Summary of the Ph.D. thesis

The study of the membrane microdomain localization of the prion-family proteins, prion and Shadoo and their interaction with calnexin

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2021

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

Publications related to the Ph.D. thesis

I. **Divya Teja Dondapati**, Pradeep Reddy Cingaram, Ferhan Ayaydin, Antal Nyeste, Andor Kanyó, Ervin Welker and Elfrieda Fodor. Membrane Domain Localization and Interaction of Prion-Family Proteins Prion and Shadoo with Calnexin. (2021) *Membranes* 11(12), 978. IF: 4.106.

II. Pradeep Kumar Reddy Cingaram, Antal Nyeste, **Divya Teja Dondapati**, Elfrieda Fodor, Ervin Welker. Prion Protein Does Not Confer Resistance to Hippocampus-Derived Zpl Cells against the Toxic Effects of Cu^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} Not Supporting a General Protective Role for PrP in Transition Metal Induced Toxicity. (2015) PLoS ONE 10 (10): e0139219. IF: 3.3

Conference abstracts related to the thesis

I. **Divya Teja Dondapati**, Ferhan Ayaydin, Pradeep Kumar Reddy Cingaram, Andor Kanyó, Ervin Welker, Elfrieda Fodor, "Interaction of prion-family proteins prion and shadoo with calnexin in membrane microdomains", in Straub Days Conference, Biological Research Center, Szeged, Hungary, 2019.

II. **Divya Teja Dondapati**, Pradeep Kumar Reddy Cingaram, Ferhan Ayaydin, Ervin Welker, Elfrieda Fodor, "Study of the membrane micro-domain localization of prion protein family members in N2a cells using a detergent free density gradient method" in the International Prion Conference, Edinburgh, Scotland, 2017.

III. **Divya Teja Dondapati,** Pradeep Kumar Reddy Cingaram, Ervin Welker, Elfrieda Fodor, "Study of the membrane micro-domain localization of overexpressed GPI-anchored proteins in mammalian cells", in the Recombinant Protein Technology-2016 ELRIG International Conference, Gothenburg, Sweden, 2016.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) represent a class of incurable, fatal neurodegenerative disease of humans and animals. In human most known prion diseases include Creutzfeldt–Jakob Disease (CJD), kuru, Gerstmann Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), whereas in animals, bovine spongiform encephalopathy (BSE), scrapie of sheep and chronic wasting disease (CWD) of deer and elk. Characteristic symptoms of the disease include degeneration of the tissues of the central nervous system (CNS) and appearance of spongiform lesions, absence of immunological responses, astrogliosis leading to behaviour and movement disturbances, dementia and ultimately death[1]. These diseases are rapidly progressive and incurable, currently, there is no effective therapy available, and all are invariably fatal.

Prion diseases are known to be caused by nucleic acid-free, proteinaceous infectious particles termed "prions"[2,3]. The fundamental event of the prion diseases is the conformational conversion of cellular, non-infectious prion protein (PrP^{C}) to misfolded, pathologic isoform (PrP^{Sc}), which accumulates with the progression of disease and neurodegeneration[3–5]. In prion diseases, the abnormal isoform of the host prion protein is essential for the pathogenic processes. There is an increasing evidence that the other neurodegenerative diseases (and their hallmark proteins), most notably Alzheimer's disease (Aβ and tau proteins), Parkinson's disease (α-synuclein), frontotemporal dementia (TDP43, tau or FUS proteins) and motor neurone disease (TDP43) exhibit at-least some of the misfolded prion protein's properties[6]. Although the template assisted model and the nucleation-polymerisation model had been proposed as mechanisms to explain the conformation conversion of PrP^{C} to PrP^{Sc}, to-date the exact mechanism of developing of disease still remain elusive. This is in major part due to the fact that even the healthy cellular PrP^{C}'s role is not yet fully understood and despite many decades of research, its exact function still remains enigmatic.

The cellular prion protein and its structure

PrP^{C} is a cell surface, glycosylphosphoinosityl (GPI)-anchored glycoprotein expressed abundantly in the CNS particularly by neurons and to a lesser extent in other non-neuronal tissues. It is highly conserved across all the mammalian species preventing its evolutionary

elimination, which suggests beneficial functions. PrP^{C} adopts a physiological structure with an N-terminal unstructured domain (about half of the protein) and a C-terminal globular domain. The N-terminal domain of the mature protein consists of important functional regions such as a polybasic terminal patch, an octapeptide repeat region (OR) that binds copper and other divalent cations and followed by a second positively charged segment together with which exerts regulatory effects upon transmembrane proteins, and hydrophobic domain (HD) where many binding partners had been mapped and which connects to the C-terminal domain of the protein. In the globular part, one disulphide bond and two N-glycosylation sites are located upstream of the sialylated GPI-anchor signal peptide[7]. Prion protein (PrP) is synthesized in ER, undergoes post translational modifications in Golgi apparatus (GA) and it is transported to the plasma membrane (PM) to predominantly localize to the specialised membrane domains rich in cholesterol and glycosphingolipid called "lipid-rafts" or "membrane microdomains" with its GPI-anchor. Some of the functions attributed to PrP^{C} in the nervous system include synaptic plasticity, learning and memory as well as sleep patterns; neurite growth, anti-apoptotic roles (during oxidative stress-induced cell death), pro-apoptotic roles (ER-stress), copper (Cu^{+2}) metabolism and homeostasis of trace elements[8].

PrP- a copper binding protein

The octapeptide repeat region of PrP is well known to bind divalent cations. Among them especially Cu^{2+}, which binds to some other sites on the protein as well, is well documented and characterized[9,10]. Binding of Cu^{+2} to PrP is considered to influence its conformational transition to PrP^{Sc}[11]. Several studies have shown PrP^{C} is directly implicated in the uptake/transport of metals, notably copper, zinc, and iron, albeit there is no direct proof that PrP transports these metals. The cell surface PrP^{C} on the PM, is constitutively internalized via Cu^{+2} induced clathrin-dependent[12,13] and also via raft/caveolae-dependent endocytosis[14], by the exact mechanism of these is still unclear. Internalization and shedding of PrP and also changes in its structure and folding property have been reported also in response to transition metal induced stimuli, which may influence the biology of PrP^{C}. Protection against transition metal induced toxicity exerted by PrP had been reported by a few studies[15]. However, although PrP^{C}'s metal binding is well-documented and characterised, there is no consensus on its exact role in either the normal physiology of the cellular PrP^{C} or in the development of TSEs.

Shadoo protein, the shadow of PrP

Two other genes belonging to prion family, *PRND* and *SPRN*, were uncovered in 1999 and early 2000s, encoding doppel (Dpl) and Shadoo (Sho) proteins. Each mimics one of the PrP's halves, with Dpl being the structured and Sho being unstructured and flexible, and both possess GPI-anchors and are complex glycosylated as PrP. It is unclear, if any functional relationships exist between PrP and its paralogs and on their involvement if any in the formation of PrP^{Sc}. Like PrP^{C}, Sho is mainly expressed in CNS (contrary to Dpl, which is absent and toxic if ectopically expressed in CNS) and they share a number of common structural features. Beside the GPI-anchor, sites for complex N-glycosylation and resemblance to the flexible unstructured N-terminal domain of PrP^{C}, Sho similarly harbors a highly conserved hydrophobic domain (HD). Sho HD region is its only homologous sequence to PrP and PrP's HD, to which most of PrP^{C}'s functions are linked including homo-dimerization and is also a proven interaction site with Sho. Similar to the PrP's α-cleavage site at the N-terminal, Sho also has an endo-proteolytic cleavage site, but it lacks an OR region, instead this is being replaced by an arginine rich tetra-repeat segments towards its N-terminal that confers different activities to Sho than the OR to PrP.

Sho is being expressed from embryonic to adult state and had been proposed to participate in embryonic developmental pathways overlapping with PrP^{C}. Additionally, Sho manifested similar neuroprotective properties as wild type prion protein (PrP) against the toxic effects of Dpl, the Shmerling deletion mutant Δ[32-121]PrP, and the HD region deletion mutant PrPΔHD in a variety of experimental conditions and protected cells against the excitotoxic stress exerted by glutamate. Many potential interaction partners have been proposed for Sho, including many from among those of PrP's, indicating that Sho also may be involved in many different processes. On the other hand, Sho was not found to be a requisite for TSE development or to prevent against infection or prion replication kinetics when overexpressed. In addition, lack of Sho in $Sprn^{0/0}$ or in the double-knockout $Sprn^{0/0}$-$Prnp^{0/0}$ mice resulted in no remarkable phenotypes, which proves that the two proteins are not functional homologs and renders Sho's cellular role even more perplexing. We had also shown previously that contrary to PrP^{C}, expression of Sho sensitizes cells to certain drugs and produces large spontaneous inward currents in certain cells. All these, confirm Sho's ability to manifest either similar or different actions compared to PrP depending on the cells and conditions, and add to a potentially less clear cellular role of Sho, highlighting

that more research data are needed to clarify their roles and to understand the two protein's interplay.

Role of lipid-rafts and ER chaperones in Shadoo and prion protein biology

GPI anchored protein including PrP and Sho are known to localized mainly to the lipid-rafts on plasma membrane to perform specialized tasks in signaling pathways. Cellular membrane -rafts or membrane microdomains are defined as small (10–200 nm), heterogeneous, highly dynamic cholesterol- and sphingolipid-enriched domains that compartmentalize cellular processes. They are reported to be also involved in various aspects of the prion protein's life cycle including in PrP^C-endocytosis, crucial factor for normal cell biology of the protein. Additionally, localization of PrP^C to rafts had been reported to have implications on prion conversion, despite the mechanism of conversion being still unknown. PrP^C within lipid rafts at the cell surface had been shown to interact with key partners to promote neurite outgrowth, cell survival and participates in neuritogenesis and neuroprotective activities. Sho being in lipid rafts had been shown to act as a cell surface receptor for hyaluronate and/or extracellular RNA that are involved in signaling processes[16–18]. Raft localisation is critical for both PrP and Sho for proper folding, as the disruption of rafts resulted in misfolding of these proteins, leading to accumulation of misfolded isoforms[19,20]. Studies had also shown that, rafts disruption increases Sho co-immunoprecipitation with calreticulin, an ER chaperone protein. In addition, one study demonstrated that PrP is able to bind to another ER chaperone, calnexin (CNX), which inhibits its thermal aggregation *in vitro* and also the neurotoxicity of the protein *in cellulo*. While finding that matured GPI-anchored proteins may be engage in binding ER chaperones at the PM is fascinating in itself, it is unknown if PrP-CNX interaction is prioritized in some membrane domains over other, as well as it is not known whether Sho would also be able to bind to CNX, like PrP, or not, and if yet whether occurs in rafts or non-raft type membranes.

AIMS OF THE STUDIES

The main objective of the presented studies is to reveal more information on the properties of the prion-family proteins, prion and Shadoo, with special focus on their localization and distribution to specific membrane microdomains of raft and non-rafts. Beside their distribution, we aim to study their binding with the ER-chaperone protein calnexin within these domains, as well as to test the protective effects of PrP against transition metal toxicity and specifically its membrane domain re-distribution if any, in response to copper treatment. In line with the goals, we proposed the following aims:

1. To compare the membrane microdomain partitioning of prion and Shadoo proteins by using a non-detergent-based fractionation method and N2a transgenic cells expressing the proteins.

Specific aims:

1.1 To establish N2a stable transgenic cells suitable for the studies, which express the Sho or PrP proteins in fusion with a fluorescent protein tag, as well as, corresponding control cells, in order to be able to monitor the proteins by confocal fluorescence microscopy.

1.2 To compare the distribution of PrP and Sho in the isolated membrane microdomain fractions of the developed stable transgenic cells, using a detergent-free, OptiPrep density-gradient fractionation method.

2. To test whether calnexin, is a binding partner of both PrP and Sho and whether the interaction is specific to the type of membrane-domain the proteins reside in.

Specific aims:

2.1 To develop transgenic Sho-expressing and corresponding control, transgenic N2a cells, where a FLAG tag is inserted in addition to the fluorescent protein, in fusion with Sho, to allow for performing co-immunoprecipitation assays.

2.2 To compare the partition of FLAG-tagged and non-FLAG-tagged Sho in the membrane microdomains of the cells developed, using the non-detergent, density-gradient fractionation method.

2.3 To study the localizations of Sho and PrP with respect to the localization of calnexin by using live-cell imaging and immunocytochemistry combined with confocal microscopy of the transgenic cells developed.

2.4 To test, whether PrP and Sho interact with calnexin, and furthermore, if such an interaction is observed, whether it is specific to the type of the membrane domain the proteins occupy, using anti-FLAG co-immunoprecipitation and Ni-NTA bead pull-down assay in the transgenic cells developed.

3. To study the effect of copper treatment on the membrane domain localization of PrP.

Specific aims:

3.1. To test the protective effects of PrP against transition metal induced toxicity using mouse hippocampus-derived wild type ZW(*Prnp*$^{+/+}$) and PrP knock-out Zpl(*Prnp*$^{0/0}$) cells without and with reintroduction of PrP gene (part not presented in details in the Thesis).

3.2. Based on the experiments at aim 3.1., to test the effect of the presence of Cu^{2+} on the distribution of PrP in the membrane fractions of the transgenic N2a cells developed above.

EXPERIMENTAL METHODS

1) **<u>DNA plasmids necessary for the studies</u>,** were either acquired or constructed using conventional molecular biology techniques. To generate N2a stable transgenic cells expressing Sho or PrP: Sho-EYFP, Sho-EYFP-FLAG or PrP-EGFP cells, plasmids were constructed to encode for mouse Shadoo (mSho) or mouse prion protein (mPrP) such that these proteins are tagged at their C-termini, but preceding their GPI-signal sequences, in fusion with either the enhanced yellow fluorescent protein alone (plasmid: p_mSho-EYFP-$GPI_{(mSho)}$) or also with a FLAG-STREP tag (plasmid: p_mSho-EYFP-FLAG-$GPI_{(mSho)}$) in case of mSho or with the enhanced green fluorescent protein in case of mPrP (plasmid: p_$SS_{(mPrP)}$-EGFP-$GPI_{(mPrP)}$). To serve as controls, similar plasmids were also made where the protein backbone of mSho and mPrP were absent, leaving only their ER- and GPI-signal sequences in with the respective protein tags in-between and in fusion them (plasmids: p_$SS_{(mSho)}$-EYFP-$GPI_{(mSho)}$, p_$SS_{(mSho)}$-EYFP-FLAG-$GPI_{(mSho)}$ and p_$SS_{(mPrP)}$-EGFP-

$GPI_{(mPrP)}$, respectively). To generate the cells expressing untagged mPrP and a soluble EGFP and its control the plasmids constructed earlier in our laboratory were used. For the expression of the red fluorescent protein tagged calnexin, the commercially available plasmid pCMV3-C-OFPSpark was acquired.

2) **Cell culturing** of the N2a parental and transgenic cells was performed using Dulbecco's modified Eagle medium with high glucose (4.5 g/l) (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin and 1% GlutaMAX at 37 °C in humidified atmosphere with 5% CO_2.

3) **Transfection and establishment of the stable transgenic N2a cells**: Sho-EYFP-, PrP-EGFP-, Sho-FLAG-EYFP- and their respective controls EYFP-, EGFP- and EYFP-FLAG-cells, as well as the transient transfection of these cells to express RFP-CNX, were performed by transfection of the corresponding DNA-plasmids using the TurboFect transfection reagent, according to the manufacturer's protocol. In the case of N2a cells expressing untagged mouse PrP protein and soluble EGFP (PrP(+EGFP) cells) and its control cells (+EGFP) expressing only soluble EGFP, the Sleeping Beauty plasmid system was used with the TurboFect transfection reagent as reported previously[21]. Transformant cells were selected using antibiotic selection followed by cell sorting to produce clean stable populations of transgenic cells. Separate clones and populations of mixed clones were also prepared in some cases.

4) **Confocal microscopy** of the fixed and immunostained cells and of the live cells were performed by employing a Fluoview FV1000 laser scanning microscope (Olympus) using 20x objective and a VisiScope CSU-W1 spinning disk (Visitron) confocal microscope using 100x oil immersion objective, respectively, applying excitation lasers of 405 nm, 488 nm and 543 nm for as follows: DAPI; EGFP/EYFP/Alexa Fluor 488 labelled antibody and RFP/Alexa Fluor 568 antibody, and using the emission filters of 425-475 nm, 500-530 nm, LP560, respectively.

5) **Golgi complex labeling** of the transgenic N2a cells was done using the using CellLight™ Golgi-RFP, BacMam 2.0 reagent as per manufacturer's instructions.

6) **Immunocytochemistry** of the transgenic Sho-EYFP-FLAG-, PrP-EGFP- cells along with their control- and parental- cells was performed on cells grown on 8-well cover glass bottom plates for 48 h prior to fixation. The cells were fixed by 4% paraformaldehyde, were permeabilized using 1% Triton-X100 and were immunoprobed with anti-prion SAF-32, α-GFP and α-CNX primary antibodies followed by staining with corresponding secondary antibodies: anti-mouse-Alexa 488 for α-SAF32 and α-GFP and anti-rabbit-Alexa 568 for α-CNX. Nuclei were labelled with DAPI prior acquiring images using a 100x oil immersion objective and a VisiScope CSU-W1 spinning disk confocal microscope.

7) **Extraction of total cell lysates** were performed from cells grown on one 100 mm Petri dish. Cells were washed and scraped in phosphate buffered saline (PBS) and lysed in cold lysis buffer to extract the total proteins. Non-lysed cells were cleared from the samples by brief pelleting and the supernatants were collected, and were used as the total cell lysates. According to their total protein concentrations measured, they were used for further experiments and/or analysis by Western blot.

8) **Detergent free separation of membrane-rafts** from established N2a stable transgenic cells was performed using the detergent free, continuous OptiPrep density gradient method of Macdonald and Pike[22]. Briefly, post nuclear supernatants were extracted from cells plated in ten 100 mm Petri dishes and 5 mg of total protein containing amount of sample was added to the Base buffer with 50% OptiPrep density medium in bottom of 12 ml ultracentrifuge tubes to give a final concentration of 25% OptiPrep, which was then layered by a continuous gradient of 20% to 0% OptiPrep in Base buffer, up to the top of the tubes. The gradient was centrifuged for 90 min at 52000 × g at 4 °C in an ultracentrifuge and the samples were fractionated to 18 aliquots of 0.67 ml from top to bottom of the gradient. The total proteins in each fraction were measured using SDS-PAGE followed by densitometry analysis using the ImageJ program.

9) **PNGase F treatment** of total cell lysates obtained from Sho-EYFP-, PrP-EGFP- and parental cells were subjected to deglycosylation of proteins with PNGase F (Peptide -*N*-Glycosidase

F) enzyme, which removes complex N-glycans, for 2 h at 37 °C followed by SDA-PAGE and Western blotting.

10) **Copper (Cu^{+2}) treatment** of the stable transgenic PrP-EGFP- and its control cells were performed on cell seeded in ten 100 mm Petri dishes, briefly as follows. 24 h prior to Cu^{2+} treatment cells were washed with PBS followed by OptiMEM-1 media supplemented by 1% GlutaMAX. Using the method optimized for the transition metal-treatment in case of ZW(*Prnp*$^{+/+}$) and Zpl(*Prnp*$^{0/0}$) cells and based on the results obtained by applying various metal concentrations, the appropriate Cu^{2+} concentration for N2a cells was tested by looking at the morphology of the cells, and a concentration of 500 μM Cu^{2+} was chosen for the treatment. Prior to the treatment of the cells, the $CuSO_4$ was mixed with glycine in 1:4 copper to glycine molar ratio in OptiMEM-1 media supplemented with 1% GlutaMAX and the mixture was incubated for 1 h at room temperature (RT). In parallel, the same media, but without the addition of $CuSO_4$ was also used for parallel plates of cells, to serve as control. The cells were incubated with these treatments at 37 °C for 30 min in the CO_2 incubator. After the incubation, cells were chilled and membrane rafts were immediately separated as mentioned in the section: Detergent-free separation of membrane-rafts and the distribution of various proteins were assessed by Western blotting.

11) **Western blotting** of the fractionated samples, total cell lysates, bead pulled or co-immunoprecipitated samples were performed against various proteins such as Sho (by α-Sho), PrP (by α-PrP), fluorescent proteins, EGFP and EYFP (by α-GFP) and FLAG (α-FLAG) along with the marker proteins Flotillin-1, transferrin receptor protein, calnexin and the nuclear pore complex protein and beta actin where needed, by antibodies α-Flot-1, α-TfRC, α-CNX, α-NPC and α-β-actin, respectively. Briefly, samples were denatured, loaded on to SDS-polyacrylamide gels and subjected to SDS-PAGE. The SDS-gels with the resolved proteins were electroblotted onto methanol-activated PVDF membranes in cold Towbin buffer for 60 min at constant current (400 mA). The membranes were blocked using low fat milk powder as blocking agent, for 60 min at RT followed by the corresponding primary antibody incubation for overnight at 4 ºC. After washing the membranes, corresponding

secondary antibodies were applied to the membranes in blocking buffer for 2 h at RT, which was followed by addition of ECL substrate and detection of bands on X-ray films.

12) **Cholesterol determination** was performed for the f ractionated samples by measuring the total cholesterol content using the Amplex® Red Cholesterol kit and assay as per manufacturer's instructions. The fluorescence from the samples was measured using Fluoroskan Ascent FL Microplate Fluorometer and Luminometer microplate reader (Thermo Fisher Scientific).

13) **Ni-NTA bead pull-down assay** was performed from total cell lysates or each gradient fractions of stable transgenic PrP-EGFP cells and its control cells, as well as from the parental N2a cells. After overnight incubation of samples with Ni-NTA beads in Tris-sucrose buffer, beads were washed and the bead-bound proteins were eluted and subjected to Western blot analysis using antibodies against PrP, CNX and EGFP.

14) **Co-immunoprecipitation** was performed in case of N2a stable transgenic Sho-EYFP-FLAG-, its control- as well as parental N2a cells from either total cell lysates or the pooled rafts and non-raft fractions using anti-FLAG affinity beads. Samples were incubated with beads in incubation buffer, overnight at 4 ºC. Next, beads were washed beads with wash buffer and the bead-bound proteins were eluted by boiling in SDS-sample buffer followed by SDS-PAGE and Western blot analysis using antibodies against Shadoo: by α-Sho, α-FLAG, α-GFP and calnexin by α-CNX.

RESULTS

I. The study of the membrane-domain localization of prion and Shadoo proteins.

Beside already existing DNA plasmids in the laboratory, a set of additional plasmids had been created for these studies. These are the plasmids encoding for full-length mouse PrP in fusion with the fluorescent protein EGFP at its C-terminus, upstream of the GPI-signal peptide, and its corresponding control plasmid encoding only the fluorescent protein flanked by the mouse PrP's ER- and GPI anchor- signal peptides. These plasmids were designed and created to follow

similar principles as the plasmids of the EYFP tagged mSho and its control protein, made earlier by our laboratory. N2a stable transgenic cells were developed, using corresponding plasmids to transfect the cells in order to generate the following stable transformant cells: Sho-EYFP and EYFP cells, encoding for the fluorescent protein tagged mSho and its corresponding control, EYFP-$GPI_{(mSho)}$ protein, respectively; the PrP-EGFP cells and its corresponding control EGFP cells, encoding for the EGFP-tagged mPrP or EGFP-$GPI_{(mPrP)}$ protein, respectively; the PrP(+EGFP) and (+EGFP) cells encoding for untagged mPrP and soluble EGFP or soluble EGFP alone, respectively. Using these cells, the following results were obtained:

1. Live-cell confocal microscopy imaging revealed correct localization and expression of the transgenes in the developed cells. They expressed Sho-, PrP- and their control proteins are predominantly present in plasma membrane (PM) and in the Golgi apparatus (GA) as confirmed by applying a co-transfection by a plasmid encoding for a red fluorescent protein-tagged GA-marker protein. Such localization is as expected for GPI-anchored proteins.

2. Western blot analysis combined with PNGase F treatment confirmed that the expressed PrP and Sho protein constructs are of the expected molecular weights as tagged proteins, and also that they are complex N-glycosylated, as shown by a shift in the respective protein's band followed by PNGase F treatment and Western blotting for the proteins.

3. Membrane-rafts and non-rafts were successfully separated from the N2a stable transgenic Sho-EYFP, PrP-EGFP, PrP(+EGFP) and their respective control cells, as well as from the parental N2a cells using the non-detergent based, continuous OptiPrep density gradient fractionation method of Macdonald and Pike, 2005[22], which was confirmed by Western blotting of fractionated samples against various marker proteins, along with the target proteins, and testing for the "true-raft fraction" criteria of Persaud-Sawin and coworkers[23]: The fractions possessing low protein- and high cholesterol content, and presence of raft protein Flotillin-1 and absence of non-raft protein transferrin receptor, TfRC, are identified as rafts. In our experimental setup, low- to mid-dense fractions (fraction numbers 1-11) fulfilled true-raft criteria and are considered as membrane rafts, whereas those from 12 to 18, the high dense fractions as non-raft type fractions.

4. Western blot analysis of the fractionated samples along density gradient fractions revealed that:

   4.1 PrP distributes into low- and mid-dense raft fractions, where Flottilin-1 is also present, and also occupies the high dense non-raft fractions where typically TfRC resides. These type of pattern for PrP was similar in both tagged-PrP expressing PrP-EGFP and non-tagged PrP expressing PrP(+EGFP) cells, as well as similar to the distribution of the endogenous PrP detected in the parental N2a cells. This also indicates that addition of an EGFP tag and/or overexpression did not disturb the natural localization of the PrP in these N2a cells.

   4.2 Sho is found to be present from low dense raft-fractions being more detectable in the mid-dense raft fractions compared to the low-dense raft-fractions. Similarly to PrP, Sho is also detected in the high-dense non-raft fractions in the Sho expressing Sho-EYFP cells. This localization pattern of Sho is qualitatively similar to the distribution found for PrP and also for the GPI-anchored control proteins in the control cells (EGFP and EYFP cells).

   4.3 CNX, the ER chaperone and also an ER-marker protein distributed also through low-, mid- and high dense fractions being more abundant in mid-and high dense fractions. The NPC protein distributed to high dense fractions indicating that the low-and mid-dense fractions are free from nuclear membranes in all type of cells.

Taken together, both PrP and Sho possessing GPI-anchors partitioned to the membrane raft fractions, which are not different from GPI-anchored fluorescent proteins, possessing GPI-anchors of the respective proteins. Importantly, the results also demonstrate that these proteins are present also in the non-raft-type membrane fractions.

II. The study of the possible interaction of prion and Shadoo proteins with calnexin.

To allow performing co-immunoprecipitation studies with Sho, another two plasmids were created where mSho is additionally in fusion with a FLAG-tag following its EYFP tag and prior to its GPI-signal sequence, and one coding for its corresponding control fusion protein, here are the plasmids: p_mSho-EYFP-FLAG-$GPI_{(mSho)}$ and p_$SS_{(mSho)}$-EYFP-FLAG-$GPI_{(mSho)}$. N2a stable transgenic cells were successfully established using these plasmids, the cells named as Sho-EYFP-FLAG- and its control, EYFP-FLAG cells. Using these cells together with the prion expressing PrP-EGFP and its control EGFP cells, the following results were obtained:

1. Confocal microscopy and Western blot analysis disclosed that subcellular localisation and expression of FLAG-tagged Sho and its control proteins in the cells are similar to Sho-EYFP-$GPI_{(Sho)}$ and EYFP-GPI(mSho) protein's localization in the Sho-EYFP and EYFP cells, the proteins being predominantly localized to PM and GA and expressed at the expected molecular weights.

2. Applying the same non-detergent based density gradient fractionation method on the transgenic FLAG-tagged Sho expressing cells, followed by Western blotting of the fractionated samples against the Sho- protein construct by α-Sho, α-FLAG, and α-GFP antibodies revealed a similar pattern of distribution of Sho as of its non-FLAG tagged Sho counterpart, being more abundant in mid- and high- dense fractions compared to low dense fractions, indicating also that FLAG tag did not disturb its preference of localisation in the membrane microdomains. The selected marker proteins (Flot-1, TfRC, CNX, NPC) distribution pattern is also similar to those found earlier.

3. Immunocytochemistry and live-cell analysis of transiently transfected Sho-EYFP-FLAG-PrP-EGFP- and their respective control cells with RFP-calnexin using VisiScope CSU-W1 spinning disk confocal microscope revealed that flourescent protein tagged-PrP and Sho and CNX localize to similar organelles and specifically colocalize with CNX in the fine, tubular and sheet-type structures of ER, as well as the nuclear membrane, but not in PM and GC.

4. Analysis of Ni-NTA bead pulled-down or anti-FLAG co-immunoprecipitated total cell lysates of PrP and Sho expressing cells revealed that CNX is pulled and precipitated by both PrP and Sho when using total cell lysates. This reveals that CNX is not only a binding partner of PrP but also of Sho.

5. Fractionating the samples of PrP-expressing PrP-EGFP and its control cells and performing Ni-NTA bead pull-down assay in each fraction, reveals that in both raft-type and non-raft type fractions PrP pulls down CNX, whereas this is not observed in the control fractions. This indicates that PrP binds CNX irrespective of its localization to the type of membrane domains.

6. Fractionating the Sho-expressing Sho-EYFP-FLAG and its control cells (similarly to PrP-EGFP cells) and pooling all raft- fractions and separately all non-raft fractions from each, then subjecting each pulled sample to anti-FLAG bead immunoprecipitation followed by Western blotting the eluted precipitate, reveals that CNX is being immunoprecipitated in the Sho-expressing cells, but not in the control cells. This shows that Sho also binds CNX irrespective of its raft- and non-raft localization.

Altogether, these studies revealed that PrP, Sho and CNX colocalize in the ER compartments and both PrP and Sho bind to CNX is both raft and non-raft type membrane fractions.

III. The study of the effect of copper treatment on PrP membrane domain localisation in N2a cells.

1. Using mouse hippocampal cells derived from wild type and PrP KO mice, ZW(*Prnp*$^{+/+}$) and Zpl(*Prnp*$^{0/0}$) respectively, and treatments by various divalent transition metals (Cu^{2+}, Zn^{2+}, Mo^{2+}, Co^{2+}), we revealed that while the PrP expressing cells are more resistant to the toxic effects of these metals, reintroduction of PrP into the PrP-null cells, does not protects the cells against toxicity (results not presented in the thesis). Based on these results and using the same protocol developed, we treated the transgenic N2a prion expressing PrP-EGFP cells (and its control EGFP

cells) by 500 μM Cu^{2+}, corresponding to the limit where morphological signs are apparent in most of the cells, but their viability still permits performing the experiment.

2. Using non-detergent based density gradient fractionation method on copper treated and untreated conditions of stable transgenic PrP-EGFP and EGFP cells followed by Western blotting of the fractionated samples a similar pattern of distribution is observed in both conditions for PrP's distribution in raft and non-raft type membrane fractions, showing no difference in its distribution pattern across the membrane microdomains.

Overall, the copper induced effects on PrP's distribution along the membrane density fractions could not be detected as well as reintroduction of PrP into the PrP-null Zpl cells alone, did not restore the cells' resistance to toxic effects of the transition metals applied.

CONCLUSIONS

In this work, in order to study the behaviour of the two prion protein-family members, prion and Shadoo proteins in the membrane microdomains, we created the DNA-plasmids and established N2a stable transgenic Sho and PrP expressing cells taking advantage of fluorescent protein-tags- and FLAG-tag, which allowed monitoring these proteins at subcellular level and their protein-protein interactions in the membrane fractions. First, we used these cell models to compare the preference of Sho and PrP's localisation in membrane raft- and non-raft domains using non-detergent based membrane-raft fractionation method. Second, we used this approach to test, the interaction of Sho and PrP with one of the ER-chaperone, CNX and specifically studied these interactions in the membrane microdomains. Thirdly, based on our parallel results on Zpl and ZW cells and applying the same method of copper-treatment we tested if any changes occur in the membrane fraction localization of PrP using the non-detergent, continuous OptiPrep density gradient fractionation method.

We report as our major findings that beside both prion protein and Shadoo occupying raft-type membrane fractions, a significant proportion of them are present in transferrin receptor-marked non-raft membrane domains. We suggest that their dual raft/non-raft distribution reflects their loose confinement to rafts and may support their multifunctional capacities. The presented

results also reveal that both Sho and PrP^C bind with calnexin, an ER chaperone, and that this interaction is present in both type of membrane domains. This allows to propose that calnexin is a binding partner of both Sho and PrP, and that at least a fraction of these proteins is bound to CNX at any given time and independent of their localization to raft-or non-rafts. It may be suggested that the unfolded structure of these proteins may necessitate chaperone assistance, among them of CNX's, in certain instances and independent of their presence in rafts or non-raft type membrane domains. Furthermore, we could not observe relocation of PrP from rafts to non-rafts upon copper-treatment of these N2a cells, in line with a lack of the rescue effect of PrP observed upon the copper- and other transition metal treatments of Zpl ($Prnp^{0/0}$) cells when PrP was reintroduced into the cells. Based on these, we propose that the involvement of PrP in diminishing the toxic effects of copper (or of the other metals studied) as well as its copper-induced trafficking, may be more complex processes than merely binding these metals, and/or may also be dependent on the cellular models used.

These results add to our understanding of the PrP and Sho's biology in terms of their membrane domain localization characteristics; confirm a new binding partner, CNX, for Sho; reveal that the binding with is present for both PrP and Sho in both raft- and non-rafts; and also, report that the rescue effect of PrP against transition metal toxicity, as well as its trafficking influenced by copper binding is not generally observable, indicating the existence of more complex process at place, and/or a possible dependence on the model system used.

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