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CALCIUM DYNAMICS AND VESICLE-RELEASE PROTEINS IN A PRION-INFECTED NEURONAL CELL LINE

AKADEMISK AVHANDLING

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Av

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To my parents

ABSTRACT

The prion particle, PrP^{Sc}, is an infectious, misfolded form of the cellular prion protein, PrP^{C} that is expressed at high levels in the central nervous system. Prions cause neurodegenerative diseases with clinical signs including dementia, ataxia and myoclonus. These diseases cause characteristic electroencephalographic changes. Neuropathological findings characteristic for prion diseases are spongiform degeneration, astrogliosis and neuronal cell death. Loss of presynaptic proteins such as SNAP-25, syntaxin 1, synaptophysin and synapsin 1 has been observed using immunohistochemistry. The aim of this thesis was to characterize alterations in presynaptic components implicated in neurotransmission that could be of pathogenetic importance for brain dysfunction in prion diseases.

For this purpose I used an immortalized gonadotropin-releasing neuronal cell line (GT1-1) that is susceptible to a mouse-adapted scrapie strain. This cell line represents a suitable system for my studies as the cells possess well characterized voltage-gated ion channels and express several proteins involved in regulated exocytosis. First, using fluorometric calcium measurements and patch clamp technique, we found reduced voltage-gated N-type calcium currents in scrapie-infected GT1-1 cells whereas the L-type calcium currents were unaffected. No change in the expression of the N-type α1b subunit could be observed indicating that the impairment in N-type calcium channel function does not reside in altered levels of this protein. Second, using Western blotting we found decreased complex formation of proteins involved in exocytosis, i.e. SNAP-25, syntaxin 1A and synaptophysin. The reduced level of complexed forms of these proteins resulted in an increased amount of monomeric SNAP-25, syntaxin 1A and synaptophysin that could be correlated to the level of PrP^{Sc}. However, when complex formation was abolished using exposure of samples to heat, a reduced expression of these monomeric proteins could be revealed in the scrapie-infected GT1-1 cells as compared to uninfected controls. Quinacrine, a compound that has been shown to clear scrapie infection from cell cultures, cleared PrP^{Sc} also from the ScGT1-1 cultures and caused a restoration, at least partially, of the N-type calcium currents and of the levels of monomeric SNAP-25 and synaptophysin. Finally, I observed that the reduced complex formation in ScGT1-1 cells was not caused by an increased excitability of the cells. Instead, long-term depolarization could surmount the reduced complex formation. Taken together, these results indicate that a prion infection can cause functional changes in presynaptic components and possibly electrochemical transmission in neurons, which may contribute to brain dysfunction in prion diseases. Finally, from the perspective of therapeutic strategies the notion that these changes are reversible is of importance.

Keywords: Prion, Scrapie, Voltage-gated N-type calcium channels, SNARE proteins, Exocytosis, Long-term depolarization,

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following original papers and manuscripts that will be referred to in the text with their roman numerals.

I. **Sandberg M.K.**, Wallén P., Wikström M.A., Kristensson K.
Scrapie-infected GT1-1 cells show impaired function of voltage-gated N-type calcium channels (Ca_v 2.2) which is ameliorated by quinacrine treatment.
Neurobiology of Disease, 2004 **15**(1):143-151.

Erratum in *Neurobiology of Disease*, 2004 **16**(2):478-479

II. **Sandberg M.K.**, Wikström M.A.
Voltage-gated N-type calcium channels in scrapie-infected and non-infected GT1-1 cells -An electrophysiological characterization.
Manuscript

III. **Sandberg M.K.**, Löw P.
Altered interaction and expression of proteins involved in neurosecretion in scrapie-infected GT1-1 cells.
Journal of Biological Chemistry, 2005 **280**(2):1264-1271

IV. **Sandberg M.K.**
Effects of chronic depolarization on expression and interaction of proteins involved in neurosecretion in scrapie-infected GT1-1 and non-infected GT1-1 cells.
Manuscript

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LIST OF ABBREVIATIONS

AHP	Afterhyperpolarization
BFA	Brefeldin A
BSE	Bovine spongiform encephalopathy
CNS	Central nervous system
CWD	Chronic wasting disease
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia neurons
EEG	Electroencephalography
EPSP	Excitatory post-synaptic potential
FFI	Fatal familial insomnia
GnRH	Gonadotropin-releasing hormone
GPI	Glycosyl phosphatidyl inositol
GSS	Gerstmann-Sträussler-Scheinker disease
GT1-1	Gonadotropin-relesing hormone neuronal cell clone 1
GT1-7	Gonadotropin-relesing hormone neuronal cell clone 7
HVA	High voltage activated
LTP	Long-term potentiation
LVA	Low voltage activated
mRNA	messanger ribonucleic acid
^{Ntm}PrP	Form of PrP^{C} that spans the membrane with its amino-terminal domain in the ER lumen
^{Ctm}PrP	Form of PrP^{C} that spans the membrane with its carboxy-terminal domain in the ER lumen
ORF	Open reading frame
PIPLC	Phosphatidyl inositol-specific phospholipase C
PK	Proteinase K
$PrP^{0/0}$	PrP knockout mice
PrP^{C}	Cellular isoform of the prion protein
PrP^{Sc}	Disease associated isoform of the prion protein
PrP27-30	Protease-resistent core of PrP^{Sc}
Prn	Gene family to which the prion protein belongs
RML	Rocky Mountain Laboratory
sCJD	sporadic Creutzfeldt-Jakob disease
sPrP	Proteinase K sensitive PrP^{Sc}
^{Sec}PrP	Fully translocated and glycolipid-anchored form of PrP^{C}
SNAP-25	Synaptosomal associated protein of 25 kDa
SNARE	SNAP receptor
ScGT1-1	Scrapie-infected Gonadotropin-Relesing Hormone Neuronal Cell clone 1
synprint	Synaptic protein interaction site
TEA	Tetraethylammonium
TM	Transmembrane
vCJD	variant Creutzfeldt-Jakob disease

INTRODUCTION

Prions are proteinaceous, infectious particles that are resistant to inactivation by procedures that modify nucleic acids [1]. Purification of the infectious particles identified a protease-resistant polypeptide, now recognized as PrP 27-30, that is enriched in infectious preparations [2]. PrP 27-30 is the core of the abnormal misfolded PrP^{Sc} and micro-sequencing of this polypeptide [3] provided sufficient partial sequence information to discover the PrP gene encoding the cellular prion protein PrP^{C} [4, 5]. PrP^{C} is a prerequisite for replication of PrP^{Sc}, since PrP^{C} knockout mice exposed to PrP^{Sc} show no signs of disease and are unable to replicate prion particles [6, 7]. Thus, PrP^{Sc} associated with disease is a misfolded isoform of a normal, cellular protein PrP^{C}. Although extensive information has been accumulated on the biophysical properties of the PrP^{C} and PrP^{Sc} the mechanisms by which the infectious particles cause a fatal brain disease remain elusive. In my thesis I will focus on the potential role of voltage-gated calcium channels and synaptic vesicle release proteins in the pathogenesis of brain dysfunction caused by prion infections.

Prion Protein and Brain Dysfunction

The Cellular Prion Protein (PrP^{C})

The PrP gene belongs to the *Prn* gene family and the prion protein is a conserved protein through mammalian species. The entire ORF resides within a single exon which excludes the possibility for alternative RNA splicing [8]. PrP^{C} mRNA is constitutively expressed in the brains of adult animals [4, 5] but its expression is strictly regulated during development [9]. The highest levels of PrP mRNA can be found in neurons [10], but PrP^{C} can also to a less extent be observed in extraneural tissues [11]. At the ultrastructural level, PrP^{C} is predominantly found on the plasma membrane of dendrites including spines, dendritic transport vesicles, endosomes, axolemma, axonal transport vesicles and myelin sheaths [12]. The pre- and postsynaptic membranes together with the membranes of synaptic vesicles are also labeled for PrP^{C} [12-15].

The PrP^{C} protein is synthesized in the rough ER and is subjected to several post-translational changes such as glycosylations, formation of a disulfid bond and the addition of a glycosylphosphatidylinositol (GPI)-anchor during its transfer via the Golgi apparatus to the plasma membrane [16-18]. The GPI moiety directs the PrP^{C} protein to lipid rafts in the plasma membrane [17] from where the protein constitutively cycles to endocytotic compartments and is exposed to proteolytic cleavage [19-22]. Kinetic analyzes have shown that PrP^{C} transits through the cell in ~60 min [23]. PrP^{C} has been shown to bind copper ions with a low micromolar affinity via the aminoterminal octapeptide repeats through a pH sensitive process [24-27].

Recently, it has been shown that PrP^{C} can be synthesized in several topological forms. These different forms can be observed in cell-free translation systems as one principle form that is fully translocated and glycolipid-anchored to the membrane (^{Sec}PrP), one form that spans the membrane with its carboxy-terminal domain in the ER lumen (^{Ctm}PrP) and one form that spans the membrane with its amino-terminal domain in

the ER lumen (^{Ntm}PrP) [28]. ^{Ctm}PrP has attracted recent interest since transgenic mice expressing ^{Ctm}PrP show neurodegenerative changes resembling those observed in some genetic prion diseases. In addition, ^{Ctm}PrP has been found in one variant of heritable Gerstmann-Sträussler-Scheinker disease (GSS) in humans [29]. However, experimental scrapie infections in mice or hamster are not associated with an increased amount of ^{Ctm}PrP in the brain [30].

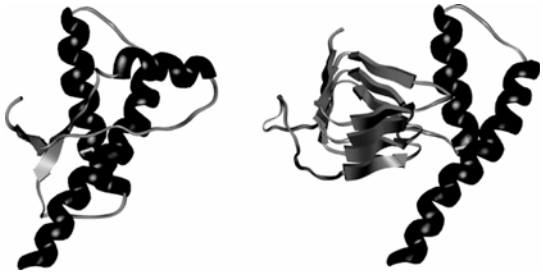
$PrP^{0/0}$ mice show no overt changes in their development or behavior [31] indicating that PrP^{C} is dispensable. No alterations are seen in the short- or long-term fear-motivated memory, anxiety or exploratory behavior compared to wildtype mice [32]. However, changes in the circadian rhythm and sleep pattern have been observed in $PrP^{0/0}$ mice [33]. Furthermore, some reports have demonstrated that neurons are more susceptible to oxidative stress in the brains from such mice (for review, see [34]).

The Prion Disease associated Prion Protein (PrP^{Sc})

PrP^{Sc} is an abnormal isoform of PrP^{C}, formed by a so far unknown post-translational event. PrP^{Sc} consists of 45 % β-structures and 30 % α-helix conformations in comparison to the 3 % β-structures and 40% α-helix conformations of PrP^{C} [35, 36]. In early studies of PrP^{C} and PrP^{Sc}, one important difference between these two isoforms was shown, which is still used in diagnostics of prion diseases, namely that PrP^{Sc} possesses a protease-resistant core named PrP 27-30 [2]. PrP^{Sc} forms rod-shaped macromolecular aggregates when extracted in detergents, while PrP^{C} is solubilized [37]. Furthermore, PrP^{Sc} is attached to the membrane by a GPI- anchor that is resistant to digestion with phosphatidylinositol-specific phospholipase C (PIPLC), while the GPI-anchor of PrP^{C} is protease-sensitive, indicating a difference in membrane topologies between the two isoforms [38, 39]. Direct interaction between PrP^{C} and PrP^{Sc} is required for the conversion of PrP^{C} to PrP^{Sc} [40-43] and this process occurs in caveolae-like domains or rafts at the plasma membrane, or in early endosomes [39, 44, 45]. Direction of PrP^{C} to clathrin-coated pits prevents the formation of PrP^{Sc} [19, 46].

Recently, structural predictions that PrP^{Sc} consists of a left-handed β-helical structure have been presented [47, 48]. Furthermore, recombinant mouse prion protein can be polymerized into amyloid fibrils that when inoculated intracerebrally into transgenic mice produce neurologic dysfunction. In addition, extracts from the inoculated mice transmitted disease to wild-type mice, which indicates that prions may be synthesized *ex vivo* [49].

Variations in glycosylation of PrP^{C} have been suggested to influence the rate at which PrP^{C} is converted to PrP^{Sc} [50], and the ratio of PrP^{Sc} glycoforms can differ between prion strains [51]. Finally, proteinase K sensitive prion protein (sPrP) have been described in the blood of infected rodents [52] using the conformation-dependent Immunoassay which simultaneously measure specific antibody binding to denatured and native forms of PrP [53].



PrP^{C} **PrP^{Sc}**

Figure 1. Models for the tertiary structures of PrP^{C} and PrP^{Sc}. *(Adapted from www.cmpharm.ucsf.edu/cohen with permission from Fred E. Cohen).*

Prion Diseases

Prions cause fatal neurodegenerative diseases, which can be of sporadic, infectious or hereditary origin. All these forms of disease are associated with the abnormal, misfolded isoform of PrP^{Sc} and can be further transmitted to other individuals.

Creutzfeldt-Jakob disease (CJD) was described by Creutzfeldt 1920 and by Jakob 1921. CJD can occur in sporadic, familial and acquired forms, but the most common is the sporadic form (sCJD) with an annual incidence of less then one case per million populations. sCJD constitutes about 85 % of all human prion diseases [54]. The principle clinical features of sCJD are rapidly progressive dementia, myoclonic involuntary movements and ataxia. Death usually follows within a few months after onset. The electroencephalogram (EEG) is abnormal in about 65 % of the patients, showing triphasic periodic synchronous discharges [55]. CJD can be **iatrogenically** transmitted from neurosurgical instruments, corneal or dura mater grafts, or pituitary-derived hormones with diverse clinical and pathological features. The familial forms of prion diseases are all associated with point mutations in PrP^{C} and include **familial CJD, Gerstmann-Sträussler-Scheinker disease (GSS)** and **Fatal Familial Insomnia (FFI)**.

Scrapie has been known to occur with a large geographical distribution in the sheep and goat populations since the eighteenth century. There is a great variability of histopathological lesion and abnormal prion protein accumulation patterns that depends on the scrapie strain and the host genotype. In addition, at least 20 laboratory mouse scrapie strains have been characterized with various pathological changes and incubation times.

Bovine Spongiform Encephalopathy (BSE) was discovered in 1986 in cattles in the United Kingdom (UK). BSE is considered to be associated with a new type of disease in humans, **variant Creutzfeldt-Jakob Disease (vCJD).** The epidemiological evidence is based on the fact that the first cases were all identified in the UK and that the timing of the vCJD onset coincided with a 5-10 years delay from the peak of the BSE epidemic. In addition, similar ratios of glycoforms and patterns of PrP deposition have been reported from transmission experiments in transgenic mice [56]. BSE has also been transmitted intracerebrally to cynomolgus macaques and these animals show similar clinical, molecular and neuropathological features to human patients with vCJD [57].

Chronic Wasting Disease (CWD) is a contagious prion disease that probably can be transmitted between captive and free-ranging mule deer, white-tailed deer and Rocky Mountain elk due to environmental contamination. As the

known distribution of CWD has expanded the concerns regarding this disease have increased.

Prion infections and histopathological changes in the brain

Pathological changes in prion diseases occur in the nervous system and includes spongiform neuronal degeneration, astrogliosis and in some cases deposition of amyloid fibrils. Spongiform changes are characterized by the presence of small vacuoles within the neuropil [58]. The vacuoles are limited by unit membranes and are mainly localized to the neuronal processes [59]. The interior of the vacuoles is electron-lucent and contains fragmented membranes [59, 60]. Focal distensions of dendritic, and less often of axonal, processes can be observed and are co-localized with vacuoles. The enlargements of the processes are often disproportionately greater than the volume occupied of the vacuoles [59, 61]. When pyramidal and nonpyramidal neurons in brains from patients with CJD were Golgi impregnated, irregular contours of dendrites, decreased number of spines per unit length and focal spherical or ovoid swellings could be demonstrated [59, 61]. Similarly, brains from scrapie-infected hamsters show spongiform changes with vacuolation, and in Golgi impregnations the dendrites of cortical neurons appeared thinner and there was a loss of spines together with focal swellings in dendritic varicosities [62].

Prion infection and axon terminal alterations

In addition to the changes in dendritic spines described in the previous section, axon terminal alterations occur early in mice infected with scrapie [63, 64]. A generalized reduction in presynaptic proteins involved in exocytosis such as synaptophysin, synapsin-1, SNAP-25, syntaxin-1 has been demonstrated in brains from CJD patients [65-67] as well as in brains from mice infected with scrapie [64, 68, 69]. It has been debated whether these changes are secondary to neuronal cell death or not. For instance, there are reports on reduced levels of presynaptic proteins that parallel the neuronal cell death in CJD cases [65-67] and in scrapie-infected mice [64]. However, no such correlation could be demonstrated in other reports on murine scrapie [63, 64, 68] and one study has shown disorganization and loss of synapses without correlation to neuronal cell death in brains from CJD patients [70]. PrP^{Sc} has been shown to co-localize with synaptophysin, a protein localized to small synaptic vesicles and used as a synaptic marker, in brains from patients with CJD [67]. Furthermore, PrP^{Sc} accumulates in synaptosomes obtained from thalamus and neocortex of scrapie-infected mice [71]. However, axonal changes may occur in areas with only subtle amounts of PrP^{Sc} [63]. Thus, several studies report axon terminal changes, but knowledge on how these changes relate to dysfunctions in prion-infected individuals is still limited.

PrP^{Sc}, PrP^{C} and electrophysiological changes

One prominent electrophysiological change in sCJD is the appearance of periodic sharp-wave complexes in the EEG over various cortical areas [72]. The EEG changes resemble interictal epileptogenic discharges and may precede or coincide with myoclonic jerks. Similar findings have been reported in sheep [73] and rats [74, 75] infected with scrapie.

Furthermore, transgenic mice infected with the Sc237 scrapie strain showed prolonged epileptiform discharges in cortex and hippocampus, which were suggested to be caused by a decreased synaptic inhibition [76]. At terminal stages, prolonged action potentials and reduced fast afterhyperpolarization (AHP) in the CA1 pyramidal cells occur and the former finding was abolished using blockers of voltage-gated calcium channels [76]. Two other electrophysiological studies showed hyper excitability in the CA1 pyramidal cells of mouse [77] and hamster [78] brains infected with the ME7 or Sc237 strain of scrapie. Changes in the AHP have been observed in several studies, but with somewhat variable results. Johnston et al. [77] found an increased fast and medium AHP in mice infected with the ME7 strain while Barrow et al. [78] found reduced medium and slow AHP in hamster brains infected with the Sc237 strain. In a later study, infection with the ME7 strain caused elimination of the slow AHP and this effect was suggested to depend on alterations in tetraethylammonium (TEA)-sensitive potassium conductances [79]. Another study showed no dysfunctions in dorsal lateral geniculate nucleus neurons in brains from ME7-infected mice at late stages of the disease when this area is subjected to marked pathological changes [80]. Finally, a loss of long-term potentiation (LTP) has been found in the CA1 region of the hippocampus in brains from mice infected with ME7 scrapie strain [81].

In order to determine whether these electrophysiological changes reflect direct effects on the neurons by PrP^{Sc} or a loss of function of PrP^{C}, electrophysiological studies on the brains from $PrP^{0/0}$ mice have been performed. Electrophysiological studies using slice preparations from the CA1 region of hippocampus from brains of $PrP^{0/0}$ mice have shown hyper excitability, weakened $GABA_A$ receptor-mediated fast inhibition and impaired LTP [82]. Furthermore, a dose-dependent facilitation of excitatory synaptic transmission has been shown to correlate with PrP^{C} expression, indicating a modulating role of PrP^{C} on neuronal excitability [83]. However, other electrophysiological studies from the CA1 region of hippocampus in $PrP^{0/0}$ mice showed no such changes [84, 85].

On the other hand, a reduced late AHP has been seen in CA1 pyramidal cells [86] and in cerebellar Purkinje cells [87] from $PrP^{0/0}$ mice. Recently, a conditional PrP knockout mouse has been produced to investigate the effects of PrP depletion on neuronal survival and function in the adult brain [88]. The animals showed a significant reduction of AHP potentials, which again indicates a modulating role of PrP^{C} on neuronal excitability. The reduced AHP observed in $PrP^{0/0}$ mice was restored by expression of human PrP 200K commonly found in inherited prion diseases, indicating that the change is caused by the loss of PrP^{C} [89]. The PrP conditional knockout mice remained healthy and showed no signs of neurodegeneration up to 15 month after PrP depletion. In addition, depletion of neuronal PrP^{C} in the adult brain of RML infected mice prevented the development of clinical prion disease and reversed early spongiform pathology in these animals [90].

Toxicity of prion protein peptides

In order to study direct effects of PrP^{Sc} on neurons the component of amyloid plaques was isolated and added to neuronal cells. GSS is an inherited prion disease associated with extensive deposition of PrP amyloid in the central nervous system [91]. When proteins from amyloid plaque cores in brains from patients with the Indiana kindred GSS were extracted and purified, the major component was determined to be a 11kd degradation product of PrP spanning residues 58 ~ 150 [92]. This degradation product corresponds to a region where β-sheet and amyloid can form [93] and is partly resistant to proteolysis [94]. Synthetic peptides homologous to segments of this fragment of PrP form fibrils *in vitro*, resembling the fibrils observed in GSS brains (PrP106-121) and the

scrapie-associated fibrils (PrP127-147) [95]. Long-term application of the peptide with the highest fibrillogenic probability, PrP106-121, to hippocampal cultures [96] and cerebellar granule cell cultures [97] cause neuronal death by apoptosis. The neuronal cell death *in vitro* has been suggested to be partly mediated by the microglia response, since co-cultivation of the two cell types increases the toxicity of the peptide [98]. The PrP106-121 fragment also mediates astroglial proliferation and this proliferation is prevented by L-type calcium channel blockers [99]. Acute exposure of the GT1-7 cell line to the PrP106-121 fragment increases cytosolic free calcium [100], while long-term exposure of primary cultures of cerebellar granule neurons or the neuroectodermal GH3 cell line to the fragment, reduces the calcium current activity mediated by voltage-gated L-type calcium channels [97, 101]. In addition, exposure to this fragment reduces the N-type calcium currents in PC12 cells, while the L-type calcium channels are unaffected [102]. Finally, full length recombinant PrP^{C} added to cerebellar granule cells causes reduced voltage-gated L-type calcium currents and this effect is dependent on the copper-binding amino-terminal domain of PrP^{C} [103]. Thus, several pieces of evidence have been provided that PrP fragment can induce disturbances in voltage-gated calcium channels. However, addition of PrP fragments mimic extracellular effects of PrP^{Sc} on neurons rather then intracellular events in an infected cell. Therefore, studies on voltage-gated calcium channels in neuronal cells harboring an endogenous infection would be informative.

Voltage-gated Calcium Channels

Voltage-gated calcium channels were discovered when neuromuscular transmission was investigated in muscle fibers of crab legs [104]. The action potential induced in these fibers was not dependant on sodium but on calcium channels [105]. Calcium channels are expressed ubiquitously in organisms varying from paramecium to humans, and their main functions are both electrogenic and regulatory. The intracellular free calcium concentration in a resting cell is within the range of 30 to 200nM, a level that is maintained due to the combined actions of an ATP-dependent calcium pump and a Na^{+}-Ca^{2+} exchange system. The low intracellular concentration ensures the fast responses of different biological processes like contraction, secretion or gating (for review, see [106]).

Nomenclature of Voltage-gated calcium channels

It has been suggested that the voltage-gated calcium channels originally evolved from voltage-gated potassium channels (for review, see [107]). Eukaryotic potassium channels are structurally based on a 6-transmembrane (TM) motif that also can be found in voltage-gated calcium and sodium channels. It is hypothesized that the calcium selectivity developed first, giving rise to low voltage activated (LVA) and high voltage activated (HVA) calcium channels, and that sodium channels evolved later from LVA calcium channels [107]. The LVA calcium channels are rapidly inactivated at depolarized holding potentials and show a tiny conductance and are therefore named T-type calcium channels (for review, see [108]). The HVA channels usually do not inactivate as rapidly as LVA channels and are subdivided into three different subtypes; 1. long-lasting and large single-channel conductant, dihydropyridin-sensitive L-type calcium channels; 2.

intermediate single channel conductant N and P/Q-type calcium channels defined by their sensitivity to ω-conotoxin GVIA and ω-agatoxin IVA, respectively, and; 3. R-type calcium channels that are resistant to these toxins.

Voltage-gated calcium channels are structurally composed of five different subunits; a principle transmembranous subunit (α_1), an intracellular subunit (β), a disulfide-linked dimer associated with α_1($\alpha_2\delta$) and finally a transmembrane subunit (γ) (for review, see [109]). The α_1 subunit defines the different voltage-gated calcium channels, and ten different α_1 subunits have been identified so far. These subunits were originally classified as A through I as referred to previously, but recently families of calcium channels more structurally and functionally related have been designated (Ca_v1, Ca_v2 and Ca_v3; see Fig. 2). The Ca_v1 family of α_1 subunits shows 75 % amino acid sequence identity with the α_1 subunit of skeletal muscle L-type calcium channels and corresponds to the HVA L-type calcium channels according to the old nomenclature. The Ca_v2 family shows less than 40 % amino acid identity with Ca_v1 α_1 subunits but higher than 70 % amino acid identity among themselves and includes the HVA calcium channels P/Q-, N- and R-type calcium channels according to the old nomenclature. Finally, the Ca_v3 α_1 subunits exhibit only 25 % amino acid identity to other known homologues and correspond to the LVA T-type calcium channels. The distant structural relationship between these channels and HVA channels as discussed previously indicates the early evolutionary divergence of these families.

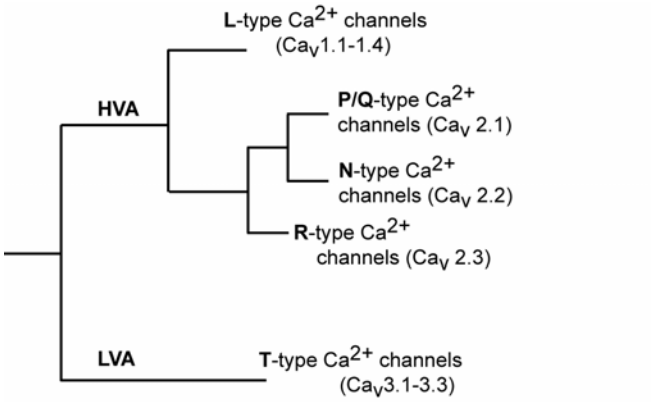


Figure 2. Voltage-gated calcium channel nomenclature. *(Modified from Ion Channels and Excitable Membranes, B. Hille, Sinauer Associates, Inc., MA, USA)*

Voltage-gated L-type calcium channels ($Ca_v1.2$-4)

Voltage-gated L-type calcium channels are the main contributors to HVA calcium currents in vertebrate cardiac, skeletal and smooth muscles. These channels also occur in several endocrine cells and are involved in the regulation of endocrine secretion (for review, see [106]). In particular, the channels mediate calcium entry in cells that respond

to long or steady depolarizations and the L-type calcium channels are also the principal source of calcium involved in the regulation of gene expression [110]. In neurons, these channels are localized primarily to cell bodies and proximal dendrites as seen in hippocampal pyramidal cells and several other classes of neurons [111, 112]. They mediate a slow persistent calcium current in comparison to the other calcium channels that show fast inactivation [113]. The L-type calcium channels can be isolated pharmacologically by 1,4-dihydropyridines such as nifedipine or nimodipine [114-116].

Voltage-gated N-type calcium channels ($Ca_v2.2$)

Voltage-gated N-type calcium channels are, together with the P/Q-type, the main channels involved in presynaptic vesicle release and transmission in CNS. This has been shown pharmacologically by using ω-conotoxin GVIA and ω-agatoxin IVA that specifically blocks these types of channels [117-119] with inhibition of synaptic transmission as a consequence [120-124]. Immunofluorescence using antibodies recognizing the principle α1 subunit of N-type calcium channels, [125] showed punctate structures on dendrites in cortical and hippocampal pyramidal neurons and on Purkinje cells [125]. In the rat brain, N-type calcium channels occur at particularly high levels in the cerebral cortex, the hippocampal formation, basal ganglia, thalamus and the posterior lobe of hypothalamus as determined by radiographically labeled ω-conotoxin GVIA [126-128].

Presynaptic calcium channels are subjected to neurotransmitter-induced inhibition (for review, see [129]). Briefly, the neurotransmitter binds to a G-protein-coupled receptor and mediates the release of the $G_{\beta\gamma}$ subunit from the G-protein complex. The $G_{\beta\gamma}$ subunit changes the gating properties of the N-type calcium channel to a reluctant state with low open probability and brief openings. The reluctant state can be converted to the normal willing state by strong depolarization and the mechanism is therefore recognized as voltage-dependent inhibition. Therefore, a single action potential may strongly inhibit synaptic vesicle release while a train of action potentials may relieve the inhibition.

The N-type calcium channels exhibit different properties depending on the repertoire of N-type subunits expressed in a cell [130]. The β-subunits are especially interesting, since they have been shown to mediate effects on voltage-dependent inhibition. When the β4 subunit and the principle subunit α1B of N-type calcium channels are co-expressed in the tsA-201 cell line, channels inactivate more slowly as compared to when the principle subunit α1B is co-expressed with the β3 subunit [131]. In addition, alternative splicing of the gene encoding for the N-type calcium channel α1B subunit has been shown to mediate functional differences by interaction with different β subunits [132].

Close functional interactions between the voltage-gated N-type calcium channels and the neurotransmitter release machinery have been described (for review, see [133]). Both mammalian presynaptic N- and P/Q-type calcium channels have a ~225 aa stretch in the intracellular domain II-III linker region of the α1 subunit where proteins involved in the docking of synaptic vesicles to active zones binds. This sequence is recognized as the synaptic protein interaction or the "synprint" site. The functional importance of the synprint site for neurotransmitter release was first acknowledged when Mochida et al. injected rat synprint peptides into presynaptic superior cervical ganglion neurons and found a 50 % decrease in the evoked excitatory post-synaptic potential (EPSP) amplitude [134]. However, invertebrate calcium channel homologs such as *Drosophila melanogaster* and *Caenorhabditis elegans* do not seem to have a synprint site

[135], but yet show a relatively rapid synaptic transmission. Thus, it has been suggested that the synprint region may not primarily serve to co-localize synaptic vesicles to the source of calcium entry, but to provide modulation and fine-tuning of calcium channel activity [133].

The turnover rate of voltage-gated N-type calcium channels in the plasma membrane varies between 15-18 hours in undifferentiated neuronal cell lines [136]. Differentiation of these cells is accompanied by an increased level of the N-type channels in the plasma membrane due to their slower internalisation and degradation rates. N-type calcium channels can be transported to the cell surface by different pathways. For instance, neuroblastoma cells exposed to ω-conotoxin GVIA or cadmium for 6-8 hours show an increased recruitment of functional N-type calcium channels to the cell membrane that is blocked by brefeldin A (BFA), a transport blocker of newly synthesised proteins through the Golgi apparatus [137]. On the other hand, Passafaro et al. have demonstrated that short-term depolarisation with potassium chloride (minutes) mediates a recruitment of N-type calcium channels to the cell surface that is BFA-insensitive in human neuroblastoma cells and rat PC12 cells [138]. Continuous depolarization for days has been reported to cause a reduction of N-type calcium channels on the surface of cultured rat myenteric neurons [139].

N-type calcium channel knockout mice ($Ca_v2.2^{0/0}$) with a disruption in the coding region of the α1B gene [140] lack N-type calcium currents in dorsal root ganglion (DRG) neurons and show no compensatory changes in the function of other voltage-gated calcium channels [141, 142]. They have an increased chemical-induced inflammatory and thermal nociceptive threshold [141-143] and they show changes in vigilance state transitions combined with hyperactivity [144]. In addition, they show altered behavioral responses to ethanol, consume less alcohol and experience reward at lower ethanol doses [145].

The function of voltage-gated N-type calcium channels in a neuronal network.

The voltage-gated N-type calcium channel blocker, ω-conotoxin GVIA, has been shown to inhibit neurotransmitter release in several tissue preparations [122-124]. In some areas of the brain, however, blocking the N-type calcium channels with ω-conotoxin GVIA also induces an increased number of population spikes. For instance, when ω-conotoxin GVIA was added to slices from the hippocampus, multiple population spikes together with spontaneous synchronous discharges could be observed extracellularly in the CA1 area [146]. Intracellular recordings demonstrated that inhibition of N-type calcium channels by ω-conotoxin GVIA reduced inhibitory synaptic transmission to a higher extent than excitatory transmission in both the CA1 and CA3 areas [146]. Similar observations were made by Potier et al. using intracellular recordings from CA1 neurons in the hippocampal slice preparation [147]. Poncer et al. concluded that GABA release was evoked by either voltage-gated N- or P/Q-type calcium channels depending on the location of the synapse-forming interneuron in hippocampus. Glutamatergic synapses located in the hippocampus, exhibit at least three types of calcium channels that participate in glutamate release even at synapses originating from a single presynaptic neuron [148].

In dissociated cultures from the hypothalamus, inhibition of N-type calcium channels by ω-conotoxin GVIA reduced the inhibitory synaptic transmission more effectively than the excitatory synaptic transmission [149] indicating differences in the relationship between calcium influx and transmitter release in excitatory and inhibitory neurons (see Fig. 3).

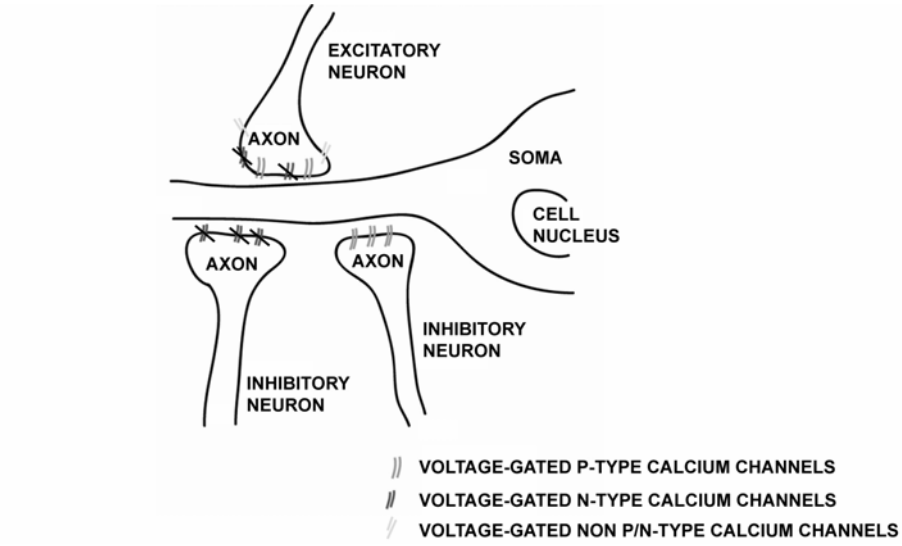


Figure 3. Distribution of voltage-gated calcium channels may modulate the excitation in a neuronal network. Axon terminals of inhibitory neurons exhibit either N-type or P/Q-type calcium channels in certain neuronal circuits while excitatory neurons show several types of voltage-gated calcium channels. Down-regulation of N-type calcium channels may therefore silence inhibitory neurons while the excitatory neurons are less affected. Thus, the net effect may be an increased excitation in the neuronal network.

Proteins Involved in Vesicle Release

The exocytotic machinery and SNARE complex assembly

Voltage-gated N-type calcium channels interact closely with presynaptic proteins involved in vesicle release. As described in a previous section, the level of presynaptic proteins such as syntaxin 1, SNAP-25 and synaptophysin is reduced in brains from patients with CJD and in mouse brains infected with experimental scrapie. This indicates that prion diseases may cause disturbances in the presynaptic vesicle release pathway.

A key feature in exocytosis is membrane fusion. Membrane fusion is a universal process occuring both extracellularly when enveloped viruses enter a host cell and intracellularly when different cellular organelles fuse with each other or with the plasma membrane. Regulated neurotransmitter release by synaptic vesicle exocytosis is fast and calcium triggers the release within less than 1 millisecond [150]. This indicates that synaptic vesicle fusion with the plasma membrane is largely prepared before the stimuli enter the synapse and evoke neurotransmitter release.

All intracellular fusion is suggested to be performed via similar core processes that are regulated by an array of cofactors (for review, see [151, 152]). Thus, it is important that the donor membrane such as an intracellular transport vesicle is attached to a suitable acceptor membrane, i.e. docking. This process has been suggested to occur via the Rab GTPases that serve as identity tags (for review, see [153]).

When fusion is initiated, a large dynamic protein complex is assembled that consists of the SNARE (SNAP receptor) proteins and Munc-18, a syntaxin-interacting protein. For the donor membrane to get in close proximity with the acceptor membrane SNARE proteins associate with both membranes. Thus, the synaptic complex is principally formed by one SNARE protein attached to the vesicles (synaptobrevin) and two SNARE proteins attached to the plasma membrane (Syntaxin 1 and SNAP-25). For this reason the SNARE proteins are termed vSNARE (vesicle) and tSNARE (target).

The SNARE proteins are defined by one homologous sequence, the SNARE motif that contains 60-70 amino acids and includes eight heptad repeats typical for coiled coils. The SNARE motifs divide all SNARE proteins into four different subfamilies depending on the interacting amino acids in the center of the helix bundle, the Qa- (syntaxins), Qb-, Qc- (homologs of the N- and C-terminal SNARE motifs of SNAP-25 and the R-SNAREs (Synaptobrevins) [154].

The SNARE motif mediates the functional SNARE complex formation by spontaneous assembly into elongated four-helical bundles in which the four different SNARE motifs (always one Qa, Qb, Qc and R subfamily of proteins together) are in a parallel orientation with their transmembrane regions emerging from the C-teminal end [155]. If assembly proceeds in a zipper-like fashion from the N-terminal end of the SNARE motif to the C-terminal membrane anchors, an extraordinarily stable trans-complex is formed with the two membranes in close proximity (for review, see [151, 152]). After the membranes have fused, a cis-complex is formed. For the SNARE proteins to be recycled and reused the cis-complex has to be disassembled and this reaction is performed by the ATPase NSF together with the co-factors NSF-attachment proteins (SNAPs) [156-158]. It has been debated whether the SNARE proteins are involved in the actual fusion or if the transition from trans- to cis-configuration catalyses an essential, but incomplete, step in the intracellular fusion pathway. These differences in opinions reside in the fact that the dependence of SNARE proteins for fusion can be circumvented, at least partly, under specialized conditions and it is still not clear to what extent other proteins are essential co-factors for the fusion [151].

As described in a previous section, regulated exocytosis at the presynaptic active zone is a calcium-dependent process. The release is believed to be triggered by calcium sensors that are localized to the sites of exocytosis. Synaptotagmin 1 is a synaptic vesicle protein that is a member of a large gene family, in which several proteins most likely are involved in the transmitter release (for review, see [159]). Synaptotagmin 1 binds multiple calcium ions within two phospholipidic domains and when one of these domains is mutated in knockin mice the resulting calcium affinity of exocytosis is reduced [160, 161]. However, the mechanism behind this process is still unclear.

I have chosen to study three different proteins involved in exocytosis, Syntaxin 1A, SNAP-25 and synaptophysin. Syntaxin 1A and SNAP-25 are interesting as they interact closely with voltage-gated N-type calcium channels located at the synapse, while synaptophysin is specifically involved in small synaptic vesicle release and confined to the presynaptic vesicle release pathway. I will therefore describe these three proteins in more detail in the following sections.

Syntaxin 1A

The syntaxin family consists of 15 mammalian proteins that are localized to the intracellular organelles (syntaxin 5-15) and the plasma membrane (syntaxin 1-4) where they mediate membrane fusion [162]. Syntaxin 1A is expressed almost exclusively in neuronal and neuroendocrine cells and has been suggested to be targeted to the plasma membrane by the interaction with Munc-18-1 (the neuronal form of Munc-18) [163]. A cytoplasmic domain of syntaxin 1A directs this protein to an intracellular location. However, when Munc-18-1 is co-expressed with syntaxin 1A the protein is targeted to the plasma membrane despite the cytoplasmic domain [163]. Furthermore, Munc-18-1 keeps the syntaxin 1A in a "closed" state [164] that prevents its participation in unfavourable SNARE complexes [151, 165] and munc-18-1 null mutants have a significant reduction in cellular syntaxin 1A [166]. Munc-18-1 has also been suggested to facilitate the interaction of syntaxin 1A with cholesterol-rich lipid rafts [167].

SNAP-25

Synaptosomal associated protein of 25 kDa (SNAP-25) contributes with two of the four α-helices that compose the exocytotic SNARE complexes. This family of proteins consists of several isoforms, but the SNAP-25a and SNAP-25b isoforms are mainly expressed in neurons and neuroendocrine cells [168]. These isoforms are homologues and differ only in nine amino acids, but this portion of the protein is a substrate for post-translational fatty acylation. This acylation (palmitoylation) is believed to cause the two isoforms to have different subcellular localisation and membrane targeting (for review, see [169]). It has also been suggested that interaction with syntaxin 1A is of importance for the plasma membrane localisation of SNAP-25. Expression of SNAP-25 in HeLa cells mediate inefficient plasma membrane targeting whereas co-expression of SNAP-25 and syntaxin 1A causes the membrane localisation of SNAP-25 to markedly increase [170]. However, mutation of the SNAP-25 binding domain for syntaxin 1A still targets this protein to the plasma membrane in neuronal cells [171]. Furthermore, syntaxin 1A requires co-expression with Munc-18-1 for plasma membrane targeting and this interaction mediates a closed configuration that is unable to interact with SNAP-25 [163, 164].

SNAP-25a has been implicated in neuritic outgrowth and fusion of vesicles delivering components to the plasma membrane of the neurites and the growth cones [168, 172], whereas the SNAP-25b isoform is important for neuronal synaptic vesicle release and neuropeptide secretion [168, 173]. However, SNAP-25a can also be involved in vesicle release [174, 175], indicating that the function of these isoforms is not totally separated. Furthermore, SNAP-25b has been shown to induce larger primed vesicle pools than SNAP-25a, indicating that these isoforms both support exocytosis, but differ in their ability to stabilize vesicles in the primed state [174].

Synaptophysin

Synaptophysin is a protein commonly used as a presynaptic marker, since it is present in virtually all nerve terminals and located at the small synaptic vesicle membrane [176]. Except for in neuroendocrine cells, this protein is not expressed outside the nervous system. The protein does not interact with large dense core vesicles. The principle function of synaptophysin is still unknown, but its abundance and uniform expression

suggests that it plays an important role in synaptic exocytosis. Although, synaptophysin knockout mice do not show an abnormal phenotype and have normal synaptic transmission [177], antibodies against synaptophysin injected into cultured spinal motor neurons significantly reduced the amplitude of evoked neurotransmitter release [178]. It has been suggested that synaptophysin is involved in synapse formation, since co-cultured neurons from synaptophysin knockout mice and wildtype mice showed a difference in the ability to form synapses [179]. Synaptophysin interacts with synaptobrevin in a complex that excludes other SNARE proteins such as syntaxin 1A and SNAP-25 [180]. This observation has led to the suggestion that synaptophysin has a regulating role in the exocytotic process. The interaction between synaptophysin and synaptobrevin is dependent on high cholesterol content in the vesicle membranes [181]. However, no complex formation between synaptophysin and synaptobrevin could be demonstrated in neuroendocrine cells [182].

N-type calcium channel function and SNARE proteins

The interaction between proteins involved in presynaptic vesicle release and the synprint site in voltage-gated N-type calcium channels are of importance for modulation of these channels. Calcium ions diffuse for only short distances in a cell and it has been suggested that only channels positioned in close proximity to vesicles prepared for release are activated. For instance, there are several reports on how co-expression of syntaxin 1A and the N-type α1B subunit or co-expression of SNAP-25 and the N-type α1B mediate lower calcium channel availability in comparison to expression of the N-type α1B subunit alone (see Fig. 4). These effects have been seen both in *Xenopus* oocytes [183, 184] and in tsA-201 cells [185], and contribute to a hyperpolarizing shift in the voltage-dependence of inactivation. Furthermore, when syntaxin 1A, SNAP-25 and N-type α1B are all co-expressed, the reduced calcium currents are normalized [184, 185], which would favor calcium entry through channels docked to presynaptic vesicles. Similar effects have been observed when P/Q-type calcium channels are expressed together with SNARE proteins in the tsA-201 cell system [186]. The reduced N-type calcium currents mediated by syntaxin 1A is abolished when this protein interacts with Munc-18-1, a synaptic protein that holds the syntaxin 1A protein conformation in a closed state and unable it to interact with other SNARE proteins [185].

The effects of long-term depolarization on presynaptic protein interactions are unknown, but of interest in context of prion diseases where epileptic discharges can be observed within longer time frames.

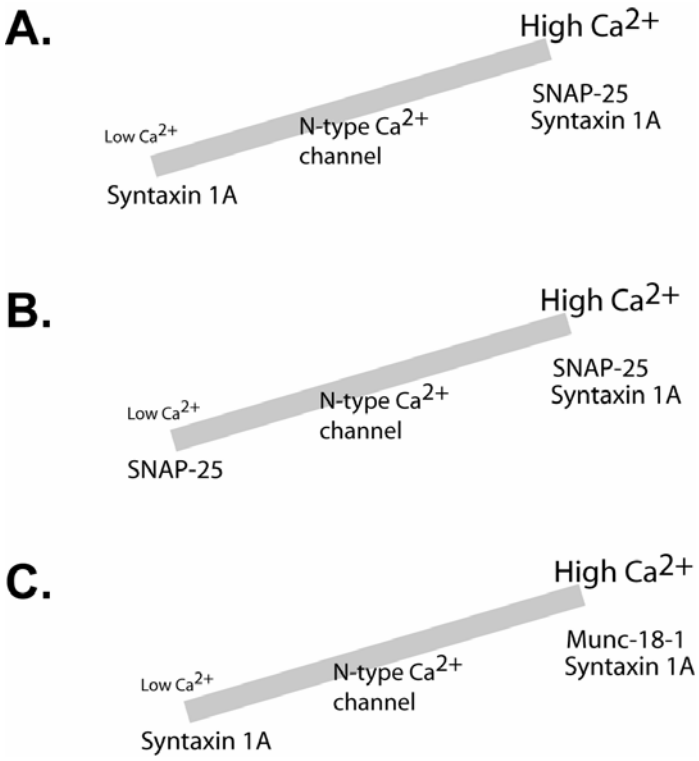


Figure 4. SNARE proteins (Syntaxin 1A and SNAP-25) show a close interaction with voltage-gated N-type calcium channels. *Co-expression of syntaxin 1A and the N-type calcium channel α1B subunit (A) or SNAP-25 and the N-type calcium channel α1B subunit (B) mediate a decreased calcium channel availability in comparison to co-expression of syntaxin 1A and SNAP-25 together with the calcium channel N-type α1B subunit (A, B). Finally, co-expression of syntaxin 1A and Munc-18-1 abolishes the decreased calcium channel availability caused by the interaction between syntaxin 1A and the N-type calcium channel α1B subunit (C).*

AIMS OF THIS THESIS

The overall aim of this thesis is to characterize alterations in presynaptic components implicated in electrochemical transmission that could be of pathogenetic importance for brain dysfunction in prion diseases.

In scrapie-infected GT1-1 cells I aim to:

I. Investigate whether a prion infection can cause changes in the function of voltage-gated calcium channels, primarily focusing on N-type calcium channels implicated in regulated vesicle release.

II. Determine whether a prion infection can affect proteins involved in exocytosis in neuronal cells.

III. Investigate whether long-term depolarization can affect complex formation of proteins involved in exocytosis.

METHODOLOGICAL CONSIDERATIONS

Detailed information about the methods used in my thesis is given in the "Materials and Methods" section in the original articles. I have chosen to describe only general aspects of the methods. A summary of all methods used in the project is presented in Table 1.

Technique	**Paper**
Cell culture	I-IV
Western blotting	I-IV
Calcium fluorescence measurements	I
Electrophysiology	II
Immunocytochemistry	III
Real-time PCR	III

Table 1. Methods used in the original articles.

In vitro systems and prion diseases.

Since the experimental analysis of prion diseases *in vivo* is time consuming and expensive, an *in vitro* system for replication of the abnormal prion protein would be advantagous. In the 1960s, establishment of explant cultures from scrapie-infected brains was performed, but the infectious agent could not be transmissed beyond the initial explant culture.

The first culture that showed continuous transmissibility and replication of the infectious agent was established in 1970 by Clarke and Haig [187]. The cell line named SMB, kept its infection for over 150 *in vitro* passages. There are a number of cell lines that are susceptible to scrapie infection but most cell types including neurons have proven difficult to infect although recently, one report on primary neuronal cultures from transgenic mice overexpressing PrP^C infected with prions has been presented [188]. Using cell lines as a model system for studies of prion infection has both disadvantages and advantages. Immortalized cell lines of neuronal origin, grow and multiply continuously in contrast to neurons *in vivo*, but may have developed different properties than neurons.

The Gonadotropin-releasing hormone neuronal cell line (GT1-1)

The gonadotropin-releasing hormone (GnRH) neurons are a small population of scattered neurons in the hypothalamus. An immortalized GnRH neuronal cell line (GT1) has been developed using genetically targeted tumorigenesis. The promoter region of the GnRH gene was used to express the SV40 T-antigen oncogene in transgenic mice and from the hypothalamic tumors that were produced, clonal differentiated, neurosecretory cell lines were obtained [189]. These cell lines (subclone GT1-1, 3, 7) extend neurites and express

mRNAs for neuronal markers such as neuronal specific enolase (NSE). At the ultrastructural level, mature neurosecretory vesicles can be seen in the cell body as well as in the neuritic processes and structural specializations with "synaptic–like" morphology can be observed at points of neuritic contacts [189].

The GT1 cells secrete GnRH in a rhythmic pattern and this process is tightly coupled to depolarization via tetrodotoxin–sensitive sodium channels [189]. The electrophysiological membrane properties mediating spontaneous activity in the GT1 cells have been thoroughly characterized and several types of potassium and calcium channels have been identified besides the sodium channels [190]. The rhythmic secretion of GnRH coincides with spontaneous calcium oscillations [191, 192] and the main contributor to this process are voltage-gated L-type calcium channels [193]. Furthermore, these spontaneous calcium oscillations can be propagated as intercellular calcium waves in the GT1-1 and GT1-7 subclones and the intercellular waves are inhibited by the gap junction inhibitor octanol or by the L-type calcium channel blocker nimodipine [194, 195].

The GT1 cell lines also release GnRH upon depolarization by increased concentration of extracellular potassium, mimicking the responses seen in GnRH neurosecretory neurons in brain slices [189]. Recently, a thorough study of voltage-gated calcium channels in the subclone GT1-7 showed that these cells express functional R-, L-, N- and T-type channels. The major contributors to the voltage-gated calcium currents in these cells are the R-type and the T-type calcium channels that could be observed in all cells analyzed. No P/Q-type calcium currents could be isolated [196]. However, these results are in contrast to previous studies in GT1-7 cells where no N-type calcium channels could be isolated [197, 198]. In the GT1-1 subclone, L- and T-type calcium channels have been demonstrated, but no N- or P/Q-type calcium channels [193]. The time period during which the cells are maintained in culture is of importance, however, both for the functional expression of voltage-gated calcium channels and for the synchronization of calcium currents to intercellular calcium waves. Treatment of GT1-7 cells with nimodipine caused a large inhibition of calcium currents in cultures maintained for less than 5 days, but after longer time periods, a less strong inhibition was observed. The synchronization of calcium currents between individual cells also increases with the time of maintenance in culture [194].

The GT1-1 and GT1-7 cells express key proteins involved in exocytosis such as SNAP-25 [189, 199] and syntaxin [199] as well as key proteins specific for regulated exocytosis such as VAMP/synaptobrevin [189, 199], synaptotagmin [199] and synaptophysin, which is a marker protein for small synaptic vesicles [199]. Furthermore, these cell lines have been demonstrated to express mRNA for GAD67, exhibit functional $GABA_A$ [200, 201] and $GABA_B$ receptors [200] and they release GABA upon depolarization with extracellular potassium [199]. GABA is generally recognized as the major inhibitory neurotransmitter in the mature nervous system, but in these cell lines addition of GABA has excitatory effects such as increased frequency of action potentials and calcium oscillations [202]. Increased levels of intracellular calcium are seen upon activation of $GABA_A$ receptors [202-204] and these effects coincide with the secretion of GnRH [199, 205]. However, prolonged activation of $GABA_A$ receptors or application of $GABA_B$ receptor agonists results in a reduced GnRH secretion [199, 205] indicating two interacting loops involving $GABA_A$ and $GABA_B$ receptors that may inversely regulate GnRH and GABA secretion [199].

This cell line represents a suitable system for my studies since the cells possess well-characterized voltage-gated ion channels and express several proteins involved in regulated exocytosis, in particular synaptophysin, which is specifically

implicated in small synaptic vesicle release. However, whether the GT1-1 cell line is equipped with voltage-gated N-type calcium channels remains to be determined.

GT1-1 cells and prion infection

The GT1-1 cell line is susceptible to infection by the mouse-adapted scrapie Rocky Mountain Laboratory strain (RML) [206]. The RML scrapie strain was prepared by inoculation of the "drowsy" scrapie strain from goat into mouse brain [207]. In many, but not all experiments in the original paper, a number of the scrapie-infected GT1-1 cells showed reduced viability and morphological signs of neurodegeneration [206]. However, there was no apparent increased cell death in ScGT1-1 cells in our experiments. The cells were incubated with 0.1% brain homogenate in the media for 3 days in 32°C and thereafter washed and cultured at 37°C. The cells were cultured for at least 5 passages before determination of PrP^{Sc} to avoid contamination of homogenate. We have used two methods for detection of PrP^{Sc}, Western blotting combined with Proteinase K (PK) treatment and immunohistochemistry combined with guanidinthiocyanate treatment. The antibody used was the D13 HuM-Fab antibody that recognizes the residues 96-106 of the prion protein (Fig. 5) [208, 209]. PK treatment digests PrP^{C}, the cleaved PrP^{C} C1 fragment and PK sensitive PrP^{Sc} ($sPrP^{Sc}$). Thus, PrP^{Sc} in these studies is defined as PK-resistant PrP^{Sc} (C2; PrP 27-30). Western blotting revealed three bands due to the different number of glycosylations in the PrP 27-30 fragment. Treatment with guanidinthiocyanate denature PrP^{Sc} and expose the epitopes recognized by D13 in the protein [210]. The ScGT1-1 cells can maintain the scrapie infection up to 30 passages and when immunohistochemistry was performed up to 85 % of the cells can be infected.

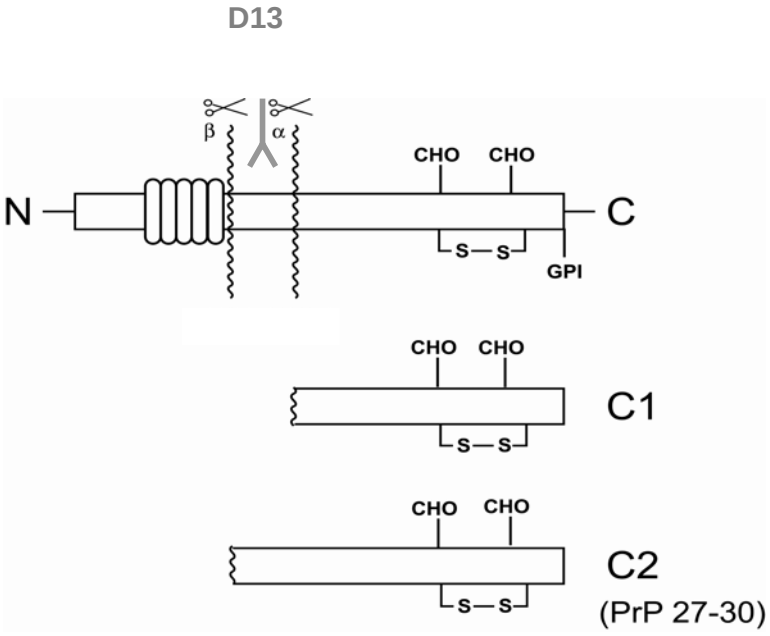


Figure 5. Cleavage sites of the prion protein isoforms and localization of the D13 antibody epitope. *Cleavage of PrP^{C} generates the C1 fragment, while processing of PrP^{Sc} generates the C2 fragment PrP27-30.*

Synaptophysin and SNARE protein complexes

The complex formation between proteins is difficult to analyze by Western blotting, since the risk of dissociating the interacting proteins during the protein assays is large. However, the SNARE protein complex has been demonstrated to be resistant to SDS denaturation [211] and in our hands even resistant to SDS treatment combined with heating in 100°C for 10 min. Therefore, these complexes can be investigated without using immuno-precipitation, although this method is more reliable. The SNARE proteins consist of SNAP-25 and syntaxin 1 localized to the plasma membrane and synaptobrevin localized to the vesicular membrane. Different experiments using synthetic liposomes, chromaffin cells or permealized PC12 cells all support the hypothesis that SNARE complex formation is a late step in the docking/fusion process [155, 212, 213]. However, the precise mechanism behind SNARE assembly is still unknown and *in vitro* biochemical methods are partly limited to reproduce the cellular SNARE complex assembly process due to the absence of a variety of proteins.

Several different complexed forms of syntaxin 1 have been described in different cell systems. The SNARE complex is normally recognized as a ternary interaction between SNAP-25, syntaxin 1 and synaptobrevin [173]. However, binary complexes consisting of 1 SNAP-25 and 2 syntaxin 1 proteins have been described in the cytoplasm of PC12 cells [214-216], which were suggested to precede the ternary complexes. Furthermore, SNARE complex cooperation and oligomerization have been observed and suggested to be important for synaptic vesicle membrane fusion [217-219].

Since the SNARE complex formation occurs spontaneously and is irreversible without recruiting the ATPase NSF and co-factors, this process must be tightly controlled. Syntaxin 1 forms an additional complex with munc-18, which has been shown to abolish the syntaxin 1 interaction with SNAP-25 and synaptobrevin [220, 221] and therefore munc-18 has been suggested to be such a control protein. Another suggested control protein is synaptophysin, which forms a complex together with synaptobrevin and thus abolishes the synaptobrevin interaction with the syntaxin 1/SNAP-25 complex [180, 222]. Due to the limitations of our method we can not establish what the complexed forms of syntaxin 1A, SNAP-25 and synaptophysin represent in our cells. Further investigations need to be performed on this matter as will be pointed out in the Results and Discussion section.

RESULTS AND DISCUSSION

I. Functional expression of voltage-gated N-type and L-type calcium channels in uninfected and scrapie-infected GT1-1 cells (paper I and II).

Since voltage-gated L-type calcium channels are the main contributor to spontaneous calcium oscillations, the role of other voltage-gated calcium channels have not been well characterized in the GT1-1 cells. To investigate elicited calcium responses, we depolarized GT1-1 and ScGT1-1 cells with local droplets of KCl using a pressure pulse ejection system. Calcium influx was analyzed in individual cells by fluorometric calcium measurements and confocal microscopy. We found that depolarization of ScGT1-1 cells elicited a decreased level of intracellular calcium when compared to uninfected GT1-1 cells, indicating a dysfunction in voltage-gated calcium channels (Paper I). When different types of voltage-gated calcium channels were isolated pharmacologically using nimodipine (L-type calcium channel blocker) and ω-conotoxin GVIA (N-type calcium channel blocker), we observed that the reduced level of intracellular calcium was abolished when voltage-gated N-type calcium channels were blocked. However, the inhibitor of L-type calcium channels had no effect on the difference in elicited intracellular calcium levels in ScGT1-1 cells compared to uninfected GT1-1 cells. From these experiments (Paper I), we conclude that the reduced level of elicited intracellular calcium in ScGT1-1 cells resides in a dysfunction of voltage-gated N-type calcium channels. Combined nimodipine and ω-conotoxin GVIA treatment demonstrated a small but significant decrease in the elicited intracellular calcium level in ScGT1-1 cells compared to uninfected GT1-1 cells. However, when a combination of both blockers were used, only a small current remained, which in amplitude was close to the limit for correct analysis and therefore no further isolation was performed. Thus, we can not exclude that a small dysfunction may reside in other voltage-gated calcium channels, too.

In paper II, we used patch clamp techniques to assure that the reduction in elicited intracellular calcium levels in the ScGT1-1 cells was not caused by a dysfunction in intracellular calcium stores. It has been shown that calcium influx through voltage-gated N-type calcium channels mediate calcium-induced calcium release from intracellular store while influx through L-type calcium channels does not mediate this process [223]. From these experiments we confirmed the functional expression of voltage-gated N-type calcium channels in GT1-1 cells. Furthermore, we found a marked dysfunction in voltage-gated N-type calcium channels in ScGT1-1 cells that most likely underlies the reduced intracellular calcium concentration observed in these cells due to depolarization with KCl in Paper I. We could also confirm that voltage-gated non-N-type calcium channels seemed unaffected by the scrapie infection.

These results are interesting in several aspects. We can conclude that the principal HVA channels functionally expressed in GT1-1 cells are L-type calcium channels and N-type calcium channels. This contradicts a previous report of this cell line, in which L-type, but no N-type calcium currents could be isolated [193]. The subclone GT1-7 has recently been demonstrated to express functional R-, L- and N-type calcium channels [196], although no expression of N-type calcium channels was previously reported [197, 198]. Vasquez-Martinez et al. demonstrated that the time period of maintenance in culture is of importance for the development of voltage-gated calcium

channels in GT1-7 cells [194]. Thus, variations in culture conditions may be one explanation for the different functional expression of voltage-gated calcium channels in these cell lines.

The GT1-1 and GT1-7 cells have recently been demonstrated to release GABA upon depolarization with KCl [199]. Furthermore, these subclones express proteins involved in regulated exocytosis, such as synaptobrevin and synaptotagmin, and synaptophysin which are localized to small synaptic vesicles [189, 199]. The voltage-gated N-type calcium currents observed in GT1-1 cells may therefore be involved in the regulated GABA release.

The dysfunction observed in voltage-gated N-type calcium channels in ScGT1-1 cells could reflect a reduced expression at the protein level or changes in the channel distribution. The dysfunction could also reside in changed modulation of the channels or be an adaptive change of the infected cells to favor their survival. In paper I, we used Western blotting to determine the protein expression of the N-type α1B subunit but no differences in the expression could be demonstrated in ScGT1-1 cells compared to uninfected GT1-1 cells. The voltage-gated N-type calcium channels have been shown to down-regulate upon neurotransmitter interaction with G-protein coupled receptors, (for review, see [129]). The reduced N-type calcium currents in ScGT1-1 cells might therefore be caused by a neurotransmitter-induced inhibition via the G$\beta\gamma$ subunit that mediates a reluctant state of these channels. It would be interesting to measure the possible release of neurotransmitters in the ScGT1-1 cells. The abnormal conversion of the prion protein has been suggested to occur in lipid rafts in the plasma membrane [39, 44, 45] where several signaling pathways are implicated. The effect of PrP^{Sc} on these domains is unknown but since no G$\beta\gamma$ subunit interaction with the raft domains has been revealed, disturbances through this pathway seem less likely to occur.

Voltage-gated N-type calcium channels have a functional role in neurotransmitter release which is a complex and tightly regulated process. These channels appear later during development than the L-type calcium channels in the sister clone GT1-7 [194]. Similar to certain viral infections such as lymphocytic choriomeningitis, the prion infection may mediate dedifferentiation of the cells to decrease their energy demand [224]. As an alternative explanation to the reduced N-type calcium currents observed, dedifferentiation might have changed the distribution of N-type calcium channels in the cells [136].

Finally, the interaction between presynaptic proteins involved in exocytosis and voltage-gated N-type calcium channels is of importance for the voltage-gated N-type calcium channel availability and this interesting notion will be further scrutinized in the discussion referring to specific aim II.

The observed disturbances in voltage-gated N-type calcium channels in ScGT1-1 cells are interesting since early signs of synaptic changes due to prion infection has been observed *in vivo* [63, 64, 68]. Electrophysiological studies performed in slice preparations from mouse and hamster brains infected with experimental scrapie have revealed hyperexcitability in the hippocampal CA1 region [76-78] but these effects are believed to be caused by disturbances in AHPs following action potentials [77-79]. Interesting studies have been made using ω-conotoxin GVIA that blocks voltage-gated N-type calcium channels and neurotransmitter release. In hippocampus and hypothalamus, inhibition of N-type calcium channels reduced inhibitory synaptic transmission to a higher extent than excitatory transmission with an increased number of population spikes as a consequence [146]. In addition, GABA release was evoked by either voltage-gated N- or P/Q-type calcium channels depending on the location of the synapse-forming interneuron in hippocampus. Glutamatergic synapses located in hippocampus, exhibit at least three types of calcium channels that participate in

glutamate release even at synapses originating from a single presynaptic neuron [148]. These results indicate that a dysfunction in voltage-gated N-type calcium channels might cause increased excitation in a neuronal network and this imbalance may underlie characteristic signs for CJD such as EEG abnormalities consisting of periodic sharp wave complexes [72]. Furthermore, continuous depolarization has been reported to cause a reduction in N-type calcium channels localized to the plasma membrane [139], which may mediate a feed forward loop in this system exaggerating the excitation further.

One important issue concerning the use of cell lines for studies of cellular properties is the selection of cells with particular properties that may arise as a consequence of the infection. Thus, the control experiment using quinacrine to clear the PrP^{Sc} from the cultures (paper I) was important to show that the reduced voltage-gated N-type calcium currents were reversible and not caused by cell selection. Quinacrine is an anti-malaria drug which has been shown to clear neuroblastoma cells from scrapie infection [225, 226]. Long-term treatment with this drug has been shown to impair voltage-gated calcium currents [227], but in our experiments no systematic reduction in the calcium currents was observed.

As described in the method section, the neuronal cell line GT1-1 has been thoroughly characterized in terms of electrophysiological properties related to spontaneous activity. Propagation of sodium-dependent action potentials activates voltage-gated L-type calcium channels that mediate GnRH secretion. The synchronization of spontaneous calcium oscillations into intercellular calcium waves depends on voltage-gated L-type calcium channels and functional gap junctions, and causes the rhythmic pattern of GnRH release. In paper I, we confirmed by fluorometric calcium measurements and confocal microscopy that the GT1-1 cells exhibit spontaneous calcium oscillations that are synchronized within large groups of cells. When spontaneous calcium oscillations were analyzed in ScGT1-1 cells and compared to uninfected GT1-1 cells, no difference in either their frequency or amplitude could be observed, indicating that the voltage-gated L-type calcium channels were preserved in the infected cells. Furthermore, the synchronization of calcium oscillations in ScGT1-1 cells was comparable to the synchronization in uninfected GT1-1 cells, showing that the infected cells have functional gap junctions as well as functional expression of the L-type calcium channels.

In paper II, we confirmed the observation of preserved non-N-type calcium currents using patch clamp recordings. These results contrast previous reports *in vitro* where acute exposure of the PrP106-121 fragment causes increased intracellular calcium levels in GT1-7 cells or prolonged exposure causes reduced voltage-gated L-type calcium currents in primary cultures of cerebellar granule neurons or in the GH3 cell line [97, 101]. However, our results agree with observations in PC12 cells exposed to PrP106-121 where decreased voltage-gated N-type calcium currents, but unaffected L-type calcium currents were seen [102]. These differences could depend on experimental conditions. PrP106-121 fragments mediate extracellular effects while our GT1-1 cell system may more reflect an endogenous progressive PrP^{Sc} production. The GT1-1 cells can maintain a stable prion infection for over 30 passages and the number of infected cells is high in the cell population. The PrP 106-121 fragment is produced using a template isolated from amyloid plaque cores in patients with GSS [95] and furthermore these fragments causes neuronal cell death upon chronic application [96, 97].

It has been reported that grafting neuronal tissue overexpressing PrP^{C} into the brain of PrP-deficient mice and infect these animals with experimental scrapie causes large accumulations of PrP^{Sc} in the graft and the accumulated PrP^{Sc} are transferred into the surrounding tissue. However, no neuropathological changes could be revealed in the

tissue surrounding the graft, indicating that extracellular accumulation of PrP^{Sc} does not seem to mediate such changes *in vivo* [228].

In conclusion, I have shown that the GT1-1 cell line expresses functional voltage-gated N-type calcium channels in addition to the L-type and T-type calcium channels previously reported. Furthermore, impairment in N-type calcium currents could be demonstrated in ScGT1-1 cells whereas the L-type calcium currents were unaffected. From my results, I conclude that a prion infection can affect voltage-gated N-type calcium channels and that these changes can be reversible upon treatment.

II. Functional Interaction and expression of proteins involved in neurosecretion in scrapie-infected and uninfected GT1-1 cells (Paper III).

In paper III, we confirmed the expression of SNAP-25 and syntaxin 1A in the GT1-1 cell line. These proteins are involved in neurosecretion as well as small synaptic vesicle release. We also confirmed the expression of synaptophysin in the GT1-1 cell line. This protein is specifically implicated in small synaptic vesicle release [176].

When an antibody recognizing the complexed forms of syntaxin 1A was used together with suitable conditions for complex formation, e.g. lysates exposed to 4°C (paper III, IV), we found 1-3 different syntaxin complexes. It has been suggested that various SDS-resistant complexes occur in living cells [215, 217]. However, their different roles in exocytosis are unknown (see the Methodological consideration section). When the lysates from GT1-1 cells were heated at 100°C for 20 min the complexed forms of syntaxin 1A were dissociated and a corresponding increase in the monomeric level of syntaxin 1A was observed. A similar trend could be seen when the monomeric forms of SNAP-25 and synaptophysin were determined in GT1-1 lysates kept in 4°C and compared to the protein levels in lysates heated in 100°C for 20 min. By these experiments we learned that SNAP-25, syntaxin 1A and synaptophysin are all implicated in complex formation in GT1-1 cells. It seems reasonable that the syntaxin 1A and SNAP-25 may form either binary or ternary complexes, or both. In paper III, only one or two complexed forms of syntaxin could be observed probably due to incomplete transfer of large proteins to the blotting membranes. However, in paper IV we could detect three different complexed forms of syntaxin 1A indicating that several different complexed forms of this protein occur in these cells. It is more difficult to interpret the complexed form of synaptophysin, but this protein has been shown to interact with synaptobrevin [182], which is expressed in GT1-1 cells [189]. However, no complex formation between synaptobrevin and synaptophysin has previously been demonstrated in GT1-7 cells [182]. Further experiments are needed to scrutinize this interaction.

In ScGT1-1 cells, however, no difference in the level of monomeric SNAP-25, syntaxin 1A or synaptophysin could be demonstrated when lysates exposed to 4°C where compared to lysates exposed to 100°C (paper III). These results indicate that the complexed forms of these proteins were dissociated or not formed in the first place. Several experiments support the hypothesis that SNARE complex formation is a late step in the docking/fusion process [155, 212, 213].

When the protein level of SNAP-25 and syntaxin 1A was determined in ScGT1-1 cells after complex formation was abolished, a marked reduction could be found compared to the expression in uninfected GT1-1 cells (Paper III). In addition, the

expression of synaptophysin was reduced in ScGT1-1 cells. When SNAP-25 and synaptophysin protein expression was determined at different time points after infection, a small reduction could be observed 32 days *p.i.* that was more pronounced 45 days *p.i.* The protein level of syntaxin 1A was also reduced but to a much lower extent and not at these early time points *p.i.* These observations of reduced levels of SNARE proteins and synaptophysin agree with those described previously in brains from CJD patients [65-67] and in mouse brains infected with experimental scrapie [64, 68, 69]. It has been debated whether the loss of presynaptic proteins in prion-infected brain tissue reflects neuronal cell death. However, several studies have shown that axon terminal changes precede neuronal cell death [63, 64, 68]. Furthermore, axon terminal changes has been demonstrated in atypical cases of CJD where no signs of neuronal cell death could be observed [70]. We did not see any marked neuronal cell death in our cell system, but these cells probably never reach late stages of infection since they are dividing continuously.

In paper III, we also used real-time PCR to determine the mRNA level of SNAP-25. In addition, this method gave us the advantage of being able to distinguish between the two isoforms SNAP-25a and b as described in the introduction. SNAP-25a has been implicated in neuritic outgrowth and fusion of vesicles delivering components to the plasma membrane, while SNAP-25b is involved in synaptic vesicle release and neuropeptide secretion. We found that the relative mRNA levels corresponding to both SNAP-25 isoforms were increased in ScGT1-1 cells compared to uninfected GT1-1 cells, indicating that both isoforms were affected by the prion infection. Similarly, the relative synaptophysin mRNA level was increased in ScGT1-1 cells (data not shown). However, the increased level of mRNA coding for these proteins may be a compensatory effect caused by an increased degradation rate of these proteins.

The reduced complex formation in ScGT1-1 cells may reflect changed distribution or expression of important proteins implicated in this process as described in detail previously. Syntaxin 1A switch between an opened and a closed conformational state that regulates the protein interaction with SNAP-25. Munc-18-1 in complex with syntaxin 1A mediates a closed conformation in which no interaction between syntaxin 1A and SNAP-25 can occur. Furthermore, syntaxin 1A requires Munc-18-1 co-expression for correct transportation to the plasma membrane [164]. It is therefore also possible that the reduced complex formation reflects changes in SNARE and synaptophysin protein expression. SNARE complex formation is favored when the SNARE proteins are in close proximity, e.g. expressed in high concentrations [213]. On the other hand, the decreased levels of SNAP-25 and syntaxin 1A may be caused by the disturbed SNARE complex formation (paper III). Furthermore, SNARE complex formation as well as the expression of SNARE proteins increases with maturation of cerebellar granule cells [214]. The turnover rate of SNAP-25 decreases in mature neurons when the SNARE complex formation increases [229].

The reduced level of synaptophysin in ScGT1-1 cells (paper III) deserves a special interest, since this effect indicates that synaptic vesicles containing classical neurotransmitters and, thus, neurotransmission may be affected in prion infected brains. Synaptophysin has been shown to interact with synaptobrevin in a complex, and in this complexed form interaction between synaptobrevin and the other SNARE proteins is excluded, indicating a mechanism for controlling the exocytotic machinery [180]. From our experiments we learned that the level of synaptophysin in complexed forms was reduced in ScGT1-1 cells compared to the level in uninfected GT1-1 cells (paper III). The decreased expression of synaptophysin may, similarly to the SNARE proteins, reflect disturbances in the exocytotic machinery in ScGT1-1 cells. Furthermore, the decreased level of SNARE and synaptophysin complex formation in ScGT1-1 cells may

lead to a changed distribution of the monomeric synaptophysin and SNARE proteins, with increased degradation of these proteins as a consequence. In addition, attempts to promote cellular differentiation by db-cAMP and IBMX revealed no further changes in the distribution of proteins in ScGT1-1 and uninfected GT1-1 cells and the two cell populations responded similarly with increasing protein levels of SNAP-25 and synaptophysin.

Synaptophysin interacts specifically with cholesterol in the synaptic vesicle membrane and depletion of cholesterol using methyl-β-cyclodextrin blocks synaptic vesicle endocytosis in PC12 cells [230]. Furthermore, the interaction between synaptophysin and synaptobrevin has been shown to depend on the cholesterol content in the vesicle membrane. Depletion of endogenous cholesterol using lovastatin in primary cultures from mouse hippocampus caused a down-regulation of the synaptophysin-synaptobrevin complex [181]. SNARE proteins have also been shown to be highly enriched in lipid rafts [231], indicating that these signaling platforms are important for functional and spatial control of regulated exocytosis. The prion protein is a GPI-anchored protein that similarly to SNARE proteins is localized to lipid rafts [17, 44]. In addition, conversion of PrP^{C} into PrP^{Sc} may occur in association with lipid rafts since cholesterol depletion or replacement of the GPI-anchor with a residue targeted to clathrin-coated pits prevents the PrP^{Sc} formation [19, 46, 232]. The abnormal conversion of PrP^{C} in rafts may therefore cause functional disturbances in protein signaling pathways residing in these domains.

The reduced complex formation observed in ScGT1-1 cells was correlated to the amount of PrP^{Sc}. When populations of GT1-1 cells were infected at two different time points and the level of PrP^{Sc} was determined using Western blotting after a number of passages, a marked difference in the level of PrP^{Sc} could be observed between those batches (paper III). When immunohistochemistry combined with guanidinthiocyanate treatment was used to determine the number of infected cells in the cultures, a clear difference could also be found. The results indicate that less number of cells was infected in the batch with the lower amount of PrP^{Sc}. Furthermore, when the levels of monomeric SNAP-25, syntaxin 1A and synaptophysin were determined, an increased level of the monomeric forms of these proteins could be seen that corresponded well to the level of PrP^{Sc} (paper III). When the ScGT1-1 cells were exposed to quinacrine that previously has been shown to clear PrP^{Sc} from cell cultures, the increased level of monomeric SNAP-25 and synaptophysin was reversible (paper III). In addition, we observed from these experiments that the dysfunctions caused by PrP^{Sc} in these cells were at least partially reversible upon quinacrine treatment.

The reduced complex formation implies disturbances in the exocytotic machinery in ScGT1-1 cells. Referring to our results in paper I and II, we suggest that the dysfunction seen in voltage-gated N-type calcium channels and the disturbed complex formation between proteins involved in exocytosis are related. For instance, it has been shown that the balance between SNAP-25 and syntaxin 1A is of importance for the channel availability. Co-expression of the N-type α1B subunit together with either SNAP-25 or syntaxin individually reduces N-type channel availability when investigated in Xenopus [183, 184] or in tsA-201 cells [185]. However, combined co-expression of all three proteins normalizes the channel availability, which would favor calcium entry through channels prepared for docking and release of presynaptic vesicles.

Our findings indicate disturbances in the synaptic vesicle release pathway. Interestingly, synaptosomes derived from cortical and thalamic areas in hamster brain infected with experimental scrapie have been shown to release less GABA in response to KCl [71]. Furthermore, these animals showed an abnormal aggregation of synaptic vesicles in the presynaptic boutons at the terminal stage of the disease. The level of

presynaptic proteins such as syntaxin 1A, SNAP-25 and synaptophysin is reduced in brains from patients with CJD [65-67] as well as in mouse brains infected with experimental scrapie [64, 68, 69]. In addition, PrP^{Sc} has been revealed to accumulate in synaptosomes [71] and PrP^{Sc} and synaptophysin co-localize in brain tissue from patients with CJD, indicating a synaptic distribution of PrP^{Sc} [67]. Furthermore, several studies have supported the notion that synaptic alterations occur early in mice brains infected with experimental scrapie[63, 64]. These results all indicate similarly to our study that the changes in presynaptic protein are directly related to the presence of PrP^{Sc}.

Taken together, I have found a reduced level of complexed SNAP-25, syntaxin 1A and synaptophysin in ScGT1-1 cells as well as a reduced level in the total amount of these proteins. My results indicate that a prion infection can cause disturbances in the interactions of proteins involved in synaptic vesicle release and that the changes can at least be partially be reversible upon treatment.

III. Chronic depolarization in Scrapie-infected and uninfected GT1-1 cells (Paper IV).

An abnormal pattern of periodic sharp wave complexes resembling interictal epileptiform discharges can be observed, mainly at the terminal stage of two thirds of the sCJD cases [233]. The extensively increased, synchronized bursting activities during epileptiform discharges have been demonstrated to cause an enhanced level of interstitial potassium [234] and it has been suggested that this enhancement of extracellular potassium may induce further epileptic seizures [235, 236]. In paper I-II we observed that the voltage-gated N-type calcium currents were impaired in ScGT1-1 cells. Furthermore, in paper III we describe how the complex formation between proteins involved in neuronal exocytosis was reduced in these cells. Both these changes in the ScGT1-1 cells could hypothetically be attempts of the infected cells to dampen an increased excitation.

In paper IV, I exposed uninfected GT1-1 cells to long-term depolarization for 72 hours using increased concentration of extracellular potassium and found that the level of complexed syntaxin 1A forms was markedly decreased. No morphological signs of toxicity could be found in the cells. Several reports support the hypothesis that SNARE complex formation is a late step in the docking/fusion process and catalyse the fusion of vesicle release [155, 212, 213]. These results indicate that long-term depolarization mediates a disturbed/reduced vesicle release that is reflected by a reduction in SNARE complex formation. The reduced SNARE complex formation may either be caused by a depletion of vesicles due to an overload of the exocytotic machinery or reflect a protective mechanism against increased excitation in the cell. For instance, long-term depolarization has been shown to cause internalisation of voltage-gated N-type calcium channels from the plasma membrane [139] and a reduced calcium channel availability may decrease SNARE complex formation. Furthermore, Munc-18-1 has been shown to function as a chaperone and hold syntaxin 1A in a closed conformation, which abolishes this protein's interaction with other SNARE proteins [164]. However, it is unknown what directs the Munc-18 interaction with syntaxin 1A.

Reduced SNARE complex formation has also been described in other conditions. Chronic morphine treatment of mice has been described to decrease SNARE complex formation in synaptosomes prepared from hippocampus [237]. Furthermore, primary adrenal medulla chromaffin cells derived from SV2A knock out mice show a

decreased level of SNARE complexes [238]. The SV2A knock out mice exhibits a fatal epileptic phenotype with severe seizures. The specific function of SV2 however, is still unknown.

The reduced level of SNARE complex formation in GT1-1 cells exposed to long-term depolarization resembles that observed in non-depolarized ScGT1-1 cells. Thus, one possible explanation for the reduced level of complexed syntaxin 1A in ScGT1-1 cells is that it reflects an increased degree of excitation. However, unexpectedly, the ScGT1-1 cells exposed to long-term depolarization showed an increased level of complexed syntaxin 1A (paper IV). No changes in cell morphology could be seen. From these experiments, I could exclude the possibility that the reduced complex formation was caused by an increased excitation in ScGT1-1 cells. The increased level of complexed syntaxin 1A in ScGT1-1 cells exposed to long-term depolarization may therefore instead indicate disturbances in the exocytotic machinery or vesicle recycling pathway which may be at least partially surmounted by the increased excitation. Alternatively, the increased level of complexed syntaxin 1A could reflect an increased expression of SNARE proteins due to the increased excitation. It has been shown that SNARE complex formation is favoured by increased proximity of SNARE proteins e.g. increased concentration. PC12 cells and cerebellar granule cells exposed to extracellular KCl during 96 hours showed an increased expression of SNAP-25 mRNA and protein [239]. A slightly increased level of syntaxin 1A and SNAP-25 could actually be demonstrated in ScGT1-1 cells exposed to long-term depolarization.

In summary, these experiments show that long- term depolarization causes reduced levels of complexed syntaxin 1A in uninfected GT1-1 cells, while the level of complexed syntaxin 1A in ScGT1-1 cells was increased. Thus, I conclude that increased excitation does not cause the reduced levels of complexed forms of exocytotic proteins in ScGT1-1 cells.

CONCLUDING REMARKS

Morphological and clinical features characteristic for prion diseases indicate disturbances in synaptic transmission. Early signs of axon terminal changes with decreased levels of presynaptic proteins such as synaptophysin, SNAP-25, syntaxin 1 and synapsin 1 are important observations that may give clues to an understanding of the pathogenesis behind dysfunction in the prion-infected brain. The present thesis provides new information supporting the notion that the presynaptic structures may be a main target in these diseases. In particular, we found a marked dysfunction in voltage-gated N-type calcium channels in ScGT1-1 cells. These channels are in mature neurons principally located at the presynaptic terminals. Furthermore, no changes could be observed in the L-type calcium channels that are localized also to the cell soma and dendrites in mature neurons. We could not demonstrate any changes in the level of the N-type α1b subunit indicating that the impairment in voltage-gated N-type calcium currents is caused by either changes in their modulation or distribution. Voltage-gated N-type calcium channels interact closely with presynaptic proteins involved in vesicle release in mature neurons. In our cell system, we also found that a prion infection caused decreased levels of complexed syntaxin 1A and SNAP-25, proteins involved in neurosecretion. In addition, we found similar disturbances in the levels of complexed synaptophysin, a protein confined to small synaptic vesicles and implicated in regulated vesicle release were found. The change in levels of monomeric forms of these proteins was correlated to the amount of PrP^{Sc} present in the cells. When the complex formation was abolished using exposure of samples to heat, a decreased level of SNAP-25, synaptophysin and syntaxin 1A could be observed. No changes in the cellular distribution of these proteins could be seen. An increased level of SNAP-25 and synaptophysin mRNAs was seen, which indicates increased turn over of these proteins. Finally, treatment with quinacrine, a drug that has been shown to clear PrP^{Sc} from cell cultures, cleared PrP^{Sc} also from the ScGT1-1 cells and partially reversed the effects both upon voltage-gated N-type calcium channel function and levels of monomeric SNAP-25 and synaptophysin. These results show that the effects are not caused by a cellular selection favored by the prion infection but rather caused by the presence of PrP^{Sc} in the cells.

The reduced voltage-gated N-type calcium currents and decreased level of complexed forms of proteins involved in exocytosis in ScGT1-1 cells could be an attempt from the infected cells to dampen an increased excitation, which frequently occurs *in vivo* during prion infections. Unexpectedly, however, ScGT1-1 cells exposed to a long-term depolarization did respond differently in comparison to uninfected GT1-1 cells, showing an increased level of protein complex formation. These results indicate that the observed effects are not caused by an increased excitation. The long-term depolarization could instead at least partially surmount the decreased level of protein complex formation, which indicates that the ScGT1-1 cells are subjected to disturbances in the synaptic vesicle release pathway.

Recently, electron microscopy has revealed abnormal aggregation of synaptic vesicles in hamster brain at late stages of scrapie infection. Furthermore, a decreased level of GABA release could be observed in synaptosomes derived from thalamic and cortical areas of the brain. These findings together with the data presented in this thesis all indicate disturbances in the presynaptic machinery, caused by the presence of PrP^{Sc}.

Taken together my results indicate that a prion infection can affect the vesicle release pathway, which may be one pathogenetic mechanism underlying brain

dysfunctions caused by prion infection. Furthermore, from the perspective of therapeutic strategies it is encouraging that these changes seem to be reversible upon treatment.

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