNARE proteins. The synaptobrevin 1 antibody used for all experiments is a polyclonal antibody raised against the cytosolic domain of *S. rosetta* synaptobrevin 1 (Paper I and Paper II). For the formation of the SNARE complex, the cytosolic domain of synaptobrevin 1 is important (Pobbati et al., 2006). Conversely, this may also mean that a fully assembled SNARE complex hides the antigens recognised by the synaptobrevin 1 antibody and therefore cannot be enriched by co-IP.

To improve binding specificity and reduce non-specific binding, the co-IP experiment was repeated several times and optimised in various ways (intermediate optimisation experiments not shown). For the first co-IP experiment, all samples were in-gel digested for mass spectrometry analysis (**Figure S3**). In the second, optimised co-IP experiment, on the other hand, samples were in-solution digested, which usually leads to a higher yield of identified proteins than in-gel digested samples (Havliš & Shevchenko, 2004; Klont et al., 2018). However, we measured a larger number of proteins in the in-gel digested samples of the first co-IP experiment (**Table S2**, **Figure S3**). The lower number of proteins identified in the second, optimised co-IP experiment could therefore mean that by optimising the ratio of lysate to beads, non-specific binding proteins were successfully excluded (**Table S3**). As an additional optimisation, we also performed a co-IP with affinity-purified synaptobrevin 1 antibody in the second, optimised experiment. Since the concentration of the affinity-

purified antibody was greatly reduced by the purification process, smaller amounts of this antibody had to be used for the co-IP. The ratio of antibodies to beads also had to be reduced by ~57.62% in the co-IP with affinity-purified antibody. The smaller amount of antibody-coupled beads to larger amounts of *S. rosetta* cell lysate may have led to the substantially lower number of identified proteins. Considering that the co-IP with affinity-purified antibody identified only a few proteins that were not present in all other synaptobrevin 1 co-IPs, we cannot exclude that the affinity purification process affected the stability and functionality of the antibody.

In order to further verify potential interaction partners in the future and to avoid dependence on antibodies, competitive pull-down experiments with recombinantly expressed *S. rosetta* synaptobrevin 1 could be performed. For example, *S. rosetta* cell lysate could be added to biotinylated, glutathione S-transferase- or His-tagged synaptobrevin 1 coupled to streptavidin, glutathione Sepharose or nickel nitrilotriacetic acid beads, respectively (Di Giovanni et al., 2010; Xie et al., 2017). Additionally, proteins in *S. rosetta* cells could be cross-linked with membrane-permeable cross-linking agents such as disuccinimidyl suberate before cell lysis. Such an approach would allow us to study the *in vivo* interactions and to assess weak and transient protein-protein interactions in *S. rosetta* (Dettmer et al., 2013; Edelmann et al., 1995; Söllner et al., 1992). Individual potential interaction partners could also be investigated by *in vivo* co-localisation studies (Booth et al., 2018; Wetzel et al., 2018). Moreover, thermal proteome profiling, which is currently under development in our laboratory, may reveal unexpected interaction partners of synaptobrevin 1 and other synaptic protein homologues in *S. rosetta* (Franken et al., 2015; Reinhard et al., 2015; Savitski et al., 2014).

Nonetheless, our results suggest successful enrichment of *S. rosetta* synaptobrevin 1 by all synaptobrevin 1 co-IPs (**Figure 4 B-C**, **Table S2**, **Table S3**). This could be useful for numerous future experiments that require native synaptobrevin 1. As mentioned above, native *S. rosetta* synaptobrevin 1 could be used for comparative cleavage assays, or an attempt could be made to reconstitute it in artificial liposomes and investigate it in fusion experiments (Weber et al., 1998). Although the specificity

of the identified interaction partners remains to be verified, our results indicate that *S. rosetta* synaptobrevin 1 can be immunoprecipitated together with potential interaction partners.

The immunostainings in paper I indicate that synaptobrevin 1 is localised at the vesicle-rich apical and basal pole of *S. rosetta* cells (**figure 2 c-f**). To further assess the subcellular localisation of synaptobrevin 1 in *S. rosetta*, we used a detergent-free subcellular fractionation protocol. According to our results, synaptobrevin 1 appears to be present in the cytosolic and membrane fraction, where we also suspect putative secretory vesicles to be present (Huttner et al., 1983; Pearse, 1975; Walworth & Novick, 1987; Whittaker et al., 1964; Zinser et al., 1991). We therefore hypothesise that, as in animals and yeast, synaptobrevin 1 may be localised on secretory vesicles in *S. rosetta* (Baumert et al., 1989; Protopopov et al., 1993). Further studies in the future are needed to confirm this assumption. For example, the described subcellular fractionation protocol could be combined with a sucrose density gradient and ultracentrifugation as performed by Hake (2019) and Sigg et al. (2017) to enrich the membrane fraction. In combination with immunoisolation, this could allow the enrichment of secretory vesicles from choanoflagellates, similar to the isolation of synaptic vesicles (Burger et al., 1989; Grønborg et al., 2010; Morciano et al., 2005; Takamori et al., 2000). In addition, electron microscopic analysis and expansion microscopy, possibly even in combination with super-resolution microscopy, using the affinity-purified synaptobrevin 1 antibody, could help to verify the isolation of vesicles and to determine the exact localisation of synaptobrevin 1 in *S. rosetta* (Burger et al., 1989; Chen et al., 2015; Chen et al., 2021; Gao et al., 2021; Grønborg et al., 2010; Halpern et al., 2017; Klimas et al., 2023; Morciano et al., 2005; Takamori et al., 2000; Wang et al., 2018; Zhuang & Shi, 2023).

In the future, it will be important to also raise antibodies against *S. rosetta* synaptobrevin 2. This would allow us to investigate the localisation and potential interaction partners of *S. rosetta* synaptobrevin 2 in a similar way as synaptobrevin 1 was studied (Paper I: **figure 2 c-f**, Paper II: **Figure 4** and **Figure 5**).

5. Conclusion and future perspectives

In the two studies conducted, the evolutionary origin of the machinery important for regulated synaptic vesicle exocytosis was investigated. The first study showed that most of the investigated core proteins of neurosecretory vesicles predate neurons, as they are also present in the genomes of unicellular organisms. Moreover, 3D reconstructions revealed diverse vesicular landscapes in the two choanoflagellate species *S. rosetta* and *M. brevicollis*, where vesicles could be assigned to distinct classes. Immunostainings of single and colonial *S. rosetta* cells indicated the presence of synaptobrevin at the vesicle-rich apical and basal pole of the cells. There, synaptobrevin staining appeared to overlap with tubulin antibody staining. In summary, in the first study we found features in choanoflagellates that are also present in specialised secretory cells such as neurons, including polarised vesicle landscapes, plasma membrane contact sites, a polarised shape and potentially a directed transport system.

The second study aimed to investigate putative secretory synaptobrevin homologues in more detail in *S. rosetta*. Since little was known about the function and localisation of synaptobrevin in choanoflagellates, I investigated the role of synaptobrevin in *S. rosetta*, which allowed comparisons with exocytotic synaptobrevin from animals. In *S. rosetta*, there are two putative secretory synaptobrevin homologues that seem to be essential for choanoflagellates, similar to those in animals. One of these synaptobrevins from *S. rosetta* has specific amino acids at certain positions, similar to animal synaptobrevin 2, which are thought to be important for substrate recognition by rBoNT/D-LC. However, unlike animal synaptobrevin 2, recombinant synaptobrevin from *S. rosetta* is not cleaved by rBoNT/D-LC. In addition, potential interaction partners of synaptobrevin in *S. rosetta* were identified by co-immunoprecipitation. Over 20% of the identified potential interaction partners were also found in proteomic studies on isolated synaptic vesicles. Moreover, subcellular fractionation experiments of *S. rosetta* indicate the presence of synaptobrevin in the cytosolic and membrane fractions. These findings support the hypothesis that synaptobrevin is localised on the membrane of vesicles in *S. rosetta*. In conclusion, this study has elucidated previously

unknown properties of putative secretory synaptobrevin in choanoflagellates and allowed a comparison with animal secretory synaptobrevin.

My findings lead to many new questions and speculations, such as: What do synaptobrevin-positive vesicles contain and what is their function? Do choanoflagellates communicate? And if this is the case, do unicellular choanoflagellates communicate via secretion, or do only colonies communicate via intercellular bridges? Are budding, tethering, docking and fusion events of secretory vesicles in choanoflagellates regulated by the same machineries as in neurons? And do choanoflagellates have the molecular set-up for highly regulated secretion? All these questions remain a conundrum that may be solved in the future by isolating putative secretory vesicles and studying their protein composition and content.

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7. Papers

Paper I:

Göhde, R., Naumann, B., Laundon, D., Imig, C., McDonald, K., Cooper, B. H., Varoqueaux, F., Fasshauer, D. and Burkhardt, P. (*submitted version*)

"Choanoflagellates and the ancestry of neurosecretory vesicles."

This is the submitted version, which may contain minor deviations from the final, published version.

Paper II:

Göhde, R. and Burkhardt, P. (in preparation)

"Characteristics of secretory synaptobrevin homologues in the closest unicellular relative of animals."

Paper I

I

Choanoflagellates and the ancestry of neurosecretory vesicles

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Paper II

Characteristics of secretory synaptobrevin homologues in the closest unicellular relative of animals

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