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Differentiation of SH-SY5Y-cells and proteolytic cleavage of the prion protein

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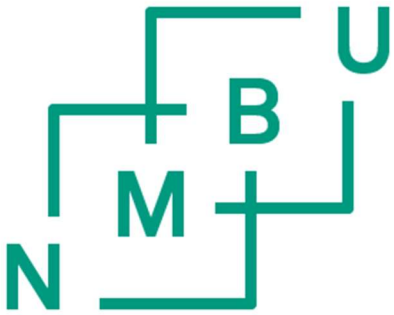

Norwegian University of Life Sciences

Department of Preclinical Sciences and Pathology (PREPAT)

DIFFERENTIATION OF SH-SY5Y-CELLS AND PROTEOLYTIC CLEAVAGE OF THE PRION PROTEIN

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At last I would like to dedicate this thesis to Günther, who was there for me from the beginning and almost to the end.

DEFINITIONS

PrP, PrP^{C}	Prion protein, cellular
PrP^{Sc}	Misfolded scrapie conformer of PrP, a marker for prion disease and prion infectivity
Undiff, SH-SY5Y, diff-	Undifferentiated SH-SY5Y cells
Diff, SH-SY5Y-diff, diff+	Differentiated SH-SY5Y cells
C1	C-terminal fragment of the prion protein after α-cleavage
N1	N-terminal fragment of the prion protein after α-cleavage
FL-PrP^{C}	Full length prion protein (not cleaved)
Shed-PrP^{C}	Shed PrP^{C}
Poly I:C	Polyinosinic:polycytidylic acid
BDNF	Brain Derived Neurotropic factor
RA	Retinoic acid
FBS	Foetal Bovine Serum
6H4	PrP^{C} Antibody 6H4
GFP	Green Fluorescent Protein
Complete cell medium	Cell maintenance medium, used for non-differentiating cells
PNGase	N-glycosidase F, enzyme used to deglycosylate proteins, removes N-glycans
huPrP, huPrP+	Cells transfected with human PrP^{C}
huPrP-GFP	Cells transfected with human PrP^{C} and GFP
pCI-Neo, Mock-SHSY, huPrP-	Control cells transfected with a “mock” protein
dsRNA	Double stranded RNA
TLR	Toll Like Receptor
IFN	Interferon
IRG/ISG	Interferon Responsive Gene/Interferon Stimulated Gene
ER	Endoplasmic reticulum

SUMMARY IN ENGLISH

This study focuses on the prion protein (PrP^C) and its processing in the cell and physiological functions. The study's main goal is to contribute to the knowledge of what role PrP^C plays in the cells.

We have investigated PrP^Cs expression in the neuroblastoma cell line SH-SY5Y. We have successfully differentiated these cells into neuron-like SH-SY5Y-diff cells that are more homogenous and probably a better model for adult neuron cells, using a combination of Retinoic Acid, Brain Derived Neurotrophic Factor (BDNF) and serum deprivation. We experimented with different concentrations of BDNF and serum to find optimal differentiation conditions.

PrP^C can be proteolytically processed after translation in several ways, the most common being α-cleavage of the protein, resulting into two fragments; C1 and N1. Our main finding concerns the expression of PrP^C and the changes in proteolytic cleavage after differentiation as well as the effect of the immunostimulant Poly I:C on differentiated SH-SY5Y cells.

Our results imply that differentiation may alter the PrP^C expression both directly through translation and through the proteolytic cleavage of the protein, altering the C1 proportion of the proteolytic fragments in differentiated cells. We have also found that exposure to Poly I:C does not seem to alter the expression of PrP^C nor the C1-proportion in differentiated cells, but the differentiated cells had a weaker response to Poly I:C within the measured timeframe of 24 hours.

The experiments are pilot studies, and the results are to be regarded as preliminary. The statistical strength is limited by the low number of replicates. The nature of the cell clones with a highly variable expression of PrP^C themselves complicate the interpretation, and especially the mRNA-results are challenging to interpret due to the highly variable mRNA values between cell clones.

Difficulties with the differentiation consumed valuable time at the start of the project and made it challenging to replicate the experiments sufficiently in our given timeframe, as well as proceed with other follow-up experiments.

Further replicates would be needed to clarify if the results were statistically significant or not. It would also be interesting to work with other cell types, for example cells from the Norwegian dairy goats with the *PRNP* $^{Ter/Ter}$ genotype that are naturally devoid of PrP^C.

To conclude, this study has contributed with new information about differentiation of the SH-SY5Y cell line. Our data suggest that these cells have higher levels of the C1-fragment of PrP^C, indicating alterations in the posttranslational proteolytic α-cleavage. The cells did not seem to respond differently to the immunostimulant Poly I:C when differentiated in regards to PrP^C expression.

SUMMARY IN NORWEGIAN

Denne studien tar for seg prionproteinet (PrP^C) og dens prosessering i cellen og fysiologiske funksjoner. Studiens hovedmål er å bidra til kunnskap om hvilken rolle PrP^C spiller i cellene.

Vi har sett på PrP^Cs ekspresjon i neuroblastomcellelinjen SH-SY5Y. Vi har differensiert disse cellene til nevronlignende celler kalt SH-SY5Y-diff som er mer homogene og sannsynligvis en bedre modell for modne nevroner. For å differensiere dem brukte vi en kombinasjon av retinolsyre, hjernederivert neurotrofisk faktor (BDNF) og serumsulting. Vi prøvde oss frem med flere ulike konsentrasjoner av BDNF og serum for å finne den optimale differensieringsprotokollen.

PrP^C kan bli prosessert proteolytisk på flere måter etter translasjonen, men den vanligste er den såkalte α-kløyvingen som resulterer i to fragmenter kalt C1 og N1. Vårt hovedfunn angår prosesseringen av PrP^C og endringene i proteolytisk prosessering etter differensiering, i tillegg til effekten av immunstimulanten Poly I:C på differensierte SH-SY5Y-celler.

Resultatene våre indikerer at differensiering kan muligens øke PrP^Cs ekspresjon enten direkte via translasjon og/eller ved øking av C1-andelen av fragmentene via proteolytisk prosessering. Vi har også funnet at stimuli med Poly I:C ikke ser ut til å endre ekspresjonen av PrP^C eller C1-andelen i differensierte celler, men at de differensierte cellene hadde en svakere respons til Poly I:C innen 24 timer.

Disse eksperimentene er pilotstudier, og resultatene må derfor ses på som midlertidige. Den statistiske styrken begrenses av det lave antallet replikater. Variasjonen i PrP^C- og mRNA-ekspresjonen mellom celleklonene kompliserer også tolkningen av resultatene.

For å kunne si om studiene er statistisk signifikante trengs flere replikater. Det ville også vært interessant å utforske andre celletyper, for eksempel celler fra norsk melkegeit som har en naturlig forekommende mutasjon, *PRNP* $^{Ter/Ter}$ som gjør at de ikke uttrykker PrP^C.

For å konkludere har denne studien gitt oss ny informasjon om differensiering av cellelinjen SH-SY5Y. Vi har også indikasjoner på at disse modne cellene har høyere nivåer av C1-fragmentet av PrP^C, som kan indikere endringer i den posttranslasjonelle proteolytiske α-kløyvingen. De differensierte cellene ser ikke ut til ha endringer i PrP^C-ekspresjonen når stimulert med Poly I:C.

INTRODUCTION

PRIONS AND THE PRION PROTEIN

Prion diseases constitute a group of fatal neurodegenerative diseases including Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, Bovine spongiform encephalopathy (BSE) in cattle and Chronic wasting disease (CWD) in cervids (4).

These diseases are important both for human and animal health due to their severity, the susceptibility of several mammals as well as their possibly zoonotic potential. Currently, there is no treatment nor vaccine against the diseases and the prions are extremely resistant in the environment, making it difficult to clean and disinfect contaminated areas and materials. The detection and eradication of prion disease is extremely expensive, as the BSE outbreak in Great Britain in the 1980s and the current CWD outbreak in Norway are examples of. The prion diseases are experimentally transmissible and characterized by a sponge-like appearance of the affected nervous tissue, therefore originally called transmissible spongiform encephalopathies (TSEs) (5).

In transgenic mice, host expression of the cellular prion protein PrP^{C} has long been known to be an absolute requirement for development of prion disease (6-8). This was also recently demonstrated in a line of Norwegian dairy goats that lack PrP^{C} due to a naturally occurring nonsense mutation (6).

Although the pathogenesis of prion diseases is not fully understood it involves misfolding of PrP^{C} into a protease-resistant conformer known as the "scrapie conformer" PrP^{Sc}. During the misfolding, the alpha helical rich PrP^{C} is transformed into PrP^{Sc} which is enriched in beta-sheet secondary structures. Already misfolded PrP may then act as templates and induce further misfolding of the other PrP^{C}s allowing propagation of the prion (4).

The development of clinical disease might be explained by either a *loss of function* when the prion protein no longer maintains its normal function, and/or by a *gain of toxicity* elicited by tissue accumulation of misfolded PrP conformers. In order to gain further insight into the molecular pathogenesis of prion diseases and develop strategies for disease intervention, it is fundamentally important to understand the normal functions of the prion protein. These proposed functions will also be discussed later. In this study there has been used a human neuroblastoma cell line (SH-SY5Y) to investigate the proteolytical processing of the protein.

Diseases associated with an accumulation of misfolded proteins in the form of amyloid fibrils or plaques are sometimes called conformational diseases, the most famous probably being Alzheimer disease that is associated with the build-up of misfolded Tau-proteins. These diseases, including the TSEs, may be sporadic, familial or infectious (5).

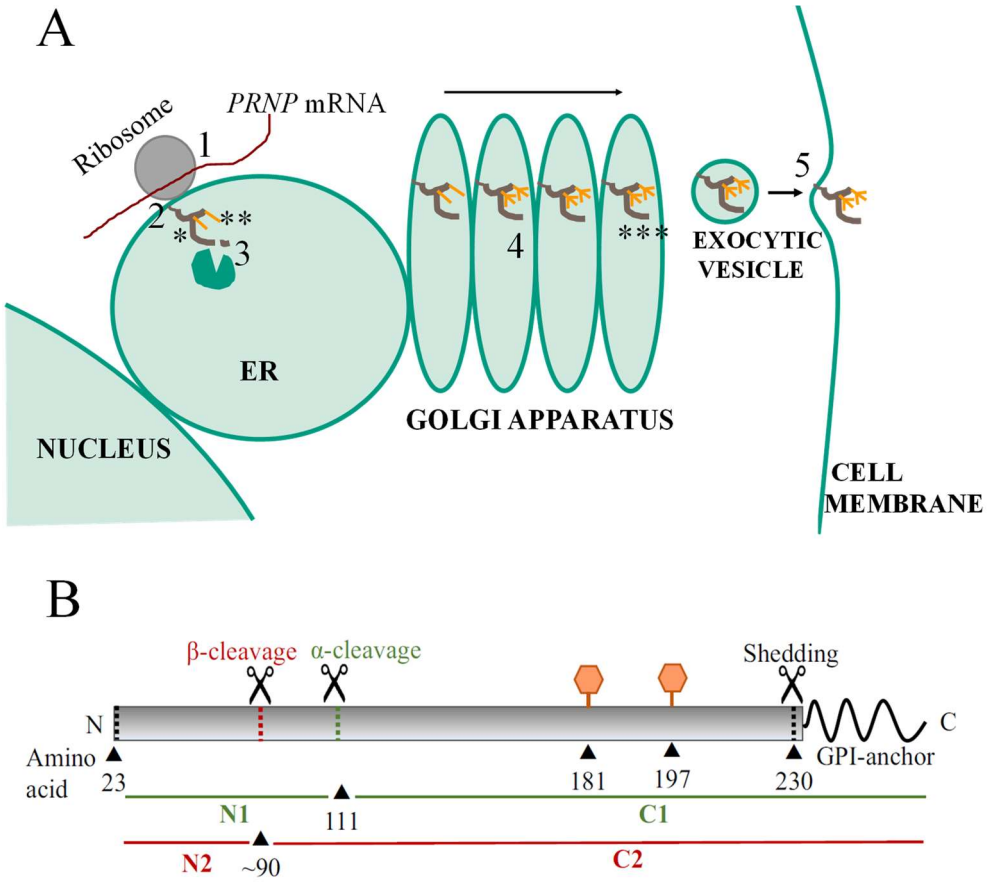


Figure 1: Prion protein in the secretory pathway and PrP^C processing. (A) The secretory pathway of the human prion protein. 1: *PRNP* mRNA is translated directly into the endoplasmic reticulum (ER). At the same time, the glycan groups may be placed at codon 181-183 and/or 197-199. The final PrP can be un-, mono- or diglycosylated. 2: GPI-anchor replaces codons 231-253. 3: Signal peptide codons 1-22 cleaved by signal peptidase. 4. Glycans modified, extended. 5. Exocytic vesicle fuses with cell surface.
*: Disulfide bond C179-C214. **: Glycans at N181, N197. ***: DXE signal at 144-146 targets to exocytic vesicle. Figure 1A Adapted and used with permission from Eric Vallabh Minikel / cureffi.org.(2) (B) Schematic presentation of the prion protein with α, β and shedding cleavage sites as well as the two possible glycosylation sites (hexagons).

The synthesis of PrP^C (Figure 1 A) starts with the transcription of the *PRNP* gene, followed by processing and nuclear export of its mature mRNA to the cytoplasm. When the mRNA is translated to protein by the ribosome, the N-terminal sequence of PrP^C emerges first and its endoplasmic reticulum (ER) sorting signal is recognized by a protein complex that ensures docking of the ribosome to ER-membrane and the translocon which enables the newly synthesized protein to be translocated into the lumen of the ER during synthesis. Upon entry into the ER, the N-terminal ER-signalling sequence (N-terminal 22 amino acids) is removed by an ER-signal peptidase that resides in the ER. During its transit through the secretory pathway, PrP^C is further modified by establishment of a disulphide bond, addition of two asparagine-attached glycan groups (N-glycans) and a C-terminal

glycosylphosphatidylinositol (GPI) membrane anchor, tethering the protein to cellular membranes (9).

In order to be synthesized, *PRNP* is first transcribed into mRNA which in the cytoplasm binds to ribosomes and protein synthesis starts. Upon recognition of PrP^C's N-terminal signal sequence, the protein synthesis complex docks onto the endoplasmic reticulum (ER) to ensure co-translational translocation into the ER-lumen (Figure 1A) (9). As many proteins, PrP undergoes several posttranslational modifications before arriving at its destination. These modifications include glycosylation, glypiation (adding of the GPI-anchor), formation of the disulphide bond and proteolytic cleavage. After passing through the secretory pathway, the protein may be anchored in the cell membrane via its GPI-anchor, shed N-terminal parts into the extracellular matrix, or traffic between recycling endosomes and the cell membrane. When anchored in the cell membrane, PrP^C is associated to lipid rafts but may also cycle between endosomes and the cell membrane (10, 11).

Glycosylation

In the ER, oligosaccharyl transferase adds glycan groups on the still translating prion protein, a process called glycosylation. Human PrP^C has two glycosylation sites; at asparagine residues (N)181 and (N)197 (12). PrP^C can have three major glycosylation states i.e., di-, mono- or unglycosylated. We are able to distinguish unglycosylated proteins from their glycosylated counterparts, e.g. on a western blot due to the extra mass contributed by the glycans. Enzymatic removal of the N-glycans can be achieved by treatment of protein preparations with Peptid:N-glycosidase F (Pngase-F) allowing discrimination of full length PrP (FL-PrP) from the C-terminal fragment generated by proteolytic processing (C1 fragment) in western blot analysis (13).

Glypiation

The GPI anchor is structurally conserved among eukaryotes and is present on 10-20% of all plasma membrane proteins. It allows membrane attachment without the protein itself spanning the lipid bilayer. In addition, GPI-anchored proteins can be packed more tightly and therefore take up less space than proteins with a transmembrane domain. GPI is synthesized and transferred to the protein in the ER and is further modified in the ER and Golgi apparatus. The site where the GPI attachment signal is cleaved off and replaced with the GPI anchor is called the ω-site and is believed to be situated at amino acid 230 in huPrP (10, 14).

Both PrP^C and PrP^{Sc} are bound to the membrane through a GPI-anchor. In a study by Chesebro et al in 2005, scrapie-infected transgenic mice lacking the GPI-anchor did not deposit the non-amyloid form of PrP^{Sc} but developed amyloid plaques instead and minimal symptoms of clinical disease (15).

Proteolytic processing of the prion protein

Several proteolytic modifications of PrP^C have been described: α-, β- and γ-cleavage and shedding. These processes result in different fragments (Figure 1B) that most likely have different functions and may therefore influence the protein stability and degradation time, its distribution and putative signalling differently. The proposed physiological functions of PrP^C are many and will be addressed later. In general, PrP^C is thought of as a multivalent scaffolding protein that can bind multiple extracellular and transmembrane ligands, and thus be able to influence many cellular functions. The proteolytic processing of PrP^C may be a

way of regulating PrP^Cs associated functions, and cleavage products may also act as soluble ligands. Since the cleavage processes influence PrP^Cs halftime in the cell, these processes may be a way of regulating PrP^C itself (13).

α-cleavage

In 1993, Harris et al reported that the chicken homologue of mammalian PrP underwent a cleavage within or just N-terminal to the hydrophobic domain (16). This was later specified to be between amino acids 110/111 or 111/112 and termed α-cleavage (17). The result of this cleavage is the release into the extracellular environment of the N1-fragment of approximately 11 kDa, leaving the membrane bound C1-fragment of approximately 16 kDa attached to the plasma membrane. Early reports suggested that the α-cleavage occurred in acidic endosomal compartments, but it is now thought to happen during transit through vesicles along the secretory pathway (Figure 1A)(18) or during the shuttling between the plasma membrane and endosomes, and is catalysed by an unidentified enzyme, generically called "α-PrPase". Several enzymes from the ADAM (**a** **d**isintegrin **a**nd **m**etalloprotease) family have been considered as responsible for the α-cleavage such as ADAM8, ADAM 17, ADAM 9 and ADAM10 (13, 17), although recent studies with ADAM10-knock out animals suggest that ADAM10 is probably not the culprit (19).

The GPI-anchor binds the C-terminal part of the prion protein to the plasma membrane. Therefore, the C-terminal cleavage products (C1, C2, C3) will remain attached to the membrane whereas N-terminal fragments will be liberated to the extracellular matrix.

The α-cleavage site is just N-terminal to a hydrophobic region of the protein, and it liberates the un-structured, copper-binding N-terminal tail of PrP^C. The products resulting from this cleavage have different properties than the full-length protein (fl-PrP^C); C2 has a higher stability and persistence at the cell surface than fl-PrP^C and will therefore increase the turnover time of PrP^C in the cell (18), indicating that α-cleavage might be a way for the cell to regulate PrP^C, increasing cleavage when PrP^C is needed. Importantly, the C1 remnant cannot form PrP^{Sc} (18), making it an interesting factor in ways of halting prion disease development. Mice with overexpression of C1 show delayed development of prion disease (13). In addition, cell lines with a higher C1 percentage have shown a lower susceptibility of PrP^{Sc} conversion (20). Table 1 gives an overview of anti-PrP antibodies that can be used to detect various fragments of the protein.

One of the best characterized functions of PrP^C is myelin maintenance of peripheral nerves, which has been proposed to involve α-cleavage of axonal PrP^C. The liberated N1 may diffuse to a receptor on the myelinating Schwann cell membrane in order to elicit a myelin maintenance signal, thus functioning like a mediator of paracrine signalling. N1 is required for important interactions and is released when the vesicle fuses with the cell membrane (17, 21). It may act neuroprotective as a consequence of it binding to a surface receptor and acting as a signal molecule (13).

C1 percentage in different brain regions and genotypes

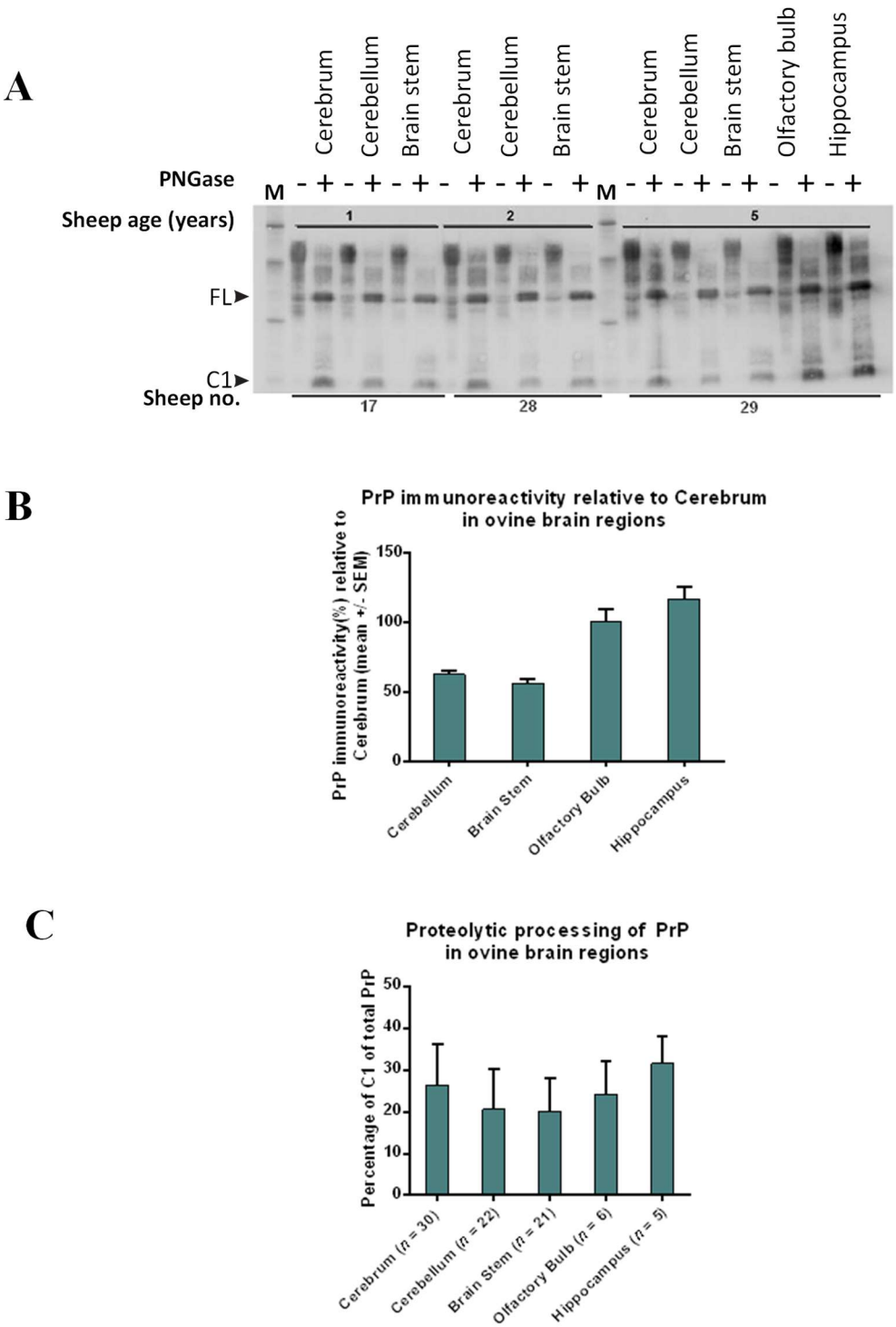


Figure 2: PrP protein in ovine brain regions. (A) Western Blot of PrP^C in different ovine brain regions. (B) PrP immunoreactivity relative to cerebrum (cortex). The immunoreactivity in different regions varied considerably between animals but was consistent within single animals. The levels are therefore presented relative to cerebrum. (C) C1 percentage of total PrP in different regions. Figure based on the work from Campbell et al in their article "The PrP^C C1 fragment derived from the ovine $A_{136}R_{154}R_{171}$ *PRNP* allele is highly abundant in sheep brain and inhibits fibrillisation of full-length PrP^C protein in vitro"(3).

The different fragments of PrP^C are rarely at the main area of investigating when studying PrP^C, even though increased production of C1 is, as mentioned above, associated with resistance to prion disease. A few years ago, our laboratory ("Prion Lab" NMBU) investigated levels of PrP^C processing in various brain regions of sheep of various age- and *PNRP* genotype categories. Data from this analysis was included in a publication in 2013 (3). An example from this analysis is shown in Figure 2, where the relative C1 content in different ovine brain areas as well as in different *PRNP* genotypes associated with varying resistance to prion disease was examined. They show that relative C1 levels in different sections of ovine brain vary considerably between animals but are consistent within a single animal.

Campbell et al also state that the relative amount of C1 in ovine cortex varies with *PRNP* genotype, some of which are associated with different resistance to classical scrapie. Because the levels vary so little between brain areas in one animal, the cerebrum (cortex) is used to compare different PrP genotypes in sheep. One of the genotypes (ARR/ARR) is associated with resistance to classical scrapie while two other genotypes (ARQ/ARQ and VRQ/VRQ) are associated with susceptibility. In this study, sheep with the ARR/ARR genotype expressed significantly more relative C1 and less C2 compared with the two other genotypes. The authors proposed that the increased α-cleavage in ARR/ARR sheep contributes to the disease resistance in these sheep (3).

β-cleavage

β-cleavage occurs around amino acid 90, producing the C2- and N2-fragments. The N2-fragment is approximately 9 kDa and is released. The C2 fragment is 18-20 kDa, depending on the glycosylation status, and is bound to the cell membrane in the same manner as C1/fl-PrP^C. It is not completely clear which processes or enzymes that are involved in the β-cleavage.

Unlike C1, C2 is easily converted from PrP^C to PrP^{Sc} *in vivo* and *in vitro* and is possibly a promoter of prion disease. β-cleavage is described to occur mainly under pathological conditions (21). Understanding the β-cleavage of PrP^C is an important topic of investigation, but this was not within the aims of this study and will therefore not be elaborated further.

γ-cleavage

This cleavage process was first described in 2016 by Lewis et al and termed γ-cleavage (22). It is still not clear where in the cell this happens, but possible locations are the secretory pathway, similar to the α-cleavage and/or at the cell surface such as the β-cleavage. Unglycosylated PrP^C is favoured and can be cleaved by a γ-PrPase within the far C-terminal part, rendering a soluble N3 and a short C3 fragment. The γ-cleavage site is still unknown but considering the size of C3 and N3 it is probably between amino acids 176 and 200 (22). Both β- and γ-cleavage is also considered increased under pathological conditions, although C3/N3s roles in prion disease remain unclear (13).

Shedding

Shedding denotes the most C-terminal proteolytic cleavage of PrP^C, leaving the GPI-anchor and a few amino acids attached to the plasma membrane while the rest of the protein (shed-PrP) is released. ADAM10 cuts the protein between amino acids 228 and 229 in murine PrP^C, but the exact cleavage site in human PrP^C remains unclear (23).

Shedding is also the only proteolytic cleavage that releases glycosylated fragments, as the three other cleavages happen distally to the glycosylation sites. Some studies indicate that in addition to fl-PrP^C, C1 and C2 may also be cleaved by ADAM10 and shed from the plasma membrane. Interestingly, ADAM10 is also responsible for the cleavage of the amyloid precursor protein involved in Alzheimer disease (13).

The functions of shed-PrP^C remain elusive, although the fact that is has been conserved in several species and makes up a high proportion of the total cell PrP^C argue for it being of importance. It has been suggested that soluble shed-PrP^C may interact with membrane bound PrP^C forming a ligand-receptor complex and that shed-PrP^C is the PrP fragment with most importance for neuroprotection, neurotrophism, chemotaxis and in the inflammatory mast cell response (13).

Trafficking and turnover

As mentioned, PrP^C may cycle between the plasma membrane and endosomes. This shuttle takes approximately 60 minutes, and during every cycle an estimated 1-5% of the PrP^C molecules undergo α-cleavage. The N-terminal fragment is then released when the shuttle reaches the cell membrane (11). This shuttle is likely to be a form of regulation of the cell's surface pool of PrP^C (24).

PROPOSED FUNCTIONS FOR THE PRION PROTEIN

PRNP has some properties that are typical for housekeeping genes: It has a GC-rich region next to the transcription start site as well as several evolutionary conserved binding sites for transcription factors, the latter maybe to adapt PrP^C expression to various stimuli. As mentioned, PrP^C can shuttle between the plasma membrane and endosomes, but the functional form is believed to be at the cell surface(24). PrP^C is expressed at various levels in several organs; analyses of PrP^C expression reveal that the highest expression of PrP^C is found in the central and peripheral nervous system, intermediate levels in thymus, intestine, heart and spleen and lower levels in lung, muscle, kidney, lymph node, skin, pancreas and liver (25).

Even so, animals that do not express the protein, either knock-out animals or animals with a naturally occurring mutation, do not seem to be markedly affected by the lack of prion protein(26). The fact that PrP-null mouse strains seem to develop normally suggests that PrP is either unnecessary for normal development, or that its functions may be replaced by other protein(s) (27). In any case, these animals show that PrP^C is not essential for life.

A review from Castle and Gill in 2017 (24) state that the current literature supports that PrP^C is strongly associated with myelin maintenance (28, 29) and cellular differentiation, and might be associated with modulation of circadian rhythms, glucose homeostasis, immune function and iron uptake. Earlier studies support PrP^C with functions such as stress-protection, copper homeostasis and neuronal excitability, but these functions seem to be less relevant than anticipated according to Castle and Gill. They also mention the theory of PrP^C being a scaffolding protein, and therefore being able to control/influence a multitude of functions in the cell. Since PrP^C is not a transmembrane protein it cannot directly pass signals into the cytosol but depends on other binding partners in order to form signalling complexes

(27). Another review from Wulf et al, also from 2017, supports that PrP^Cs main functions regards myelin maintenance and neural development and possibly a neuroprotective role (30).

PrP^C is expressed in immune cells, in particular T-lymphocytes, Natural Killer cells, macrophages and mast cells (24). This has led to a putative association with immune function, both in ways to protect the cell and to dampen the immune response as PrP^C is highly expressed in organs with an immunological quiescence such as the brain, eye, placenta, testes and pregnant uterus. This is part of the picture indicating that PrP^C helps protect the cells from inflammatory damage (31), although a definitive immunological function for PrP^C is still uncertain (32, 33).

SH-SY5Y-CELLS

About the cell line

A cell line has its origin from an organism but has been immortalised and further grown in the laboratory. The SH-SY5Y cell line is a subclone from the heterogenic neuroblastoma cell line SK-N-SH, which was established in 1970 from a bone marrow biopsy of a 4 year old female patient (34). These tumours derive from neural crest cells (35).

The SK-N-SH cell line consists of at least two different sub-types: Neuroblastic (N-type) and substrate adherent (S-type), and some studies also report a third, I-type cells that are intermediate variants of N- and S-type cells (35). The SH-SY5Y cell line is derived from N-type cells, with neuroblastic properties and a low proportion of S-type cells, and *in vitro* differentiation may only target one of the types as described further below (36).

The SH-SY5Y cell line is widely used in Parkinsons's Disease research, a disease characterized by death of dopaminergic neurons (37). Xicoy et al report that 393 out of 962 analyzed original publications state that SH-SY5Y-cells have a dopaminergic phenotype without actually showing supporting evidence (38). Differentiation of SH-SY5Y cells can induce both dopaminergic, adrenergic and cholinergic phenotypes, the differentiation protocol being an influencing factor of which phenotype the cells turn into (39, 40), making the cell line quite versatile and widely used. The cells may even respond differently to the same treatment depending on cell source (36).

There are both advantages and disadvantages in using established cell lines compared with other models and tools for investigation. It is easier to get a homogenous culture consisting of only one cell type, e.g. by using differentiation, compared with primary cell cultures. The use of cell lines can replace some of the research conducted on animals, and using human cell lines t will mimic human conditions better than animal cell cultures, to a certain extend. The use of cell cultures is an important step in replacing, reducing and refining the use of research animals when possible, an important principle in scientific studies. Moreover, many analytical imaging tools, for instance live imaging and methods for transgenics are easily available in cell culture. This makes detailed investigations of protein properties and functions easier and less costly.

Nevertheless, it is important to keep in mind that cell lines are genetically manipulated cells or cancer cells and will therefore not always represent the original tissue correctly (39). Cell cultures may reveal crucial molecular interactions and functions, but not completely replace the use of whole organisms. When using a cell culture, our reference population is still other cell cultures of similar type, not an intact animal or animal populations. In addition, one may

experience problems such as contamination of the cell culture with other cells or pathogens when working with cell cultures, similar to problems with pathogens in research animals.

Differentiation of SH-SY5Y-cells

Cells go through different stages in a cell cycle; G1-phase, S-phase, G2-phase and M-phase, where they grow, synthetize new DNA and divide, respectively. When the cells differentiate, they exit the cell cycle and enter the G0-phase (Figure 3). In G0, the cell is neither dividing nor preparing for division. This phase was earlier wrongfully called a resting phase, but the cell is metabolically active and specialized to perform its duties. Cell cycle arrest usually means arrest in G0 although arrest in G2/M-phase is also described (41). The cell cycle interval is the time it takes for one newly divided cell to divide again. For SK-N-SH and SH-SY5Y cells, this is reported to be 27-48 hours (42, 43).

As damage to the cell's DNA can be lethal both to the cell itself and the organism, the cell has several cell cycle checkpoints. These are mechanisms where the cell cycle is slowed and the DNA replication and mitosis are checked to see whether they are complete without any damages. The most important checkpoints are typically at transitions into a new phase; G1, G2 and the Spindle assembly checkpoint (SAC) at respectively the G1/S-transition, the G2/M-transition and the metaphase/anaphase-transition (1, 44).

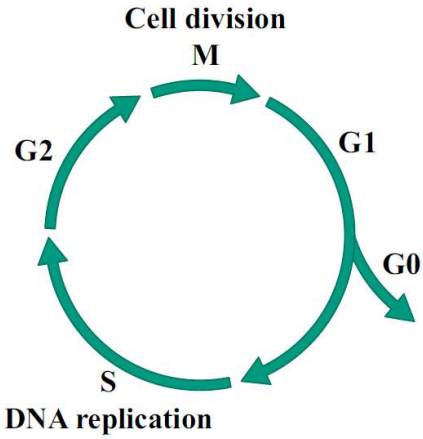


Figure 3: Cell cycle. Simplified version of the cell cycle with the different phases, not showing the checkpoints. G1-phase: The cell is growing, and biosynthesis is resumed at a high rate. The cell can now either enter G0 continue to S phase. G0-phase: The cell has left the cell cycle, stopped dividing and can differentiate, as is the case with SH-SY5Y cells that differentiate into SH-SY5Y-diff. S-phase: The cell is preparing for division with DNA-synthesis. Transcription and translation is downregulated. G2-phase: The cell continues with preparation for division, and is now growing, synthetizing proteins and forming a spindle in order to separate the chromosome pairs. M-phase: Cell growth stops and the cell is dividing (mitosis)(1).

Cells from cancerous cell lines are being differentiated for several reasons: Differentiation will normally make the cell population more homogenous, it may better represent a specific cell type or tissue which may oftenbe desired (42). Undifferentiated and differentiated SH-SY5Y cells (SH-SY5Y and SH-SY5Y-diff, respectively) differ in many ways, and cells used for *in vitro* experiments should be differentiated to get more accurate results for *in vivo*

models. There are many protocols to ensure differentiation, some of which combine multiple methods to obtain fully differentiated cells (45).

Because cell cultures often are rapidly dividing cancer cells, an undifferentiated cell culture will have cells representing the whole spectrum of cells with regards to differentiation. One of the key purposes of differentiating the cells is to synchronize the majority of cells into the same phase; namely the G0 in which the cells are non-dividing.

During the differentiation process the cells undergo a series of profound genetic, morphological and functional alterations which manifest with a neuron-like morphology with neurite outgrowth and synaptophysin-positive synapses. Synaptophysin is a glycoprotein expressed by neurons and neuroendocrine cells, localized to synapses and used as a synaptic marker. Further, expression of several neuron specific enzymes, neurotransmitters and corresponding receptor repertoire (42).

The principal step in differentiating the cell population is to expose, in a controlled way, the culture to specific chemicals or signalling molecules to which the cells respond. Some of the earliest reports of differentiation of SH-SY5Y-cells come from Påhlman et al in 1980, who differentiated SH-SY5Y-cells with tetradecanoylphorbolacetate (TPA), a chemical known to differentiate hematopoietic cell lines. In 1984, they described differentiation of SH-SY5Y with Retinoic Acid (RA) which is a derivative of vitamin A and known to have profound effects on cell growth and differentiation (46). Other methods include different neurotrophins such as BDNF (38), Silver Nanoparticles and different combinations of these(47), and some have also used conditioned media derived from *other* differentiated cells (48).

Today, RA is a common differentiation factor that is broadly used both alone or in combination with other factors or protocols. Alone, RA may be insufficient to achieve lasting differentiation of the SH-SY5Y cell line into a homogenous cell population of N-type cells. Encinas et al., report that when treating the cells with RA alone, the cell culture will initially consist of an N-type population with neurite outgrowths; however, with time the S-type cells will emerge and dominate the culture. They concluded that RA treatment alone is not suitable for acquiring homogenous cultures (36). Others have reported that the S-type cells outgrow the N-type more quickly if the foetal bovine serum (FBS) used has not been heat-inactivated, which is done in order to inactivate complement that can destroy cells under certain conditions (40).

Encinas et al investigated the distribution of cells during differentiation with RA and found that several cells where not in cell arrest in G1, but were still in the S, G2 and M-phases. Further treatment with BDNF in addition to RA resulted in 90% of the cells being arrested in G1 and progressive withdrawal from the cell cycle and entry into G0. Withdrawal of BDNF could induce an attempt to re-enter S-phase, following apoptosis (36).

RA initiates neural differentiation and inhibits cellular proliferation and its effects can be assessed both morphologically and biochemically. The morphological changes in the cell culture include decreased cellular density and acquisition of long processes and a more elongated shape. The proliferation rate and apoptotic tendency decreases, and the cells obtain a more neuronal-like morphology (49, 50). Biochemical changes include higher levels of antioxidant enzymes, lower levels of H_2O_2 and increased resistance to H_2O_2 as well as susceptibility to some oxidative stress from exposure to 6-hydroxydopamine (51). In addition

to these changes, RA-differentiated cells upregulate genes associated with cell cycle arrest at the G1-checkpoint, such as cyclin-dependent kinases (CDK)-inhibitors (50) and promote survival by activating the phosphatidyliositol-3-kinase(PI3K)/Akt signalling pathway that is important in cell cycle progression. They also upregulate the antiapoptotic Bcl-3-protein (42). All these changes are part of keeping the cell alive as well as exiting the cell cycle into G0 and differentiation.

BDNF is a neurotrophin, a family of proteins that are crucial for survival and differentiation of neurons during development, and among else important for synaptic transmission in the adult brain. In brain tissue the levels of BDNF are normally high, but can fluctuate significantly according to neuronal activities, underscoring its functional importance (52).

Several differentiation protocols combine BDNF and RA, as BDNF seems to enhance the effects of RA. When establishing synaptic connections during differentiation, it is normal that half the cells receive insufficient neurothrophic survival signals to induce differentiation. When differentiated with BDNF, the cells depend completely on neurotrophins for survival, maybe because the cells are trying to re-enter S-phase as a response to withdrawal of BDNF, causing apoptosis (36). This neurotrophic dependence supports the observation that the cell culture is more homogenous since all the cells are BDNF-dependent, alike mature neurons (40).

Another differentiation method is to expose the cells to nutrient-poor medium, also called serum starvation or deprivation. This usually involves keeping the cells in medium with a low serum concentration, typically between 0,05%-0,5%, but sometimes as high as 5%. The cells first response is typically to stop proliferating and enter cell cycle arrest and under certain conditions undergo apoptosis (53). Withdrawal of growth factors combined with serum deprivation may induce apoptosis, as was observed in our experiments (data not included).

Cell cycle arrest achieved using only serum starvation can be reversible. Human dermal fibroblasts (HDF) and Adipose stem cells (ASC) that were starved for 18-24 hours entered the G0/G1-phase and re-entered the cycle when normal serum concentration was restored. The cells were not exposed to differentiation factors such as RA, which might explain why the cell-arrest is reversible (54). SH-SY5Y-diff, on the other hand, are unable to re-enter the cell cycle once differentiated, and removal of neurotrophic factors such as BDNF rapidly induces apoptosis (36).

As mentioned above, differentiation may promote only one cell type in the cell culture. Substances such as BDNF will promote the N-type kind of cells, but also normal cell culture maintenance will select against S-type cells with splitting and harvesting because they attach more strongly to the plate (36). The differentiation will select against epithelial cells(40) as well as driving all the cells into the same stage in the cell cycle; G0. When combining RA and BDNF in their protocol, Encinas et al. observed a prolonged period were the culture is N-type dominated. When combining RA, BDNF and serum depravation they achieved a homogenous cell population of N-type cells with only a few (almost undetectable) S-type cells (36).

In order to verify that the cells have differentiated to a more neuronal phenotype, various cellular markers are used, which are typically proteins with altered expression during and/after differentiation. A number of neuronal markers exist, including: Grown-associated

protein (GAP-43), Neuronal Nuclei (NeuN), Synaptophysin (SYN), Synaptic vesicle protein II (SV2), Neuron Specific Enolase (NSE), Synaptosomal associated protein (SNAP25), Microtubule Associated Protein 2 (MAP2) and lack of expression of glial markers such as Glial Fibrillary Acidic Protein (GFAP) (40). In this thesis, we have used SNAP25 and MAP2 as markers of neuronal differentiation.

Occasionally, differentiated cells may convert to another type of differentiated cells, an irreversible process called trans-differentiation(55). Many cell lines derived from SK-N-SH, including SH-SY5Y, appear morphologically homogenous of neuroblast-type cells when plated, but gradually developing a more epithelial-like character over time (56). The phenotype of a continuous neuroblastoma cell line such as SH-SY5Y is considered to be caused by slower rates of transdifferentiation rather than the inability to develop into the other phenotype (36).

PrP^Cs effect on differentiation:

One of the proposed functions of PrP^C concerns cellular differentiation, as several studies have shown that PrP^C promotes neurite outgrowth(24, 57). Both Macedo et al and Steele et al have shown how PrP^C levels change with differentiation of different cell types. Expression of both *PRNP* (mRNA-level) and PrP^C (protein level) increased with differentiation of embryonic stem cells into neuroepithelial precursor cells, indicating a relationship between PrP^C levels and neuronal differentiation(27). The PrP^C levels directly correlate with the differentiation of nestin-positive neural precursor cells, reaching higher levels in the mature neurons. PrP^C also increases the *rate* of differentiation of neuronal cells in a dose dependent manner: PrP^C-knock out cells remain undifferentiated for longer time-periods compared with wild type and PrP^C-overexpressing cells of the same cell type (57).

INTERFERON

Interferons (IFN) are signalling molecules involved in anti-viral responses. There are three main types of interferons (type I, II and III). Type I, IFNα and IFNβ, are released by the virus infected cells, as a response to viral infections, while IFNγ, a type II interferon, is released by T-cells after activation with the viral antigen. The released interferon will then bind to receptors and start signalling cascades in order to induce an antiviral state of the host cells (58).

Type I interferons have three major functions (59):

- Induce antimicrobial states in infected and neighbouring cells
- Promote antigen presentation and Natural Killer cell function
- Activate the adaptive immune system

IFNα and IFNβ bind to the IFNAR receptor on the cell surface and activate JAK1 and TYK2 (kinases). The kinases phosphorylate STAT1 and STAT2, transcription factors and signal transducers. STAT1 and STAT2 then dimerize and enter the nucleus, where they together with IFN-regulatory factor 9 (IRF9) form the ISGF3-complex. ISGF3 is a direct transcription activator and upregulates the transcription of interferon responsive genes (IRGs). There are several hundred IRGs, many that help the cell to enter an antiviral state (59).

The Toll Like Receptor (TLR)-family is one of two families involved in induction of type I interferons, the other being RIG-I-like helicases (60). Polyinosinic–polycytidylic acid (Poly

I:C) is a double-stranded RNA (dsRNA) used to simulate virus infections by stimulating TLR. Poly I:C is associated with viral infection since dsRNA is produced by most viruses at some point during their replication and is a strong immunostimulant because dsRNA is normally not supposed to be found in a healthy eukaryote cell. Activation of Toll Like Receptor 3 (TLR3) leads to various downstream signals, among else activation of interferon regulatory factor 3 (IRF-3), which in turn leads to the induction of type I IFN and cytokine production such as interleukin-1β and -6 and Tumour Necrose Factor-α(61, 62). The effect of Poly I:C can therefore be assessed by measuring the change in IRGs. There are more than 300 type I IFN responsive genes (63), most of which can be found in a database over IRGs; Interferome (64). Some of these are essential to the immune system, in particular those with direct antiviral activity such as ISG15, Mx GTPases, RNase L and PKR. The Mx family consists of MxA/MxB in humans, and Mx1/Mx2 in mice. They are all considered to increase antiviral resistance, although MxA is the only Mx family member with demonstrated antiviral activities. Unlike RNase L and PKR, the Mx family and ISG15 are not expressed at constitutive levels and depend on type I and III interferons for their expression (63).

Many of the tentatively assigned functions of PrP^C regard immune functions. A study done by Malachin et al (65), by the same research group as this study, looked at the effect of interferon-α on peripheral blood mononuclear cells naturally devoid of PrP^C (PrP-null cells) compared with normal, PrP^C expressing cells. They found an increased expression of 86 genes, 70% being IRGs in the PrP-null cells. They suggest that PrP^C might contribute to the fine-tuning of IRGs in these cells, arguing that development of prion diseases is associated with upregulation of interferon-responsive genes in several animals, and that animals without PrP^C (knock-out or naturally occurring mutations) have an upregulation of some interferon responsive genes. They also speculate whether these gene alterations reflect an induced loss of PrP^C function. They also performed studies of IFN-α exposure on SH-SY5Y cells with high and very low hu-PrP^C expression, showing that PrP^C-expressing SH-SY5Y cells had an inhibited response to IFN-α (65). With this study in mind, we wanted to investigate whether the same effect could be seen in differentiated SH-SY5Y cells.

AIMS AND HYPOTHESES

This project was partly based on earlier studies of the effect of Poly I:C on SH-SY5Y cells, where PrP^C was postulated to have a modulatory effect on the interferon signalling pathway.

The initial aims of the project were therefore to explore a possible relation between PrP^C-expression and expression of interferon responsive genes. More specifically, the aims were to:

- Study the proteolytic processing of PrP^C during differentiation in SH-SY5Y cells using western blot.
- Study the distribution and expression of PrP^C during differentiation in SH-SY5Y using fluorescence microscopy.
- Study the processing and expression of PrP^C in SH-SY5Y-diff after stimulation of the interferon signalling pathway.

With these aims in mind, we established the following hypothesis: "*Expression of PrP^C in SH-SY5Y-cells downregulates the expression of interferon responsive genes"*.

Due to difficulties with some methods, especially immunocytochemistry, the project plan had to be adjusted. Another reason for re-direction was that our initial experiments revealed a clear difference in C1-levels in the differentiated cells, which we found very interesting. We therefore decided to investigate this further and a new hypothesis was established: "*Proteolytic α-cleavage of PrP^C in SH-SY5Y cells increase with differentiation"*.

We also pursued the third aim concerning PrP^Cs potential regulatory effect in the interferon signalling pathway and our preliminary observations are also discussed in this thesis.

MATERIALS AND METHODS

The cell cultivation and differentiation and western blot were the author's primary laboratory work tasks and will therefore be explained in greater detail. Cell transfections and mRNA analyses were done by other members of the research group but are included as they were crucial for the experiment.

All procedures were performed at room temperature unless otherwise is stated.

CULTIVATION AND DIFFERENTIATION OF SH-SY5Y-CELLS

As already mentioned, cells *in vitro* may either be primary cell cultures or continuous cultures. While primary cell cultures are typically cells taken from a tissue and grown *in vitro*, the continuous cultures or cell lines are cells that have been passaged and grown several times. Continuous cultures can be limited or indefinite. The limited continuous cultures can only be passaged a limited number of times, but the indefinite are typically tumour cells that continue to replicate.

Culturing SH-SY5Y cells

The cell line used was SH-SY5Y (Merck/Sigma-Aldrich, MO, USA). These cells normally have an extremely low expression of PrP^C, and were transfected in order to achieve SH-SY5Y clones with a high PrP^C or Green Fluorescent Protein (GFP) expression:

- huPrP-transfected: These cells are transfected with a plasmid encoding the human prion protein; huPrP (Figure 4). They have a 5 to 150 times higher *PRNP*-expression than the negative control, depending on the clone (Figure 7C).
- Mock-transfected: These cells have also gone through the transfection process but are transfected with an empty vector in order to act as the negative control that has also been through the transfection process. These cells will therefor express similar PrP^C-levels as wild-type SH-SY5Y cells.
- huPrP-GFP-transfected: Transfected with both human PrP^C and Green Fluorescent Protein (GFP). The GFP protein is visible in fluorescent imaging and allows us to watch PrP^C without staining the cells and is used to investigate PrP^Cs location in the cell or medium. In the chimeric protein, GFP is inserted N-terminally to the α-cleavage site in PrP and will therefore allow tracing of N1 or FL-PrP^C (66).

Transfected cells were grown under selection pressure of the antibiotic Geneticin (Thermo Fisher Scientific) that is toxic to both prokaryotic and eukaryptic cells by inhibiting protein synthesis. Resistance to Geneticin is transferred to the cells in the NEO-gene, thereby selecting the transfected cells. Single clones with variable levels of PrP^C (SH-SY5Y PrPhigh) or GFP (SH-SY5Y GFPhigh) were then isolated.

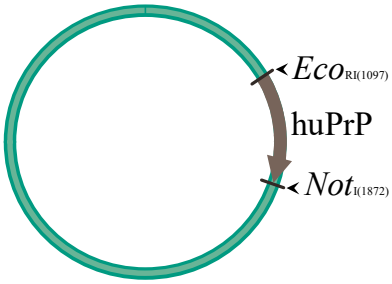


Figure 4: Transfection of huPrP in pCI-neo. The expression vector pCI-neo is used to introduce a specific gene into a target cell, in our case SH-SY5Y cells. The vector contains other regions as well; an enhancer/promoter region, an intron and a neomycin phosohotransferase gene that ensures resistance to antibiotics such as Geneticin (G-418). In our study, human PrP-cDNA was inserted between the cloning sites *EcoRI* and *NotI* at basepair 1097-1872.

Growing and maintaining the cells

The cells were cultured in the manufacturers recommended culture medium consisting of Ham's F12:EMEM (EBSS) (1:1), 2 mM Glutamine, 1% Non-Essential Amino Acids (NEAA) (all from Sigma-Aldrich) and 15% Foetal Bovine Serum (FBS) (ThermoFisher). This medium, henceforth called complete cell medium, was used for cultivating undifferentiated SH-SY5Y-cells, whereas cells undergoing differentiation (diff-SH-SY5Y) received a "differentiation medium", as detailed below.

The cells were plated in either T25-/T75-flasks (Sarstedt, Nümbrecht, Germany) or cell cultivation trays of 6/24 wells (Sarstedt) depending on whether they were destined for protein, mRNA analyses or immunocytochemistry. All cells were grown at 37 °C and 5% CO_2.

The cells were inspected every third day to monitor the level of confluence as well as the general morphology and the colour of the medium, an indicator of the remaining nutrients. When they reached a confluency of 70-80% cells were split and passaged by removing the cell culture medium, washing with PBS, adding 0,05% trypsin (Gibco, ThermoFisher), incubated for 2 minutes at 37 °C before terminating the trypsin action by adding 3x complete cell medium. All solutions that were added to the cells were heated to 37 °C. The cells were then plated again at a dilution of either 1:20 – 1:30 depending on expected/planned harvest. Cells that had been passaged more than 9 times were discarded.

Harvesting

The cells destined for western blot and qPCR were washed with PBS at 4 °C, scraped off the surface and centrifuged at 1000 rpm for 5 minutes at 4 °C. The cell pellet was then frozen at -80 °C.

Freezing

The cells were detached from the flask with trypsin, counted and diluted to a concentration of 1-1,8 * 10^6 cells/ml in complete cell medium with 10 % Dimethyl Sulfoxide (Acros Organics, ThermoFisher), which protects the cells against intracellular ice crystals during the subsequent freezing process. The cell suspension was then partitioned in 1 ml cryogenic vials

and frozen for 16 hours at -80 °C before transfer to liquid nitrogen at -196 °C. After some days a vial was thawed and cultured to examine cell viability.

Thawing

The frozen cell suspension was removed from the nitrogen tank and thawed in a water bath at 37 °C. When the suspension was almost completely thawed it was diluted 1:15 with cell medium and centrifuged at 800 rpm for 5 minutes. The cell pellet was then ready to be dissolved and plated and inspected after some days to see if the cells developed normally.

All clones differ, and not all clones will handle the transfection, freezing and thawing processes. Clones that have an atypical morphology or develop abnormally are discarded.

Differentiation

A protocol using a combination of Retinoic Acid, Brain Derived Neurotrophic Factor and serum deprivation was adapted and used to differentiate SH-SY5Y to SH-SY5Y-diff. The protocol expanded over 10 days from day -1 to day 9 (Figure 5A). First, the wells or flasks were coated with 0,01% poly-L-Lysine (Sigma-Aldrich) and cells were plated on the following day. Before plating, the cells were stained with 0,4% Trypan Blue (ThermoFisher Scientific, Waltham, MA) and counted using an automated cell counter (Countess™ II, ThermoFisher Scientific), then plated at 30000 cells/cm^2 with complete cell medium until the next day (day 1). At day 1 and 3 of the differentiation the media were changed to differentiation media with RA consisting of Ham's F12:EMEM (EBSS) (1:1), 2mM Glutamine, 1% NEAA, 1% FBS and 1‰ RA (R2625-50MG, Sigma Aldrich). At day 6 the media were changed to differentiation media with BDNF consisting of Ham's F12:EMEM (EBSS) (1:1), 2mM Glutamine, 1% Non-Essential Amino Acids (NEAA) and 25 ng/ml Recombinant Human BDNF (PHC7074, Gibco, ThermoFisher) and incubated for three days to day 9. Undifferentiated control cells were plated at 1000 cells/cm^2 and received complete cell medium at every media change. Cells that were plated for immunocytochemistry were plated on glass coverslips in 24-well plates.

Measurements of *MAP2*- and *SNAP25*-mRNA levels were performed to verify that the cells had differentiated.

Exposure to Poly I:C

The cells were exposed to 100 µg/ml polyinosinic-polycytidylic acid (Poly I:C) (Sigma-Aldrich) for 1, 3, 6 or 24 hours at day 9 of the differentiation. The Poly I:C was first diluted in dH_2O to 10 mg/ml and heated for 10 minutes at 50 °C, then diluted in complete cell medium to 100 µg/ml. Differentiated cells received Poly I:C diluted in differentiation medium with BDNF.

WESTERN BLOT

Principles of Western blot

Detecting proteins with western blot requires many steps:

Measuring protein concentration

The Bio-Rad Protein Assay is based on the Bradford Assay. The principle is to measure the protein concentration in the sample by adding an acidic dye to the lysated cell- or tissue sample as well as several standards and use a spectrophotometer to measure the absorbance at

595 nm wavelength. The absorbance is then compared with the standard curve and is used to calculate the concentration of the samples.

As an extra measurement of the protein concentration, a loading control is used when doing the western blot. This is to ensure that observed differences are the result of experimental manipulations, and not different loading quantities. The loading control proteins are typically proteins that have a high and stable expression regardless of cell type and manipulation, e.g. actin, COX-4, GAPDH and Tubulin (67).

Separation of proteins with electrophoresis

Western Blotting is a type of immunoblotting where the proteins from a sample are separated according to size using electrophoresis. The protocol (details below) makes use of the detergent sodium dodecyl sulphate (SDS) which binds do denatured proteins, and separation gels of polyacrylamide (PAGE), hence the term SDS-PAGE. Heating the protein mixture to 95 °C for 5 minutes ensures disruption of tertiary and secondary protein structures and uniform binding of SDS to the rod-like proteins, which are thereafter separated according to their molecular mass. Small proteins travel faster through the gel than large proteins, and the electrophoresis is stopped at a time where it is possible to distinguish the protein bands according to how far they have migrated. They are then compared with a benchmark protein with different known sizes.

Some proteins may require specific treatments before separating them. PrP^C is a glycosylated protein, and may be expressed as di-, mono- or unglycosylated forms. Glycosylated proteins are naturally heavier than their unglycosylated forms, and it is difficult to distinguish between the different PrP-fragments (C1, N1, FL) because the bands are smeary due to the glycan groups. They are therefore deglycosylated enzymatically prior to the electrophoresis using Peptide-N-glycosidase F (PNGase F).

Transfer of proteins from the electrophoresis gel to polyvinylidene difluoride (PVDF) membranes (semi-dry protein transfer)

The proteins in the gel are then transferred to a membrane/blotting paper. There are various techniques for this, among else a semi-wet electrophoresis technique. The principle is the same as the gel electrophoresis but instead of making the proteins travel vertically downwards through the gel, they are travelling horizontally out of the gel and onto a protein-binding membrane. This membrane will then have a copy of the protein pattern that was on the gel.

Immunoblotting

Prior to incubation with the primary antibody, the membrane is exposed to protein solution (blocking solution) to occupy sites unspecific binding of antibodies. This blocking solution contain typically proteins such as milk or serum (albumin) and will reduce the background noise in the blot. The membrane is then washed to remove unbound reagents and consequently reduce background noise.

Antibodies are used to detect the proteins. Usually, a primary and secondary antibody is used although it is possible to use only one to several, depending on the protein one wants to detect. The primary antibody is used to bind to the protein of interest and the secondary antibody is used to bind to the primary antibody. The secondary antibody is conjugated with a tag that makes it possible to detect the protein-1° antibody – 2° antibody – complex, for

example an enzyme such as alkaline phosphatase (AP). The use of secondary antibodies, or indirect detection, will also amplify the signal. When choosing the secondary antibody, it is important to remember that they are species-specific, meaning that if the primary antibody is derived from rabbit, the secondary antibody must be "anti-rabbit" for proper binding(68).

The primary antibody normally binds to a specific sequence (epitope) on the protein. When working with proteins that are posttranslationally processed, such as the prion protein, it is important to keep in mind that the blot will only show the protein parts that still contain the binding site of the antibody. This site varies with the antibody, as shown in Table 1.

We used the monoclonal antibody (mAbs) 6H4 on the western blots, and Saf32 on the immunocytochemistry. As shown in the table, 6H4 will detect FL-PrP^C and C1-/C2-fragments while Saf32 will detect FL-PrP^C and N1/N2-fragments.

Primary PrP^C- antibody	Binding site (amino acids from N)	Protein part detected
SAF-32	50-90?	FL, N1, N2
P4	89-104	N1, (N2?), C2
3F4	112-115	FL, C2
F89	142-145	FL, C1, C2
34C4	141-145	
6H4	147-155	
L-42	145-163	
SAF-84	~160-170	
F99	220-225	

Table 1: Primary PrP^C antibodies and their binding sites. The binding site decides which part of PrP^C we are able to detect. Due to proteolytic cleavage, some fragments will remain invisible.

Signal detection and semiquantitative measurement

The blots were quantified using ImageJ, a program that makes profile plots (density scans) out of the intensity of the chosen part of the image. These plots can then be used to quantify the blots and compare them with bands in the same blot. Nevertheless, background noise can influence the profile plot and bands in areas with much background noise will be interpreted as with higher expression compared with bands with little noise. This effect is significant if the blot's background noise varies much within the blot.

To reduce the effect of background noise the selection of bands can be done manually, although this also increases the probability of human error and influence on the data. In addition to this, it is important that the bands in the western blot are not saturated, as this will influence the program's ability to quantify them.

Western Blot protocol

Each cell sample were lysed with 20-60 µl lysis buffer (RIPA) consisting of Tris HCl 50 uM, NaCl 150 mM, EDTA 1 mM, DOC 0.25% and NP40 1%. An equal amount of protease

inhibitor (Roche complete, Roche Holding AG, Basel, Switzerland) was also added. The solutions were incubated on ice for one hour, then centrifuged at 3500 rpm for 5 minutes at 4 °C. Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Deglycosylation of the protein was done by incubating 20 µg total protein overnight with 1 µl PNGase-F (New England Biolabs, Ipswich, MA). For the electrophoresis, 20 µl protein (both glycosylated and deglycosylated) together with 4X SDS-loading buffer (Invitrogen, ThermoFisher Scientific, Waltham, MA) and 10X Sample Reducing Agent (Invitrogen) was heated to 90°C for 5 minutes, then centrifuged at 13000 rpm for 5 minutes. The samples were separated on 12% polyacrylamide gel (12% Criterion™ XT Bis-Tris, Bio-Rad) and Precision Plus Protein Kaleidoscope standard (Bio-Rad) was used as a benchmark and ran at 200V for 1 hour and then transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Little Chalfont, United Kingdom) with a semidry blot at 25V for one hour. The membrane was blocked in blocking buffer (5% non-fat dry-milk, Bio-Rad, in TBS-Tween) for 1-2 hours. Samples were incubated at 10°C overnight in 1% non-fat dry-milk in TBS-Tween and mouse anti-PrP^C primary antibody diluted 1:4000 (6H4, Prionics, Thermo Fischer Scientific). The membrane was then washed in TBS-Tween and incubated 90 minutes in 1 % non-fat dry-milk in tris-buffered saline with 0,1% of the non-ionic detergent Tween (TBS-Tween) and AP-conjugated anti-mouse IgG diluted 1:4000 (Novex Life Technologies, Thermo Fisher Scientific). The membrane was incubated for 5 minutes with EFC™ substrate (GE Healthcare), dried and visualized with Typhoon 9200 (Amersham Bioscience, GE Healthcare) and quantified using ImageJ software. All membranes were re-incubated with GAPDH antibody (FL-335) (Santa-Cruz Biotechnology) diluted 1:1000 and AP-conjugated Goat anti-Rabbit IgG secondary antibody, A24534 (Invitrogen) diluted 1:5000 as a loading control.

IMMUNOCYTOCHEMISTRY

Fixating and immunostaining

The cell media was removed and 4% formaldehyde (Merck, Sigma-Aldrich) in PBS added for 10 minutes on ice. The cells were then washed with PBS 3*5 minutes.

The cells were blocked with 5% Dry milk (Bio-Rad) in PBS, then incubated with the primary antibody in 1% BSA in PBST at 4°C overnight. The next day they were washed with PBST 3*5 minutes before incubated with the secondary antibody in 1% BSA in PBST at 1 hour in the dark, then washed again with PBST 3*5 minutes. The primary antibodies used were either 1:1000 Anti-Prion Protein antibody Saf32 (A03202, Bertin Bioreagent, Montigny-le-Bretonneux, France) or 1:50 Anti-NeuN antibody (MAB377, Millipore). 1:1000 Alexa-488 goat-anti-mouse (A-11029, ThermoFisher) was used as the secondary antibody for both primary antibodies.

The coverslips were then removed and mounted on Gold Antifade Mountant with DAPI (ProLong™ P36931, Thermofisher) on a microscope slide, then dried overnight in the dark.

Fluorescent imaging

Initially we tried to visualize PrP with Alexa Fluor®-conjugated antibody on fixated cells, by use of an inverted fluorescent microscope (IX81, Olympus Lifesciences, Hamburg, Germany) at 4X, 16X and 40X.

Results from the immunocytochemistry are not included in the results as we were not successful in reproducibly obtaining images of sufficient quality.

qPCR AND CELL TRANSFECTION

As already mentioned, the mRNA analysis with quantitative PCR and cell transfections were not performed by the author, but by other members of the research team. The methods for these analyses were published in the paper "Loss of prion protein induces a primed state of type I Interferon-responsive genes", by Malachin et al, 2017 (65) and the detailed methodological description below, concerning isolation of RNA and RT-qPCR is modified from that publication:

Isolation of RNA

After harvesting the cells, the cell pellet could be frozen at -80 °C or used directly. Total RNA was extracted using the Qiagen RNeasy mini plus kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. RNA concentration was then quantified using Epoch Microplate Spectrophotometer (BioTek Instruments Inc, Winooski, VT). Extracted RNA was stored at -80 °C.

Expression analysis by reverse transcription quantitative real-rime PCR (RT qPCR) analysis

cDNA was synthesized using SuperScrips III reverse Transcriptase, RNase Out, dNTP mix and random primers (all from Invitrogen, Thermo Fisher Scientific) at the following conditions: 5 min at 60 °C, >1 min on ice, 5 min at 25 °C, 1 h at 50 °C and 15 min at 70 °C. Relative expression levels were calculated using a standard curve generated from one random clone and run in triplicates and GAPDH and PDHB as reference genes.

For the Poly I:C-treatment studies on SH-SY5Y-diff, qPCR was done with Light-Cycler 480 Sybr Green I Master mix (Roche). cDNA originated from 10 ng RNA was used per reaction, and the samples were run in triplicates in a total volume of 10 µl on a LightCycler 96 system (Roche). Conditions: 5 min at 95 °C; 40 cycles of 10 sec at 95 °C, 10 sec at 60 °C and 10 sec at 72 °C; and melting curve with 5 sec at 95 °C, 1 min at 65 °C and 97 °C. Relative expression levels were calculated using the ΔΔCt method, using GAPDH and PDHB as reference genes. (65)

RESULTS

DIFFERENTIATION OF SH-SY5Y-CELLS

Changes in morphology

As mentioned in Materials and Methods, different concentrations of FBS and BDNF were tested before concluding on the final protocol presented in Figure 5A. Retinoic acid was added to a final concentration of 1‰ in all differentiation protocols.

Several pilot differentiation experiments were conducted to find out the optimal FBS concentration. As shown in Figure 5B, morphological changes were not detectable in concentrations of 5% FBS and above, but apparent at 1%. This concentration was therefore kept in the final protocol. At concentrations of 5 or 10%, the desired neural morphology was not achieved, and the cells maintained an immature morphology. At concentrations of 0,5-1% FBS the cell population showed desired morphology such as longer neurites and smaller, elongated cells. In addition, we observed halted cell division, which was the reason for why the cells destined for differentiation were plated at a higher density than their undifferentiated controls.

Initially, differentiation protocols were performed with serum deprivation and RA only. This method did not give satisfactory results (not shown) in regards to the differentiation markers MAP2 and SNAP25. BDNF was therefore added to the protocol. Similar experiments were performed to assess the difference in differentiation with 25 ng/ml BDNF and 50 ng/ml BDNF in addition to serum deprivation (1% FBS) and 1‰ RA. The observed morphological changes are shown in Figure 5C. We could detect only minor morphological differences between cells differentiated with 25 ng/ml BDNF compared with 50 ng/ml.

Changes in protein and mRNA expression

The differentiation was also evaluated using MAP2 protein assay and *MAP2* and *SNAP25* mRNA assay as differentiation markers. Western blots incubated with anti-MAP2 of two huPrP-transfected SH-SY5Y clones (huPrP2 and huPrP3) showed stronger bands at the expected mass of 150 kDa in the differentiated cells compared with the undifferentiated cells, demonstrating a higher expression of the MAP2 protein (Figure 6A). Note that the GAPDH expression, which is used to verify equal amounts of loaded total protein in the samples, is not the same in all the wells. The GAPDH-expression in undifferentiated huPrP2 (huPrP2 diff-) is higher than the others whilst the MAP2-expression is not. This difference in GAPDH is probably due to an error when loading the protein, making the difference between undiff and diff of the huPrP2-clone underrated.

The *MAP2* mRNA expression was also measured in huPrP2 and huPrP3. The relative expression varied between clones, but both clones showed an increased *MAP2*-expression in differentiated cells (Figure 6B). The mRNA expression correlates quite well with the protein expression shown in Figure 6A. *SNAP25* mRNA expression was also measured to assess the differentiation in addition to verify that differentiation with 25 ng/ml BDNF gave similar results as 50 ng/ml BDNF. The relative *SNAP25*-expression shows that both concentrations gave a relatively higher *SNAP25* expression compared with the undifferentiated cells (Figure 6B).

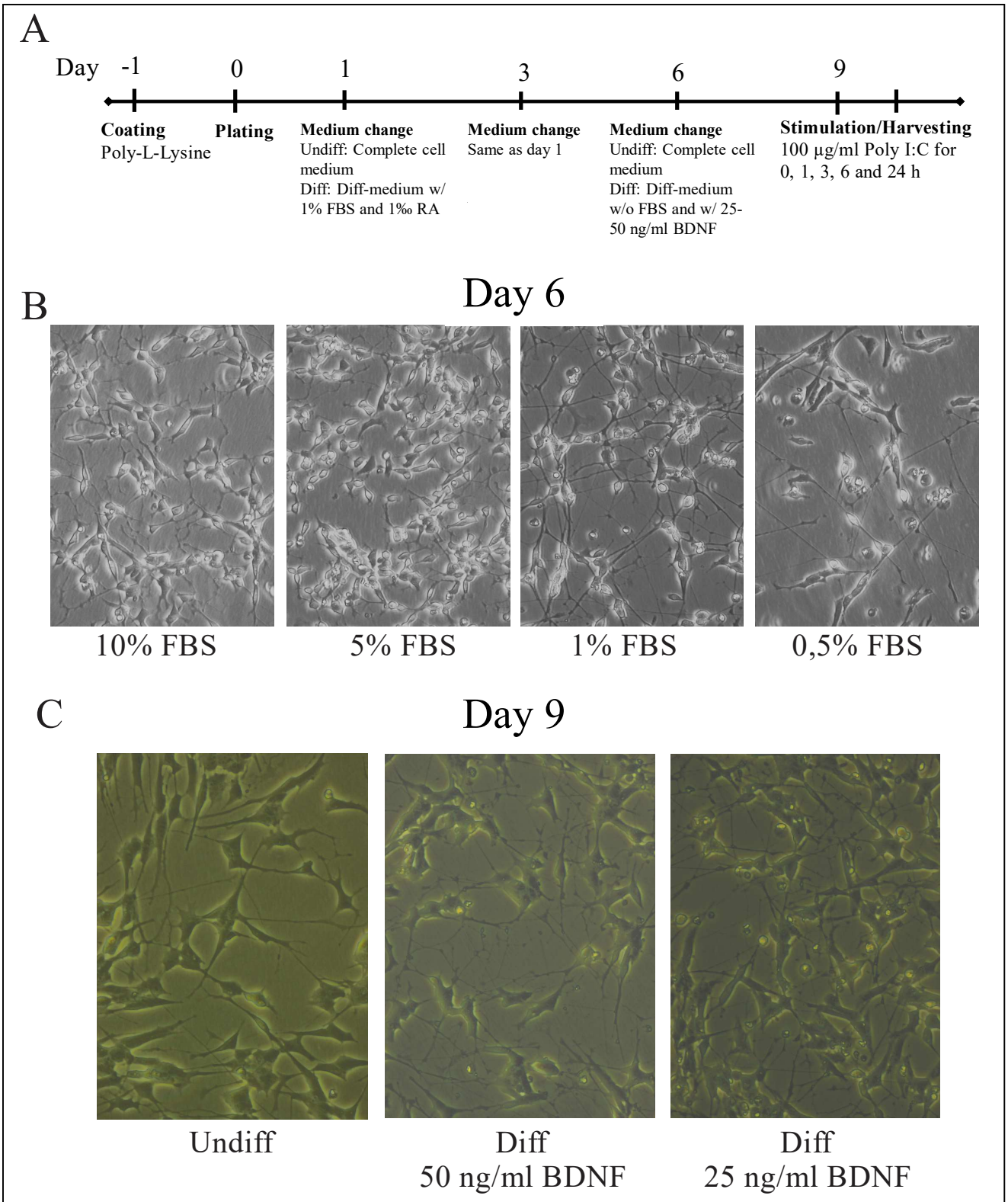


Figure 5: Differentiation of SH-SY5Y-cells. (A) Timeline of the differentiation protocol using medium enriched with FBS and RA. The medium was changed three times before harvesting the cells nine days after plating. Any stimulation with Poly I:C was done 0, 1, 3, 6 or 24 hours before harvesting. (B) Morphological changes of the SH-SY5Y-cells differentiated with 0,5-10% FBS and 1‰ RA at day 6 in the protocol. (C) Differentiated SH-SY5Y-cells at day 9 in the protocol using 1% FCS and either 50 or 25 ng/ml BDNF, negative control to the left. All the pictures were taken with Olympus IX81 inverted fluorescent microscope at 16X.

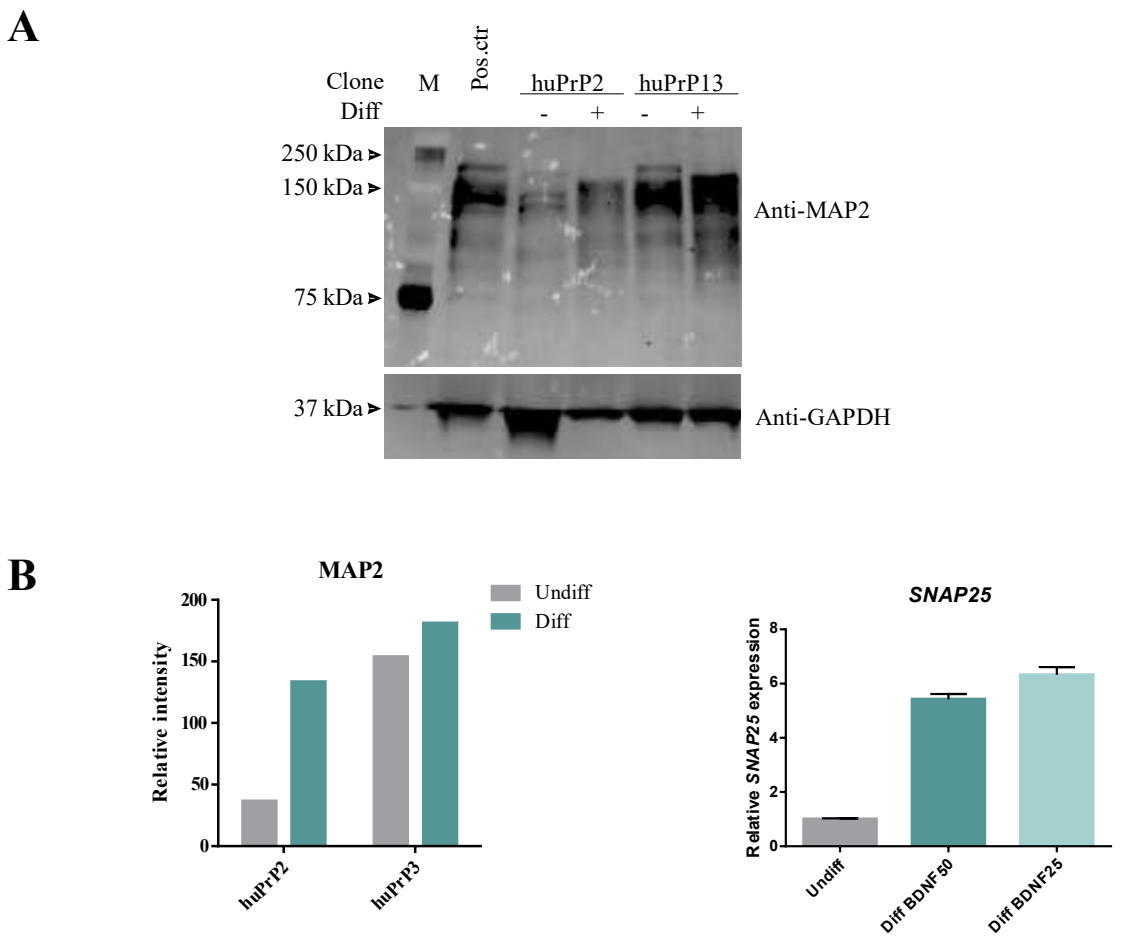


Figure 6: Changes in protein and mRNA expression with differentiation. (A) Expression of MAP2-protein in two clones of huPrP-transfected SH-SY5Y-cells, undifferentiated (-) and differentiated. A stronger expression is seen at 150 kDa in the differentiated cells. The difference between undiff and diff of the huPrP2-clone is underrated due to the higher level of protein in the undifferentiated sample (as shown in the GAPDH-blot). (B) mRNA-expression of *MAP2* and *SNAP25* in undifferentiated and differentiated SH-SY5Y-cells. The relative *MAP2*-expression is higher in differentiated than undifferentiated cells of the same clones but vary considerably between the clones. The *SNAP25*-expression is relatively higher in differentiated cells compared with undifferentiated, regardless of the BDNF concentration being 25 or 50 ng/ml. Error bars show standard error of the mean of three clones.

PrP^C-EXPRESSION IN SH-SY5Y AND SH-SY5Y-DIFF

PrP^C protein and *PRNP* mRNA expression was measured in both SH-SY5Y and SH-SY5Y-diff. The PrP^C protein expression was higher in SH-SY5Y-diff compared with SH-SY5Y, and there is a higher expression at approximately 16 kDa which represents the C1-fragment (figure 7A). The difference is most evident in the huPrP-transfected (huPrP +) cells but is also slightly visible in the "Mock"-transfected (huPrP-) showing endogen PrP, although this was not quantified. This is also evident in other blots such as in figure 9A and 9B.

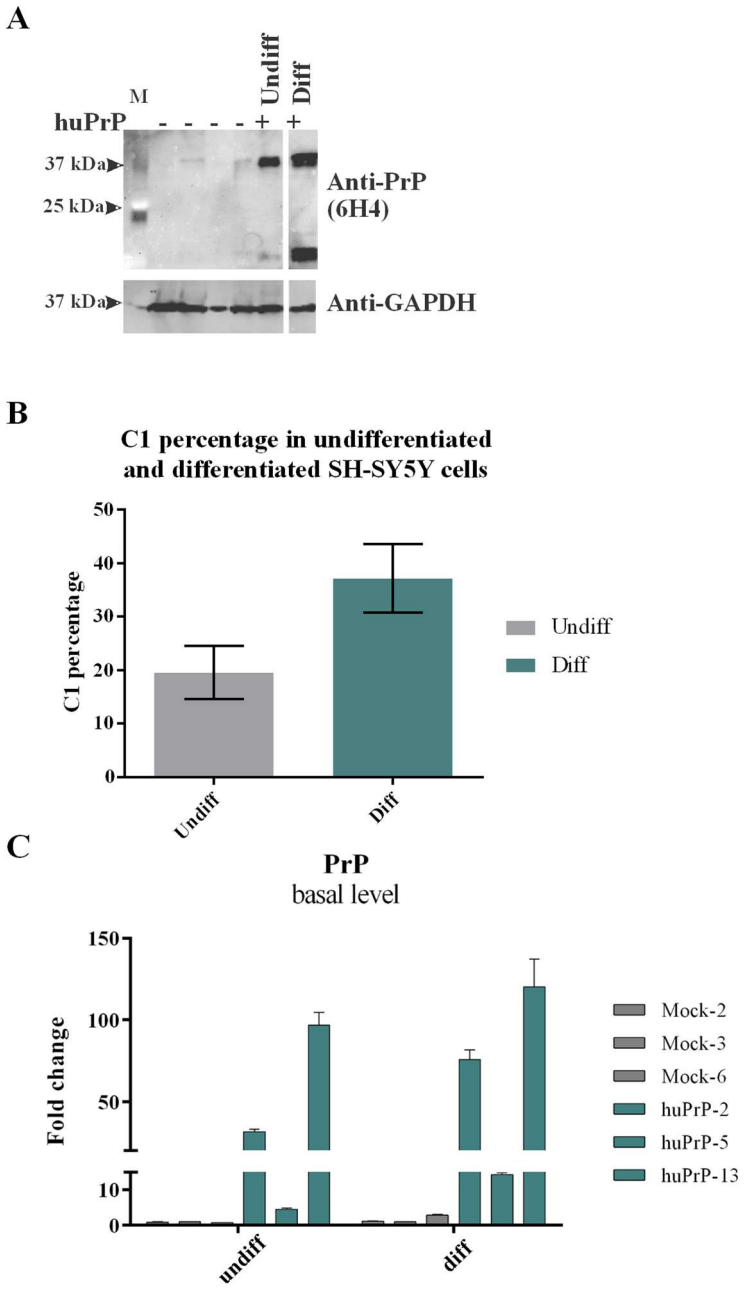


Figure 7: Changes in PrP^C expression with differentiation. (A) Changes in PrP^C protein expression in huPrP-SH-SY5Y (diff-) and huPrP-SH-SY5Y-diff (diff+). FL-PrP^C is shown at 37 kDa, while C1 is at approximately 16 kDa. GAPDH is included to show the relative protein amount in each sample. This blot shows a stronger expression of C1 in huPrP-SH-SY5Y-diff (diff) compared with the huPrP-SH-SY5Y (undiff). The four wells on the left (well 2-5) were loaded with non-PrP transfected "mock" cells (huPrP-), the second and fourth well being non-diff and the third and fifth being diff. 7A is made from one blot although the wells between huPrP-SH-SY5Y-undiff and huPrP-SH5Y-diff are cropped away for easier reading. Error bars are standard deviation of the mean. (B) Quantification of the results from four different western blots. (C) mRNA-expression of *PRNP* in six different SH-SY5Y cell clones; three "Mock" (Mock-2, -3, -6) and three huPrP-transfected (huPrP 2, -5, -13). There does not seem to be difference in the *PRNP*-expression between undifferentiated and differentiated Mock-transfected SH-SY5Y cells, but it seems to be an increase of expression after differentiation in the huPrP-transfected SH-SY5Y cells.

Figure 7B is a graphic representation of three blots corrected for the GAPDH portion and shows the same increase of C1 percentage in SH-SY5Y-diff. The C1 percentage is calculated from the quantified total protein which consists of C1 and FL-PrP^{C}.

As shown in figure 7C, the expression of *PRNP* varies between different huPrP-transfected clones, which is expected. When comparing the undifferentiated huPrP-clones with their differentiated counterparts there seems to be a slight increase in *PRNP* expression. This is consistent with the increase in the protein expression and C1 proportion, although the increase in mRNA may not be as high as the increase in protein expression.

GFP-EXPRESSION IN SH-SY5Y AND SH-SY5Y-DIFF

As mentioned in Materials and Methods, GFP was inserted N-terminal to the α-cleavage site. The green fluorescent signal will therefore indicate presence of FL-PrP^{C} or N1 but not C1.

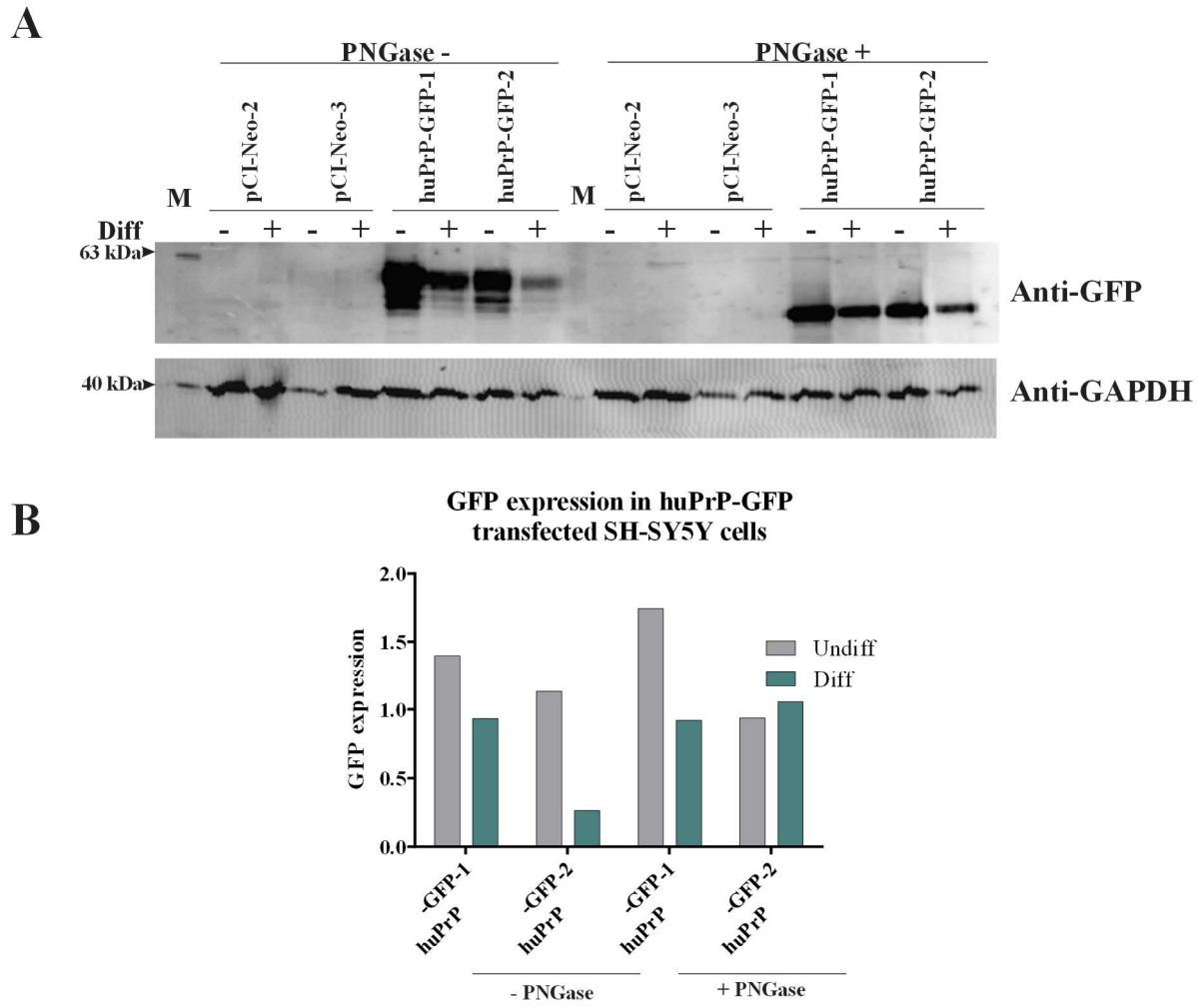


Figure 8: GFP expression during differentiation. (A) Western blot of glycosylated (PNGase-) and unglycosylated (PNGase+) SH-SY5Y and SH-SY5Y-diff. In PNGase- cells, SH-SY5Y-diff show a clear decrease in the GFP-signal compared with SH-SY5Y.This difference is not as clear in PNGase+. (B) Quantification of two different clones transfected with huPrP-GFP, PNGase- and PNGase+. The GFP-expression varies between clones but show similar results as in 8A.

Because PrP^C has three possible glycosylation states (un-, mono- or di-) we can expect three different bands in western blots with cells not treated with PNGase F (PNGase-).

We observed a decrease in the intensity of the GFP-signal in SH-SY5Y-diff, which is most evident in the PNGase- cells (Figure 8). The western blot in Figure 8A shows the expression of the clones huPrP-GFP-1 and huPrP-GFP-2 and illustrates well how the GFP-expression decreases after differentiation. This is further quantified in Figure 8B. The GFP-expression of huPrP-GFP-2 seems to decrease in the PNGase negative sample, but a slight increase is evident in the PNGase positive sample. The quantified expression of GFP is corrected for the total protein amount using the GAPDH expression. The quantified GFP-expression is therefore strongly influenced by the strength of the GAPDH-bands on the gel, and irregularities in these will reflect on the quantification. The latter should therefore be interpreted along with the blot.

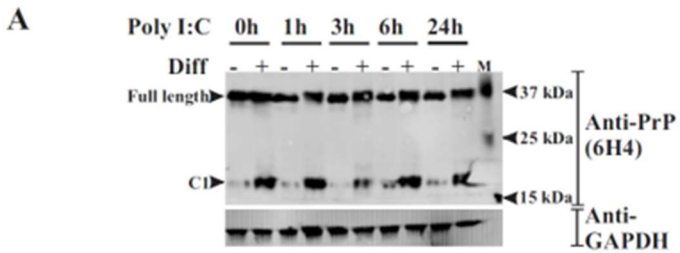
EXPOSURE TO POLY I:C DOES NOT CHANGE THE PRP^C EXPRESSION IN SH-SY5Y-DIFF

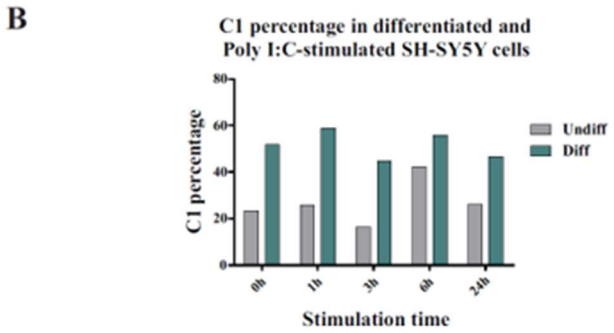
SH-SY5Y and SH-SY5Y-diff were exposed to Poly I:C for 1, 3, 6 and 24 hours. The PrP^C protein expression was assessed using western blot and the *PRNP, IFNβ* and *Mx2* mRNA expression using qPCR. In Figure 9A and 9B the PrP^C protein expression before and after stimulation of SH-SY5Y and SH-SY5Y-diff with Poly I:C is shown. Both the total amount of PrP and the relative amount of C1 appear unaltered at the investigated time points.

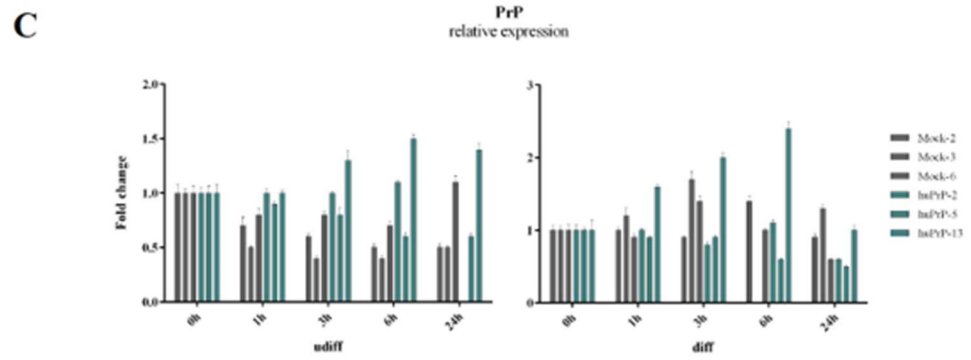
Figure 9C shows the *PRNP* expression in Mock- and huPrP-transfected SH-SY5Y cells. The fold change of non-Poly I:C-stimulated cells are almost the same as the Poly I:C-stimulated ones. Poly I:C challenge of cells seems not to influence expression levels of *PRNP* during the first 24 hours after exposure.

Expression levels of Type I interferons were assessed by analysis of *IFNβ* mRNA directly and indirectly by the interferon stimulated gene *Mx2*. Looking at the signalling cascade, we can expect an earlier response in *IFNβ* compared with *Mx2*. In SH-SY5Y, we observed an increase in *IFNβ* expression after 1 hour with a peak at 3 hours before gradually decreasing (Figure 9D). A similar reaction was seen in *Mx2* expression after 6 and 24 hours, but the decrease was not observed within 24 hours (Figure 9E). The fold change in SH-SY5Y-diff was much lower. A slight increase was seen in *IFNβ* expression and none in *Mx2* expression.

C2, one of the products from the stress-induced β-cleavage of PrP, has an estimated molecular mass of 18-20 kDa and was not observed in the blot of the Poly I:C-treated cells.


A
Poly I:C
0h
1h
3h
6h
24h
Diff
M
Full length
C1
37 kDa
25 kDa
15 kDa
Anti-PrP
(6H4)
Anti-
GAPDH


B
C1 percentage in differentiated and
Poly I:C-stimulated SH-SY5Y cells
C1 percentage
Undiff
Diff
Stimulation time


C
PrP
relative expression
Fold change
udiff
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Mock-2
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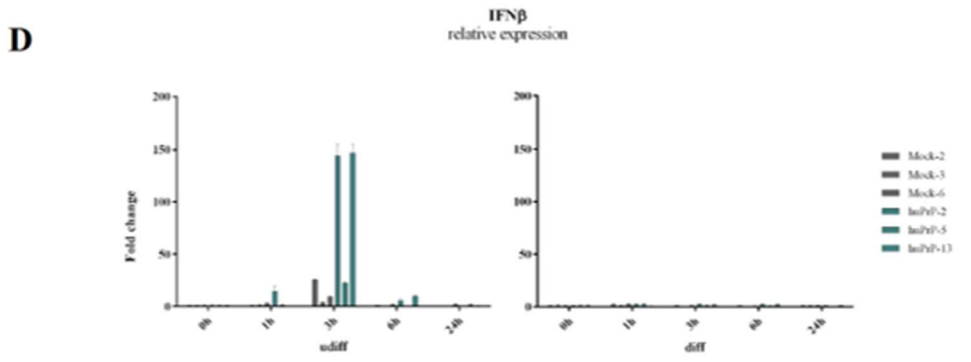
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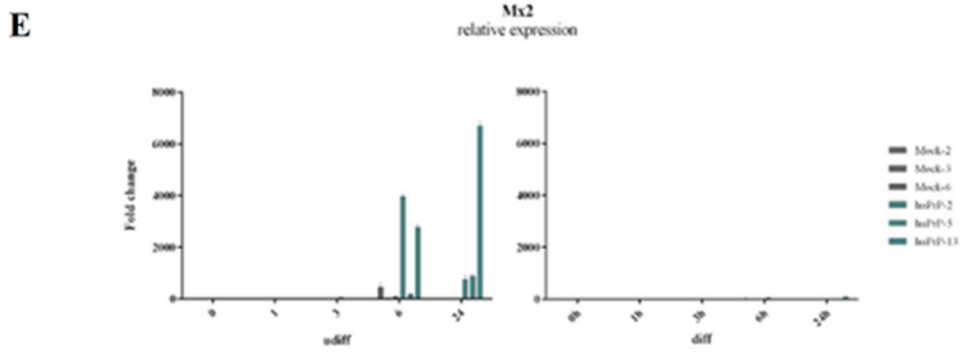
E
Mx2
relative expression
Fold change
udiff
diff
Mock-2
Mock-3
Mock-6
InfPrP-2
InfPrP-5
InfPrP-13

Figure 9: SH-SY5Y and SH-SY5Y-diff treated with Poly I:C. (A) Western Blot of SH-SY5Y and SH-SY5Y-diff exposed to Poly I:C for 1, 3, 6 and 24 hours. The blot shows no apparent difference in PrP^C-expression after exposure regardless of the time exposed. (B) Quantification of the blot from 9A, showing the C1 percentage of the total PrP^C-protein. A clear increase of C1 percentage is seen between SH-SY5Y (grey) and SH-SY5Y-diff (green), but not between the different exposure times. (C), (D), (E): Relative mRNA expression in SH-SY5Y (left) and SH-SY5Y-diff (right). "Mock" cells in grey, huPrP-transfected cells in green. (C) Relative mRNA-expression of *PRNP* in six clones of SH-SY5Y and SH-SY5Y-diff exposed to Poly I:C. The mRNA expression varies greatly within the clones, and there is no difference in expression before and after Poly I:C-exposure. Interestingly, it does not seem to be a great difference between Mock and huPrP-transfected cells either. Note that the y-axis is different in the two graphs and the expressions can therefore not be compared directly. (D) Relative mRNA-expression of *IFNβ*. SH-SY5Y show an increase after 1 hour with Poly I:C, peaking at 3 hours and decreasing at 6 hours. The fold change varies considerably between the clones. Neither Mock-SH-SY5Y-diff nor huPrP-SH-SY5Y-diff show a clear fold change at all. (E) Relative mRNA-expression of *Mx2.* These results reflect the results in 9D. huPrP-SH-SY5Y show an increase in fold change after 6 and 24 hours. SH-SY5Y-diff show little change.

DISCUSSION

The sampling and the methods

The differentiation protocol was developed during these studies and has been evaluated consecutively. Some of the adjustments that we have included in the final protocol are that 25 ng/ml BDNF give approximately the same results as 50 ng/ml BDNF, and that BDNF is needed to obtain fully differentiated cells as RA and serum starvation alone did not achieve satisfactory *MAP2*- and *SNAP25*-results. Several studies report using this combination, but usually with a concentration of 50 ng/ml BDNF although the producer does not recommend a specific concentration for differentiation(69).

The SH-SY5Y cell line is widely used in scientific studies, especially in research on Parkinsons disease, Alzheimer disease, neurovirology and of course the prion protein. Their variability is probably an important reason for their popularity, as they can be differentiated using a wide range of methods, and be selected for adrenergic, cholinergic and dopaminergic subtypes(40).

Unfortunately, a majority of the studies using SH-SY5Y cells do not differentiate them, and the ones investigating PrP^C seldomly differentiate between full length PrP^C and the C1 portion originating from α-cleavage. Given that PrP^Cs expression in different tissue is exceptionally high in neurons(57), the research of PrP^C in neuronal cells is highly relevant. Although our results are preliminary, they are an important steppingstone for further studies and intriguing ideas.

Due to the wide use of the SH-SY5Y cell line it is easily accessible and possible to compare with a variety of earlier studies. Transfection allows us to make cells expressing high amounts of PrP^C or other proteins such as GFP, which our laboratory has experience with, and it is more ethical compared with primary cultures and live animals. Some difficulties are also associated with the use of these cells as our model. The cell line consists of different phenotypes; neuroblast-like and epithelial-like, and the distribution may affect our results(42).

Regarding our methods, we evaluated PrP^Cs expression using both mRNA and protein. This showed to be necessary as they display different findings and would not have given us the same results if used alone.

Interpretation of our results

Differentiation of SH-SY5Y-cells

As there are several reports of different ways of differentiating SH-SY5Y cells(36, 40, 70) we expected to accomplish this, but we had to experiment with different protocols to achieve the optimal differentiation factors. Our cells did not fully display the typical morphology of differentiated cells when only differentiated with serum depravation and 1 ‰RA, and therefore BDNF had to be added. We do not know why they didn't fully differentiate without BDNF, as there are several reports of differentiation using only serum depravation and RA. In addition to the morphology, the differentiation was either refuted or confirmed with MAP2 protein assay and *MAP2*/*SNAP25* mRNA assay.

PrP^C expression in SH-SY5Y and SH-SY5Y-diff

As PrP^C is generally expressed at higher levels in mature nerve cells compared with neuronal precursor cells, we expected the PrP^C expression to increase as the cells were differentiated. We expected that the endogenous *PRNP* expression would increase as well, although baseline endogenous *PRNP* expression levels are very low in this cell line. We did not observe an increase in endogenous *PRNP* levels. The total *PRNP* expression in the transfected cells, on the other hand, seemed to increase slightly with differentiation, although not as much as the protein expression.

This difference in protein and mRNA expression indicate that the protein *production* has not increased as much as the total amount of protein in the cell has, and there may be other explanations for the increase in PrP protein and the increase in C1 percentage. This may be due to a difference in turnover of the protein, with a longer half-life of the protein after differentiation than before. Considering that the C1-portion has a higher stability and persistence at the cell surface compared with FL-PrP^C, the C1-proportion may very well be an indicator of the PrP^C turnover in the cell.

The GFP:PrP chimeric protein allows detection of FL-PrP^C or the N1-fragment. Because the C1-percentage increases in SH-SY5Y-diff, it is reasonable to assume that the GFP signal would decrease as the FL-PrP^C proportion decreases and the GFP-tagged N1-fragment is released into the cell culture medium. In a direct inspection of the western blot, the GFP-signal did seem to decrease in two clones, although the quantified graph of the blot suggest that PNGase treated huPrP-GFP-2 did not have a decrease in the signal, but rather an increase. As earlier mentioned in the results, we believe this apparent increase to be strongly influenced by the GAPDH-band. Due to the limited number of replicates and the variability of the results we cannot conclude whether the signal in general decreases with differentiation or not, but these results may lend support to the notion that α-cleavage is increased in the differentiated cells with consequently increased release of the N1-fragment into the media. Further experiments are needed to clarify this. The apparent discrepancy between the observed increase in total PrP-levels (un-tagged, Fig. 7a) upon differentiation, versus GFP-tagged PrP (Fig. 8a) could also indicate that the cell biology of PrP is perturbed as a result of the GFP tag. For instance, the GFP tag could alter the proteins interacting with PrP and hence its stability after differentiation.

Toll-like receptor 3-stimulation by Poly I:C did not alter the PrP^C expression in SH-SY5Y-diff

Our research group had previously performed similar studies using SH-SY5Y and IFN-α instead of Poly I:C, and found that the hu-PrP-SH-SY5Y cells were significantly less responsive to IFN-α compared with mock-SH-SY5Y(65).

Poly I:C is a powerful stimulant of the interferon signalling pathway, which in turn influences many important mechanisms in the cell. It is very interesting to test whether it also has a direct effect on PrP^Cs synthesis, processing and lifetime/turnover in the cell. It is also of value to investigate whether such effects were influenced by cellular differentiation, especially when we have observed a difference in the proteolytic cleavage and C1-percentage in addition to an increase of total PrP^C in the differentiated cells – if PrP^C and Poly I:C stimuli affect each other, then differentiated cells with more PrP^C could reveal this more clearly.

From the Poly I:C-experiments it was evident that the cells maintained stable expression levels of *PRNP,* throughout the experiment and that there were temporal differences in *IFNβ* and *Mx2* responses. As expected the *IFNβ* response was more rapid, peaking already at 3 hours, whilst the *Mx2* response, which is downstream of Type-I interferons, appeared after 6 hours and remained high also after 24 hours.

Surprisingly, the differentiated cells appeared almost refractory to the Poly I:C challenge, at least in the readouts used in this study. We observed no increase in expression of neither *IFNβ* nor *Mx2*. One possible interpretation of this observation would be that the differentiated cells have down-regulated TLR-3 receptors, which are specifically targeted by the Poly I:C treatment. Although TLR-3 receptors are expressed at high levels in brain microglial cells and astrocytes, neurons are also reported to express TLR-3, although at a lower level (71). Analysis of human neuroblastoma cells have revealed a highly variable expression of TLR-3 and hence responsiveness to Poly I:C challenge (72). It would be an interesting follow up of our data to assess TLR-3 receptor levels in SH-SY5Y cells before and after differentiation.

Whether the increased levels of total PrP in differentiated cells also contribute to the reduced responsiveness to Poly I:C cannot be excluded, but given that all differentiated clones appear refractory, regardless of PrP levels, both mock transfected and huPrP transfected, this seems an unlikely interpretation of the data.

As mentioned in the introduction; β-cleavage may increase with pathological conditions. Therefore, one could think that Poly I:C would increase the β-cleavage in the cells and therefore an increase in the C2-fragment. No bands representing this fragment at 18-20 kDa were observed in any the blots, indicating that the extensive interferon signalling pathway doesn't induce changes in the proteolytic processing of PrP^{C}. Nevertheless, we cannot exclude this change being detectable a later time than 24 hours after the stimuli.

Discussion of our results seen in comparison with the available literature

An overview of the use of the SH-SY5Y cell line in Parkinson's disease research written by Xicoy et al(38) report that the majority of studies (regarding Parkinson's disease) did not differentiate the cells. Among those who did, most studies used either only RA, RA with reduced FBS or RA and 50 ng/ml BDNF in order to differentiate them(38). From our limited examinations of the differentiation protocols used including BDNF it seems that 50 ng/ml BDNF is a common concentration, although 10 ng/ml BDNF has been reported to have been used, both in Xicoy et al's overview and by Matsumoto et al in 1995(73).

Macedo et al(27) studied which proteins accompany the alterations in the expression of PrP when differentiating. They used two cell lines; an embryonic stem cell line (ES-cells) that was differentiated to Neuroepithelial precursors (NPs) and the PK1 subline of neuroblastoma cells. The latter has a high capacity of replicating PrP^{Sc} and has a PrP-knock out form, which was also used as a negative control. The authors report that several proteins were differentially abundant with the increased expression of the prion protein during neural differentiation of embryonic stem cells, most prominently, proteins involved in myelin sheath formation, organelle membrane and focal adhesion. Their conclusion is that expression of the prion protein occurs concomitantly with changes in chaperone activity and cell-redox homeostasis.

The PrP expression level during differentiation of ES-cells to NPs was examined both at mRNA (qPCR) and protein (WB) level, similar to our studies with SH-SY5Y-cells. The *PRNP* expression increased significantly with differentiation, with a highest fold change after 6 days and in NPs and a lower expression after 9 days. PrP^C protein expression showed an increasing trend along the differentiation timeline and had a higher expression at day 6 and a clear increase in NPs. Both mRNA and WB showed highest levels of PrP in NPs. The authors suggested that PrP levels were directly associated with neuronal differentiation, playing a role both in neurogenesis and cellular differentiation. This study did unfortunately not examine the unglycosylated PrP, thus relative levels of the C1/FL-fractions could not be revealed in the Western Blot (27).

The mRNA levels will show a rapid response, while the protein levels will have a delay because it takes more time to produce and not least break down/release produced protein. Using this study as an indicator we would have expected our results to be similar with an increase in *PRNP*-expression corresponding well with increased levels of protein levels. This was, however, not so clear cut in our experiments. We observed that endogenous *PRNP* mRNA levels remained stable through the differentiation protocol, although a slight increase was observed in one of our mock transfected clones (Fig. 7c, mock clone 6). The observed difference may be explained by cell line differences.

There are various reasons for why the mRNA values do not correlate with the protein levels, such as a delay in protein synthesis /transport or modulation of the proteins half-life. If the cell is in a state transition, e.g., during differentiation, it may take longer time before the transcription changes are reflected in the protein levels (74).

Steele et al (57) investigated the role of PrP^C in neural development and in adult neurogenesis. They used mice with different expression levels of PrP^C (knock out, overexpression and wildtype) and embryonic neural precursor cultures from these mice strains to develop neurons, astroglia and oligodendroglia. They found that PrP^C is affected by differentiation; The PrP^C levels directly correlate with differentiation of nestin-positive neural precursor cells, with very low levels in the precursors and increasing levels in the immature and mature neurons. The PrP^C levels were undetectable in the glial cells. They also note that PrP^C-knock out cells remain undifferentiated for a longer period compared with wildtype and cells with PrP^C-overexpression, although the final number of neurons in the dental gyrus was unchanged by the PrP^C-expression.

Taken together, it seems well documented that PrP^C levels correlate with differentiation of these cells *in vitro*: The levels influence the neuronal differentiation in a dose-dependent manner, and the differentiation is in turn influenced by the PrP^C expression (57).

Their results align well with our observations in that PrP^C expression increases with neural differentiation. They did neither describe the C1-percentage nor the mRNA expression, making it difficult to say whether this increase in PrP^C is due to higher production and if the processing of the protein is changed with differentiation.

There are not many studies that investigate the changes of α-cleavage/C1 during differentiation, although it is seems that PrP^C levels increase in neural precursors during differentiation to mature neurons in adult neural tissue such as obex, cerebellum and spinal cord (25, 57, 75), but I have not succeeded in finding studies that address the α-cleavage of

PrP^C in differentiating cells. In some cell types, the PrP^C levels decrease as the cells mature. This is the case in humans and rodents, where early hematopoietic precursors have an expression of PrP^C but not erythrocytes and granulocytes (76).

As mentioned in the introduction, Malachin et al tested in 2017 whether the response to IFN-α differed between huPrP-SH-SY5Y and Mock-SH-SY5Y (SH-SY5Y expressing normal, low amounts of PrP^C). They found that the cells with a high expression of PrP^C showed a reduced response to INF-α. These experiments are very similar to ours regarding the Poly I:C-stimuli, although Malachin et al used non-differentiated SH-SY5Y-cells and IFN-α and not Poly I:C (65). Direct comparison of results is further complicated by the potential differentiation-induced alterations in cellular receptor repertoires.

Evaluation of the results seen in comparison to the hypotheses/topic questions

Our primary hypothesis was that "expression of PrP^C in SH-5YSY-diff cells downregulates the expression of interferon responsive genes". Our results indicate this being falsified, as exposure to Poly I:C did neither change the *PRNP*- nor the PrP^C-expression (figure 9). The α-cleavage and C1-percentage doesn't seem to be changed with Poly I:C-exposure neither.

Our second hypothesis, "proteolytic modifications such as α-cleavage of PrP^C in SH-SY5Y-cells increase with differentiation" was established after obtaining the first results. This hypothesis was not falsified, and further research is needed, but our preliminary results indicate that this hypothesis might be verified.

Limitations of the presented studies

Statistical analyses are of great importance in order to avoid biasness and to evaluate results in a more objective manner. These experiments should be regarded as qualitative pilot studies that provide us with useful insight on where to focus our research. Due to the low number of replicates, we have not calculated the statistical evidence in depth.

As mentioned in the introduction, all cell lines have limitations due to being genetically manipulated and have undergone several modifications in order to be grown "indefinitely" in the laboratory. These modifications may alter both the genotype and the phenotype, and is important to keep in mind when using cell lines as model for cells in living organisms. Some of our modifications are of course the transfection of hu-PrP and GFP, and in order to reduce the influence of the transfection process we use control cells that are transfected as well, with the empty plasmid "Mock". Another issue with the cell lines is that some values vary considerably between cell clones, for example the relative mRNA expression of *PRNP.* This is, however, also a major problem when working with tissue samples as these normally contain many different cell types with different characteristics. Indeed, cell culture with relatively homogenous cells is one way of circumventing this problem.

With this in mind, one should remember that cell culture studies are of great significance in basal science studies, as well as the ethical advantages when using less laboratory animals.

Ideas for further studies

In order to develop these studies further, and gain more information about how PrP changes with differentiation and its interactions in the interferon signalling pathway, we would like to focus on the following issues:

It would be interesting to repeat our experiments and gain more quantitative data on whether our observations can be statistically supported. In addition to using SH-SY5Y cells, it could be informative to use cells without any PrP^C expression at all, as well as primary cell lines. These cells could be PrP^C-knock out cells made using Cas9-CRISPR technology, or primary cell lines without expression of PrP^C – e.g. from Norwegian Dairy Goats homozygous with the Ter-mutation that blocks the PrP^C-synthesis, therefore not expressing PrP^C(26).

If the experiments were to be repeated, I would also reconsider the order of the wells in the western blots, rendering nicer blots that are easier to show the reader. This would of course not affect the experiments validity, only the aesthetics.

As proteins with a GPI-anchor, such as PrP^C, can be difficult to fixate, we might try using live imaging of the GFP-huPrP-SH-SY5Y clones. This would allow us to obtain more information of the localisation of PrP^C and its fragments during differentiation as well as under exposure of Poly I:C.

We would also have liked to perform studies on GFP in cell media to look for the N1-fragment. Because the cell growth obliges us to discard and change the cell medium every other-third day, it requires a different approach. GFP is mainly used to observe the object it is attached to in fluorescent microscopy, and we did also try to look at the cell medium using only GFP and not PrP^C-antibodies. It would be interesting to pursue if SH-SY5Y-diff cells also have an increase of the N1-fragment in the cellular medium, thereby confirming our findings of increased proportion of C1. Due to time constrains we were unfortunately unable to repeat this experiment to include such analysis.

CONCLUSION

This study focuses on the prion protein and its processing in the cell and physiological functions. The study's main goal is to contribute to the knowledge of what role the prion protein (PrP^C) plays in the cells.

We have investigated PrP^Cs expression in the neuroblastoma cell line SH-SY5Y. We have successfully differentiated these cells into neuron-like SH-SY5Y-diff cells that are more homogenous and probably a better model for adult neuron cells, using a combination of Retinoic Acid, Brain Derived Neurotrophic Factor (BDNF) and serum deprivation. We experimented with different concentrations of BDNF and serum to find optimal differentiation conditions.

PrP^C can be proteolytically processed after translation in several ways, the most common being α-cleavage of the protein, resulting into two fragments; C1 and N1. Our main finding concerns the expression of PrP^C and the changes in proteolytic cleavage after differentiation as well as the effect of the immunostimulant Poly I:C on differentiated SH-SY5Y cells.

Our results imply that differentiation may alter the PrP^C expression both directly through translation and through the proteolytic cleavage of the protein, altering the C1 proportion of the proteolytic fragments in differentiated cells. We have also found that exposure to Poly I:C does not seem to alter the expression of PrP^C nor the C1-proportion in differentiated cells, but the differentiated cells had a weaker response to Poly I:C within the measured timeframe of 24 hours.

The experiments are pilot studies and their results are to be regarded as indications. The nature of the cell lines with a highly variable expression of PrP^C themselves complicate the interpretations as well, and specially the mRNA-results are challenging to interpret due to the highly variable mRNA values between cell clones.

In order to improve and develop our studies we would like to perform more replicates, as well as to work with other cell lines; both PrP^C-knockout cells as well as primary cultures. We would also extend our studies with GFP-huPrP-cells, investigating the location and expression of PrP in live action, as well as the processing of the N1 fragment.

To conclude, this study has contributed with new information about differentiation of the SH-SY5Y cell line. We have also indications that these cells have higher amounts of the C1-propotion of PrP^C, indicating alterations in the posttranslational proteolytic α-cleavage. The PrP^C protein does not seem affected by stimuli with the immunostimulant Poly I:C, but the differentiated cells may have had a delayed/weaker reaction to Poly I:C compared with the non-differentiated cells.

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