4. DISCUSSION

4.1 Spectral and molecular changes during scrapie pathogenesis

In this study, thoracic dorsal root ganglia were investigated using synchrotron FTIRM in order to detect differences in protein secondary structure and distribution between control and infected animals during the progression of the disease. It was shown that the β-sheet content was constant with age in the control animals, whereas scrapie-infected animals had a significant increase in the relative amount of β-sheet rich proteins with disease progression. Immunostained adjacent slides confirmed that this increase is at least partly due to the increase of PrP^{Sc} in the tissue, supporting the findings of an earlier pilot study on terminal diseased animals (Kneipp *et al.*, 2003). The distribution of proteins rich in α-helices was variable with age, but the trends were similar for control and scrapie-infected animals. In all cases, the α-helical protein content was higher in the control animals, except for 100 dpi where the opposite was found. The relative protein content was observed to be significantly increased in pre-clinical scrapie and then declined gradually during pathogenesis ending with significantly less relative protein at the terminal stage of the disease.

Using the PrP specific antibody, 3F4, dorsal root ganglia of orally 263K scrapie-infected Syrian hamsters were shown to exhibit PrP^{Sc} accumulation as early as 76 days post infection in two of four investigated animals (McBride *et al.*, 2001). However, only ~0.1% of all proteins in the brain were estimated to be PrP^{Sc} at the terminal stage (Beekes *et al.*, 1995). In this work, results showed an increase in β-sheet protein content of about 8.3% over the course of the disease, well above the amount attributable to PrP^{Sc}. Moreover, the relative amount of protein expressed in the tissue at the terminal stage was significantly lower in infected animals than controls. Together, these results suggest that disease progression is associated with (a) an increase in proteins high in β-sheet, including but not limited to the conversion of PrP^{C} into PrP^{Sc}, and (b) the reduced expression of proteins in general due to neurodegeneration and cell death. In addition, not all cells showing elevated β-sheet stained positive for PrP^{Sc}. Thus, we suggest that other proteins in addition to PrP are responsible for the elevated β-sheet at the later stages of the disease, as has also been

suggested elsewhere (Diedrich *et al.*, 1991; Diedrich *et al.*, 1993). Diedrich and colleagues reported an increased expression of Apolipoprotein E and Cathepsin D in activated astrocytes soon after they could detect PrP accumulation in scrapie 22L infected female mice (Diedrich *et al.*, 1991). The latter protein is composed of only 4% α-helix but 50% β-sheet (Tamara, 1984) and could therefore be partially responsible for the detection of increased β-sheet in our study. Heat shock protein, transferrin, and β2-microglobulin were also increased in astrocytes during scrapie (Diedrich *et al.*, 1993) and β2-microglobulin has been shown to consist of only β-sheet and no α-helix (Okon *et al.*, 1992). However, due to the integrative nature of information provided by FTIRMS, assignment to single proteins cannot be performed.

At the pre-clinical time points, i.e. 100 and 130 dpi, the infected animals exhibited a lower β-sheet content and higher α-helical content (at 100 dpi) than the control animals. These results indicate that changes in protein composition occur long before the onset of clinical signs. These alterations could represent a downregulation of genes that encode for proteins high in β-sheet and/or an upregulation of genes that encode for proteins low in β-sheet. However, the observed increase in relative protein expression (**Figure 3.1.1C**) argues for the latter possibility, i.e. an overexpression of proteins high in helix and low in β-sheet. Recently, an FTIR spectroscopic study of serum from 263K scrapie infected hamsters at different time points during the disease was performed (Lasch *et al.*, 2006). Applying artificial neural networks, the authors were able to distinguish spectra from serum of pre-clinical infected animals from those of controls based on spectral difference in the protein Amide I region, in particular at 1630 cm^{-1}, which is assigned to β-sheet. Whether an increase or a decrease of β-sheet was responsible for the feasibility to distinguish scrapie infected from control animals at pre-clinical time points based on differences at 1630 cm^{-1} was not the focus of the study and therefore not addressed. However, together with our findings, these results suggest that, especially at early stages of the disease, the detected spectral changes may not be due to the misfolded form of the prion protein itself but rather to changes in proteins other than PrP^{Sc}.

In summary, this FTIRM study showed that dramatic protein-related changes occur also at the pre-clinical stages of scrapie, far beyond the transformation of PrP^{C} to PrP^{Sc}. PrP^{Sc} is known to induce apoptosis in neuronal cells (Chiesa *et al.*, 2000; Zhang *et al.*, 2003; Hetz *et al.*, 2003; Ye *et al.*, 2002), which involves the upregulation of pro-

and the downregulation of anti-apoptotic enzymes (Villa *et al.*, 2006; Park *et al.*, 2000). Many of these proteins, most of them members of the BCL-2 family, consist of mainly α-helices (Petros *et al.*, 2004; Letai, 2005). However, the recruitment of pro- and anti-apoptotic proteins does not always requires their de novo synthesis. Additionally to the production of new proteins, some enzymes already exist in the cytoplasm in an inactivated form. After a death signal arrives at the cell, caspases cleave parts of the protein, exposing their reactive site on the proteins surface. This involves conformational changes of the protein, for example by displacing the adapters from the pro-survival proteins (Adams & Cory, 1998) or by inserting pro-apoptotic enzymes in the mitochondrial membrane (Hsu *et al.*, 1997; Wolter *et al.*, 1997). Recently performed FTIR microspectroscopic studies of single cells at different stages of apoptosis revealed a relative increase in α-helical content relative to β-sheet or random coil (Jamin *et al.*, 2003) at both early and late apoptosis. This is in agreement with the findings of increased α-helical content and decreased β-sheet content at 100 dpi in this thesis. However, at terminal stage, the exponential increase in PrP^{Sc}, likely together with other proteins, probably causes the detected elevation of β-sheet.

When looking at the distribution of protein secondary structure, some cells at 100 dpi showed extremely low values of β-sheet (0.03) paired with high values of α-helix (0.07) in the cytoplasm (see arrowheads and inset in **Figure 3.1.2A**), which seem to be arranged in clusters. Although FTIRM cannot clarify the nature of these structures, it was shown that prion-infected cells produce transforming growth factor alpha (TGF-α) (Oleszak *et al.*, 1988), which in turn increase PrP^{C} expression in cultured human keratinocytes after exposure (Pammer *et al.*, 1998). Furthermore, studies of intracerebrally 263K infected Syrian hamsters showed significantly increased amounts of PrP mRNA in the superior and inferior colliculi at 77 days postinoculation (Li & Bolton, 1997). This type of mRNA differs from the one that was proven not to change during the disease in earlier studies (Oesch *et al.*, 1985; Kretzschmar *et al.*, 1986). Therefore, the increase in α-helix in infected neurons may partly be due to an increase in PrP^{C} expression. After leaving the Golgi apparatus, the cellular prion protein travels to the cell membrane in vesicles, where it gets attached via a GPI anchor (Robertson *et al.*, 2006; Porto-Carreiro *et al.*, 2005; Fevrier *et al.*, 2004; Fevrier *et al.*, 2005; Mironov *et al.*, 2003; Prado *et al.*, 2004). It is possible that the circular structures seen in the chemical images correspond to arrangements of vesicles filled with proteins low in β-sheet possibly together with PrP^{C} (which consists of only 3% β-sheet

but 42% α-helix) with high local density. To identify those proteins, other analytical techniques such as 2D-gel electrophoresis followed by for example MALDI-TOF mass spectrometric analysis, would be needed.

In scrapie-infected mouse brain, a total of 138 genes were found to be upregulated while 20 were downregulated during pathogenesis (Booth *et al.*, 2004). These genes were separated into four distinct clusters: genes that are (a) upregulated at 21 days post infection and later downregulated; (b) downregulated throughout the course of disease; (c) downregulated at 21 dpi and later upregulated; and (d) upregulated at 100 dpi and later downregulated. Although the main and characteristic event in prion pathogenesis is the transformation of the cellular prion protein into the pathogenic form followed by an accumulation in the central and peripheral nervous system, this study demonstrates the variety of genes encoding for proteins involved during scrapie pathogenesis besides the accumulation of PrP^{Sc}. Although assignment of spectral changes to single discrete proteins is not possible, FTIRM is a sensitive method to detect significant protein-related molecular changes during scrapie pathogenesis.

The results presented in this study show that elevated β-sheet content begins near the cell membrane in early stages of the diseases and progresses throughout the cell by the terminal state. Immunohistochemistry studies have previously shown that the deposition of pathological PrP^{Sc} starts in satellite cells and in the cytoplasm of a few neurons (McBride & Beekes, 1999). As the disease progresses, an increasing number of neurons are affected and more intense staining was observed in the cytoplasm, primarily in secondary lysosomes (Taraboulos *et al.*, 1990; McKinley *et al.*, 1991; Taraboulos *et al.*, 1994). Our finding of a possible PrP^{Sc} accumulation near the cell membrane of neurons is consistent with the previously performed pilot study reporting the accumulation of large amounts of PrP^{Sc} at the cell membrane of neurons in the DRG of hamsters (Kneipp *et al.*, 2003), as well as in scrapie-infected mouse neuroblastoma cells (Caughey & Raymond, 1991), in cultured Chinese hamster ovary cells (Lehmann & Harris, 1996), in the medulla oblongata, pons, and astrocytes of sheep (van Keulen *et al.*, 1995), and mice with natural scrapie (Jeffrey *et al.*, 1994b; Jeffrey *et al.*, 1994a). Satellite cells, which are glia cells surrounding neurons in the PNS, were also shown to accumulate the misfolded prion protein (Groschup *et al.*, 1999; McBride & Beekes, 1999).

The distribution of α-helical rich proteins in control ganglia indicates that the cytoplasm is typically higher in α-helix than the extracellular matrix and the nucleus. This is also true for neurons from infected animals at 100 and 130 dpi, and for most neurons from animals at first clinical signs and at the terminal stage. Nevertheless, some of the neurons in the later stages of the disease exhibited extremely low α-helix content, coinciding with elevated β-sheet and PrP^{Sc} deposition. A possible explanation for this observation is that, at 100 and 130 dpi, PrP^{C} is still attached to the membrane by GPI anchor or in the cytoplasm while trafficking between Golgi apparatus and plasmalemma (Prado *et al.*, 2004). However, for PrP^{Sc}-infected cells, PrP^{C} gets internalized and converted to PrP^{Sc} (Kiachopoulos *et al.*, 2004), so that these cells show less helix in the membrane and cytoplasm.

In addition to protein changes, alterations in the lipid composition, although not significant, were apparent in the intermediate stages of the disease, i.e. at 130 - 145 dpi. While the lipid composition does not change over time in the control animals, the CH_3/CH_2 ratio of scrapie infected animals gradually decreased from 100 dpi to fcs and then increased again. The relatively large error bars at 130 dpi and fcs are due to differences in individual animals, indicating variations in the extent of changes in lipid composition between animals. It has been reported that 263K scrapie infection in hamsters induces increasing intracytoplasmic proliferating membranes (Liberski *et al.*, 1989) and increased numbers of lysosomes (Choi *et al.*, 1998) at early stages in the disease, causing changes in the composition of membrane lipids. The observed changes in the composition of fatty acids in neurons of dorsal root ganglia indicate that prominent changes occur before the terminal stage of the disease. *In vivo* studies have shown that conversion of PrP^{C} to PrP^{Sc} is a posttranslational process that appears to occur in an endocytic pathway (Caughey & Raymond, 1991; Borchelt *et al.*, 1992; Taraboulos *et al.*, 1992) and that both forms of the prion protein colocalize in caveolae-like membraneous domains (Vey *et al.*, 1996). This was also shown experimentally in vitro in fibroblasts and neuronal N2Acells, where GPI anchored PrP^{C} has been localized to invaginated caveolae (Ying *et al.*, 1992) and fractionates with caveolae (Harmey *et al.*, 1995; Vey *et al.*, 1996; Naslavsky *et al.*, 1997). Therefore, changes in lipid composition could partly be the result of invaginated cellular prion protein from the surface of the neurons and therefore represent changes in the membrane system. Another possible reason could be the induction of apoptosis. Mitochondrial change, i.e. rapid depolarization of mitochondrial membranes, has

been shown to be the first detectable apoptotic event (Zamzami *et al.*, 1995a; Zamzami *et al.*, 1995b; Vayssiere *et al.*, 1994). As a result of pertubation of mitochondrial membrane permeability, cytochrome *c*, a regulator of execution caspase activity, gets released in the cytosol. A decrease in mitochondrial activity leads to changes in mitochondrial superoxide dismutases (SOD) and other enzymes which in turn increase oxidative stress and highly affect lipid peroxidation. Additionally, increased levels of α-tocopherole, dolichyle phosphate and ubiquinone as well as decreased levels of dolichol were found in scrapie infected mice (Guan *et al.*, 1996), which can affect unsaturated fatty acids. Since membranes are mixtures of lipids and proteins, changes in membrane anchored proteins, as for example caused by the internalization of PrP^{C} prior to its transformation into PrP^{Sc}, can influence lipid composition and membrane fluidity. The occurrence of cytoplasmic lamellar bodies has also been shown in scrapie-infected and normal hamster brain (Liberski, 1988). Recently, a synchrotron FTIRM study of single eukaryotic cells in early and late apoptotic cells revealed changes in the relative amount and/or type of lipids between the two stages of apoptosis (Jamin *et al.*, 2003). For example, an increased CH_2/CH_3 (i.e. a decreased CH_3/CH_2) band intensity, as was found here at preclinical time point 130 dpi and at the onset of clinical signs, was observed only in cells at an early stage of apoptosis. Therefore, the here detected changes in lipid composition at these time points compared to the terminal stage could reflect early apoptotic processes.

In the fingerprint region, most prominent differences were seen at pre-clinical time points (**Figure 3.1.6B**), indicating that alterations in phospholipids, nucleic acids and carbohydrates are early events in scrapie pathogenesis. A significant decrease in the ratio of integrated spectral regions of predominantly symmetric (1100 – 1000 cm^{-1}) by asymmetric P=O stretching vibrations in PO_2^- (~1240 cm^{-1}) was observed from 100 dpi to 130 dpi. Together with the shift towards shorter fatty acid chains (the total lower content in scrapie infected animals at 130 dpi resulted from one animal exhibiting an extremely low ratio), these results suggest early apoptotic events, occurring shortly after significant changes in protein composition and content were visible. Therefore it is possible, that the alterations in protein content and distribution are caused by the up-regulation and activation of apoptotic enzymes which then induce apoptosis, leading to characteristic changes in membrane systems, nucleic acids and carbohydrates, as was shown here.

4.2 Onset of scrapie induced changes detected in the DMNV

Immunohistological studies showed positive staining of pathological prion protein in the DMNV of orally infected hamsters with the 263K scrapie strain in all four investigated hamsters at 69 days post infection (McBride *et al.*, 2001). Furthermore, two of the 4 animals were shown to stain positive at 63 days while no PrP^{Sc} could be detected at 56 dpi. This indicates that the misfolded prion protein enters the brain and reaches titers that are detectable with the monoclonal antibody 3F4 and the used technique, i.e. immunohistochemistry, between 57 and 69 days post infection.

Early FTIR microspectroscopic experiments on orally scrapie infected hamsters at different time points revealed molecular differences between the infected and control animals based on spectral changes detected in the DMNV of the medulla oblongata (Kneipp *et al.*, 2002). In this longitudinal study, the earliest spectral changes observed occurred 90 days post-infection in the DMNV. These alterations were observed in the spectral region between 1040 and 1060 cm^{-1} and were shown to be indicative of an altered composition and/or structure of carbohydrates. By cluster analyses on DMNV spectra from diseased and control animals at 90 and 120 days post infection, spectra from infected animals could be clearly separated from controls. The early stage of the disease has been characterized by microvacuolation (Marsh & Kimberlin, 1975), membrane proliferation, structural and functional damage of mitochondria (Choi *et al.*, 1998) and DNA decomposition during apoptosis and/or a changed RNA content as the result of up- or down-regulation of genes (Riemer *et al.*, 2000).

The DMNV of sixteen 263K infected and eight age matched control animals were studied using a spectrometer coupled with a globar. Cluster analysis of first derivative average spectra of the DMNV showed that at this early time point it is not possible to separate infected from control animals. Average spectra of all infected and mock-infected animals exhibited high similarity (**Figure 3.2.3B**). However, minimal differences in the region 1100 – 1000 cm^{-1} were seen in some of the infected animals at 70 dpi, indicative of an altered composition and/or structure of carbohydrates, i.e. sugar moieties of nucleic acids or changed content of metabolic sugar molecules in the cell such as glucose. Since the investigated regions of the brain included parts of the SolN, where PrP^{Sc} is absent at this early stage, FTIRMS using relatively large apertures

and areas mapped together with averaging all animals probably dilute spectral features which might already be present.

Therefore, to enhance spatial resolution, 70 dpi infected animals were additionally mapped using a synchrotron coupled spectrometer. Here, the fingerprint region was the only spectral region that differed significantly between infected and control animals, indicating changes in carbohydrates, phospholipids and nucleic acids as being related to the first disease induced molecular events. In addition, alterations in protein content and distribution could be seen and were similar to those detected in dorsal root ganglia at 100 dpi. Specifically, the β-sheet content was decreased while the total protein content slightly increased. The lipid composition shifted minimally towards shorter fatty acid chains (in some animals more prominent than in others), the opposite of what was found in dorsal root ganglia (see **Figure 3.1.6A** on page 71), suggesting that 100 dpi DRG and 70 dpi brain are at slightly different stages of the disease.

PrP^{Sc} was shown to induce apoptosis (Giese *et al.*, 1995; Cronier *et al.*, 2004; Fairbairn *et al.*, 1994; Lucassen *et al.*, 1995; Schatzl *et al.*, 1997; Williams *et al.*, 1997), a cascade of events leading to programmed cell death. One model describes the first event as being the activation of BH-3 only proteins, members of the BCL-2 family. These proteins trigger allosteric activation of Bax and/or Bak, which oligomerize at the mitochondrial membrane. It is likely that these events lead to changes in protein and lipid composition, since they include conformational changes in the proteins, for example by displacing the adapters from the pro-survival proteins (Adams & Cory, 1998) as well as in the mitochondrial membrane, by inserting the pro-apoptotic enzymes (Hsu *et al.*, 1997; Wolter *et al.*, 1997). However, spectral indicators for changes in lipid and protein composition at 70 dpi were only small, not only compared to alterations in the region 1350 – 980 cm^{-1}. Here, molecular changes in nucleic acids, carbohydrates and phospholipids contribute to the detected findings. One of the possible contributors, bcl-2 family member Bim was linked to play a role in glucose homeostasis (Danial *et al.*, 2003; Plesnila *et al.*, 2001; Bensaad *et al.*, 2006). Moreover, perturbations of glucose homeostasis have been linked to apoptosis in several studies and therefore could possibly occur in scrapie induced apoptosis as well, contributing to the perceivable alterations (Bensaad *et al.*, 2006; Colussi *et al.*, 2000; Danial *et al.*, 2003; Gramaglia *et al.*, 2004; Malhotra & Brosius, 1999; Otsuki *et al.*,

2005; Plesnila *et al.*, 2001; Reztsova, 2006). In summary, PrP^{Sc} induces apoptosis which in turn starts a cascade of events causing molecular alterations in content and composition of macromolecules like proteins, lipids, nucleic acids and carbohydrates, all of them already present in 70 dpi scrapie infected DMNV in the medulla oblongata of the brain.

4.3 Specificity of scrapie induced spectral changes

To determine the specificity of the scrapie induced spectral changes found in earlier studies (Kneipp *et al.*, 2000; Kneipp *et al.*, 2002), similar experiments were performed on neuronal tissue derived from hamster infected with another scrapie strain, ME7, and with a virus, reovirus T3C9. For the former, DRG of terminally diseased orally 263K infected animals were compared to age-matched controls on the one hand and to terminally diseased intracerebrally ME7 infected animals on the other hand. Although the investigated controls were age-matched only to the 263K infected animals, control animals were shown to exhibit constant β-sheet content (**Figure 3.1.1A**), lipid composition and carbohydrate/phosphate profile (**Figures 3.1.6A** and **B**) during aging in the investigated time.

First it was seen that ME7 infected DRG exhibit higher amounts of β-sheet rich proteins (or proteins higher in β-sheet) than 263K infected animals, however, both are significantly increased compared to control (see **Figure 3.4.1A**). Since the Western Blot analysis revealed that there is probably not more PrP^{Sc} in ME7 ganglia than in 263K ganglia, the observed increase in β-sheet rich proteins in ME7 infected animals is probably partly caused by alterations in secondary structure of other proteins, possibly to a greater extent as in 263K. Second it was detected that the amount of β-sheet is higher when the investigated ganglion is closer to the brain (see **Figure 3.4.1B**). The fact that the misfolded prion protein in i.c. ME7 infected animals reaches the cervical ganglia before the thoracic and that they are therefore longer exposed to PrP^{Sc} could be an explanation for the different accumulation patterns.

Due to the relatively low spatial resolution (about 10 μm), the distribution of β-sheet rich proteins could not be analyzed on a subcellular level with the data taken at the Perkin Elmer Spotlight (see **Figure 3.4.2**). Therefore, additional measurements had

been carried out using a Bruker Hyperion. Different spatial resolutions can be achieved by changing the magnification cassegrain objective in the microscope. These microscopes are confocal, i.e. they have an aperture before and after the sample, improving the spatial resolution from λ to λ/2. Spectra from locations with increased β-sheet rich proteins are shown in **Figure 3.4.3**. Differences in 2nd derivative spectra between ME7, 263K and control are seen in different intensities as well as different peak locations in the amide I region, indicating differences in the secondary structures, i.e. helix, intra- and intermolecular sheet, turns and loops. As was discussed earlier (see page 107), control animals exhibit normal variation in the secondary structure of proteins reflecting the diversity of naturally occurring proteins in neuronal cells and tissue. High β-sheet rich proteins in the 263K infected animal have a slightly different secondary structure than those of the ME7 infected animal. As was shown by Thomzig et al. (Thomzig *et al.*, 2004), spectra taken from purified prion protein of ME7 and 263K infected hamster show distinct differences in their secondary structure. Due to the dilution effect of FTIRMS at the chosen spatial resolution, spectra of regions with high β-sheet still reflect the sum of all proteins. Moreover, spectra of purified prion protein were achieved on PrP27-30, only a part of the whole PrP^{Sc} molecule. Therefore, correlation between spectra derived from neuronal tissue in situ and spectra of purified proteins is not a trivial task.

In the region 1300 – 1000 cm^{-1}, ME7 derived first derivative spectra differ from 263K derived spectra in the region, as was shown in **Figure 3.4.5B**. Differences in spectral alterations were detectable in the regions of phospholipids and nucleic acids (~1240 and 1084 cm^{-1}), in oligo- and polysaccharides and/or phospholipids or cholesterol esters (1171 cm^{-1}) and in the symmetric C-O-C stretching vibrations at ~1060 cm^{-1}. These results suggest that different scrapie strains may induce different molecular alterations in the host regarding the composition of nucleic acids, phospholipids and carbohydrates. Since none of the strains exhibit peaks that can exclusively be detected in either one of them, it is very likely that the molecular changes only vary quantitatively but not quantitatively or compositionally. The detected lower absorption between 1200 – 1000 cm^{-1} in ME7 could tentatively be assigned to less glycosylation, or even higher amounts of the non-glycosylated form of ME7 PrP compared to 263K (Natalello *et al.*, 2005). It was shown earlier, that ME7 PrP27-30 exists to about 40% as mono-glycosylated and to 35-45% as di-glycosylated form, while 263K exists in about 25-35% as mono-glycosylated but 55-65% as di-

glycosylated form (Thomzig *et al.*, 2004). Moreover, studies of the glycosylation pattern in ME7 infected mice demonstrated a significant increase ($p<0.05$) of the non-glycosylated form of the prion protein in the gray matter concomitant with a decrease in the di-glycosylated form (Russelakis-Carneiro *et al.*, 2002). These results are in compliance with the Western Blot analysis performed in this thesis (see **Figure 2.4**).

The second test for determining the specificity of scrapie induced spectral changes was carried out by investigating molecular alterations in the DMNV of reovirus T3C9 infected neonatal hamster. In **Figure 3.5.8**, first derivative average spectra of reovirus infected and control animals were compared to terminal scrapie infected and control animals in the region 1300 – 1000 cm^{-1}. While the width of the peak at around 1240 cm^{-1} (asymmetric P=O stretching vibrations in PO_2 of nucleic acids and phospholipids) is slightly diminished in the DMNV of the terminally 263K scrapie infected animals, the opposite direction occurs in reovirus infected animals. In the corresponding region of the original spectra (**Figure 3.5.9**) it can be seen that reovirus infected animals exhibit a slightly narrower band width at ~1239 cm^{-1} (decreased FWHH) while terminal scrapie infected animals show a decreased peak intensity at 1239 cm^{-1} compared to control (increased FWHH). In the region 1180 – 1130 cm^{-1} both diseases lead to same changes in the corresponding average spectra, i.e. decreased intensities but almost parallel slopes. This might indicate that changes in glycogen (1151 cm^{-1}: C-O stretching (Jackson *et al.*, 1998)) and cholesteryl esters and phospholipids (1171 cm^{-1} : CO-O-C asymmetric stretching arising from ester bonds (Jackson *et al.*, 1998)) are general signs of both diseases, possibly due to inflammation, apoptosis or neurodegeneration. Here, no disease specific changes seem are observed by FTIR MS. Between 1100 and 1000 cm^{-1}, molecular alterations due to the two different diseases are more obvious. The appearance of a prominent shoulder at ~1060 - 1050 cm^{-1}, where complex sugar ring vibrations of carbohydrates absorb can only be seen in scrapie infected animals and might therefore be a specific change due to the infection with 263K scrapie. Cluster Analysis seems to support that hypothesis as shown in **Figure 3.5.10**. Advanced infectious stages, i.e. after the onset of clinical signs (150 dpi), and terminal scrapie infected animals showed high heterogeneity compared to the spectra of the other group, where virus and control, i.e. very young animals, separated from older controls and pre-clinical scrapie. This indicates that scrapie induced changes 120, 90 and 70 days after infection, i.e. before the onset of clinical signs, are smaller than age-related differences, since both control

and infected animals cluster together. However, the average spectrum of 120 dpi scrapie infected hamster clusters separately from its age-matched control. It was seen in the spectra (see **Figure 3.5.8**) that the very young animals in the region 1300 – 1000 cm^{-1} show similarities to terminal scrapie infected animals. This possibly suggests the existence of a "reverse" direction of spectral changes in late scrapie, i.e. due to neurodegeneration, while the direction from young to older animals could possibly be due to neurogeneration. Therefore, spectra from scrapie infected animals 120 dpi (150 days old) might have undergone changes in that way, making them appear more similar to 120 days old animals.

4.4 Alterations in the DMNV of reovirus infected hamster

Reoviruses of the type 3 clone 9 were shown to induce lethal meningoencephalitis in newborn but not adult mice (Morrison *et al.*, 1991; Mann *et al.*, 2002; Derrien & Fields, 1999; Clarke *et al.*, 2005; Kominsky *et al.*, 2002; Tyler *et al.*, 2001; Tyler *et al.*, 1996). Reasons for the observed age dependency have been proposed to be partly caused by differences in nervous tissue response to T3C9 replication between newborn and adult mice (Derrien & Fields, 1999). The experiments performed in this thesis prove, for the first time, that newborn Syrian hamsters are susceptible to reovirus T3C9 infection, in addition to other rodents like mice and rats, terminating in a lethal meningoencephalities. Reoviruses were shown to induce apoptosis both in vitro (Rodgers *et al.*, 1997) and in vivo (Tyler *et al.*, 1989), although with strain specific differences in their capacity (Tyler *et al.*, 1995). However, in contrast to scrapie induced apoptosis, the mitochondrial pathway in reovirus infected cells might only play a secondary role (Richardson-Burns *et al.*, 2002). One of the earliest events following T3 infection is the activation of nuclear transcription factors which seems to be required for the subsequent initiation of virus-induced apoptosis (O'Donnell *et al.*, 2005; Connolly *et al.*, 2000), which can be detected within hours after infection. The decrease in mitochondrial membrane potential and matrix condensation has been shown to be an early universal event of apoptosis (Mignotte & Vayssiere, 1998), the primary cause of which was demonstrated to be a decrease in glycolysis prior to commitment to apoptosis. Glucose degradation via glycolysis is a central part of the energy metabolism of living cells (Mair *et al.*, 2006). Glycolysis is the metabolic pathway by which a glucose molecule is oxidized to two molecules of pyruvic acid (plus two molecules ATP). Decrease in glycolysis therefore could lead to an increase in

glucose. The increased peak intensity at 1151 cm^{-1} (C-O stretching band of glycogen (Jackson *et al.*, 1998)) shown in **Figure 3.5.2** in the Reovirus infected animals compared to control could be due to increased glucose as a result of decreased glycolysis. Reovirus T3 infected newborn mice were shown to develop abnormalities in glucose homeostasis (Wetzel *et al.*, 2006). Furthermore, the ratio of the absorptions at 1151 and 1171 cm^{-1} (CO-O-C asymmetric stretching arising from ester bonds in cholesteryl esters and phospholipids (Jackson *et al.*, 1998)) in diabetic and infarcted rat heart has been shown to be reversed compared to control (Toyran *et al.*, 2006; Lui *et al.*, 1996). This is in compliance with the virus induced changes and although these are different types of tissue, the molecular alterations might be of similar nature.

Reovirus infection is associated with inhibition of DNA synthesis and cellular proliferation (Tyler *et al.*, 1996). Depending on the cells investigated and the experimental methods employed, reovirus has been reported to induce cell cycle arrest in both the G2/M phase (Poggioli *et al.*, 2002; Poggioli *et al.*, 2001; Poggioli *et al.*, 2000) and the G1 phase (Saragovi *et al.*, 1999). Arrest in G2/M phase is associated with hyper-phosphorylation (Poggioli *et al.*, 2001). The detected increase based on the calculation of integrated spectral ranges in the fingerprint region (compare 2.6.6, page 60) indicates alterations in the phosphate and carbohydrate levels of reovirus infected animals (see **Figure 3.4.5B**). In biological systems, phosphates are most commonly found in the form of adenosine phosphates, (AMP, ADP and ATP), in phospholipids and in the backbone of DNA and RNA. Furthermore, cellular protein synthesis decreases following infection with most strains of reovirus, a phenomenon referred to as host shutoff (Zweerink & Joklik, 1970; Schmechel *et al.*, 1997; Sharpe & Fields, 1982). Interestingly, the amount of total protein in the virus infected tissue is only slightly decreased. However, inner and outer capsid of the virion are composed of protein and if the virus replicates dramatically, one should expect an increase rather than a decrease. On the other hand, the induced inhibition of DNA synthesis together with protein degradation during apoptosis argues for strongly decreased protein content. Therefore, a strong decrease of proteins due to apoptosis together with an increase due to virion reproduction might total in a slight decrease as detected here.

In the lipid region, the intensity at 3013 cm^{-1} is increased in reovirus infected animals (see **Figure 3.4.4**). This peak has been assigned to the olefinic =C-H stretching band and can be used as an index of relative concentration of double bonds in the lipid

structure of unsaturated fatty acids (Takahashi *et al.*, 1991; Severcan *et al.*, 2005). These double bonds originate mainly from lipid peroxidative endproducts, such as malondialdehyde. Reoviridae are known to induce oxidative injury, a component of acute encephalitis, modulating the host cells permissiveness to viral replication (Valyi-Nagy & Dermody, 2005). An increase in the concentration of unsaturated fatty acids has also been reported in diabetic rat vein (Toyran *et al.*, 2006) and -liver microsomal membranes (Severcan *et al.*, 2005). These authors supported the hypothesis of an increased lipid peroxidation in diseased animals. Interestingly, reovirus has been shown to delay diabetes onset in neonatal nonobese mice (Wetzel *et al.*, 2006). Together with slightly decreased υ(P=O) of phospholipids at ~1240 cm^{-1} (compare **Figure 3.4.5B** and **3.5.2**), these results indicate that the fatty acyl moeities content did not change but phospholipids peroxidation occurred (Petibois & Deleris, 2004).

Comparing first derivative spectra of the DMNV in the region 1300 – 1000 cm^{-1} in control and reovirus infected animals (**Figure 3.5.1**), differences can be seen in asymmetric and symmetric P=O stretching vibrations of PO_2 groups of phospholipids and in the ribose and/or desoxyribose constitution of nucleic acids (1240 and 1080 cm^{-1}, respectively). The region between 1100 – 1000 cm^{-1} is also dominated by absorptions due to carbohydrates and could partly be explained by the increased glucose content. Furthermore, many membrane proteins are glycosylated and it is not possible to identify the causes for the detected differences in detail. Due to the integrative nature of information provided by FTIRM all molecular vibrations contribute to the complex spectral patterns detected.

At birth, the central nervous system of newborn hamsters, as well as for other rodents, is not completely developed yet. Histologically, orientation within the brain sections has been shown to be more difficult as is for adult animals. The structural and compositional differences between the distinct substructures are smaller than in older animals as can be seen by comparing average spectra of newborn DMNV to HypN on the one hand (see **Figure 3.4.2**, page 86) and of older DMNV and HypN on the other hand (see **Figure 3.2.2B**, page 73). Therefore, a correct classification of all spectra into either the cluster DMNV or HypN might not have always been accurate and could be a source of error for the misclassification of the two control animals into the group of virus infected animals in **Figure 3.4.3A**. However, cresyl violet stained sections of newborn hamster medulla oblongata (see **Figure 3.4.1A**, page 86) showed the same

localization of nuclei as was already demonstrated for older animals (Kneipp *et al.*, 2000). Variation in the average spectra of the DMNV was seen to be higher within the control animals. It has to be noted that all control animals as well as all animals in the virus group derived from the same dam. It is well known that animals of one litter are not equal to each other; some are bigger, stronger and more developed than others, which is reflected in different molecular compositions in the brain. The infection with a virus might lead to prominent biochemical changes "overlaying" the natural variation within the group of virus infected animals. These changes were shown to occur in the vibrational modes of nucleic acids, phospholipids and polysaccarides. The two misclassified control animals therefore show differences in these biomolecules compared to the remaining four controls as well.

4.5 Distribution and content of trace metals in scrapie

Several publications have reported on the involvement of trace metals in prion diseases. To understand this possible role, X-ray fluorescence (XRF) microprobe measurements were performed on the same ganglia that were studied with IR microspectroscopy. Due to the small number of individual samples analyzed here, the obtained results can only serve as preliminary observations. However, most of them are consistent with existing hypotheses about the role of trace elements in prion and other neurodegenerative diseases so that the potential and results of XRF microprobe in the investigation of prion diseases are discussed here.

Comparison of the single elemental concentrations determined from the coarse scans showed high variations between the animals and in some cases even between measurements of different regions of the same animal. Reasons for the high variability are discussed in 4.6.1.

4.5.1 Changes in the total metal content in DRG neurons during scrapie pathogenesis

As is shown in **Figure 3.6.1**, the elements copper, zinc, iron, calcium and manganese increase in scrapie infected animals (or stay constant while control decreased) at the terminal stage of the disease. However, since 2-3 scrapie infected hamster each time point are compared to only one control animal each, and it was seen that some animals exhibit overall lower concentrations of trace elements, like for example

H100.16 (terminal scrapie), it can not be determined how close the investigated control animals are for the category they represent. Nevertheless, the changes detected seem to exceed normal variation and might therefore be induced by the disease.

Copper and calcium levels were shown to be decreased at pre-clinical time points and then, while control animals decreased with age, stayed constant or slightly increased, leading to higher concentrations than in control (**Figure 3.6.1A** and **D**). In brains of scrapie infected mice, copper was shown to be significantly reduced before the onset of clinical signs, i.e. at 97 dpi (Thackray *et al.*, 2002a). Since terminal stage in that study was reached at 150 – 160 days, time points are to some degree comparable to the animals investigated here, where copper was also shown to slightly decrease at preclinical stages. However, as already mentioned, it can not be determined whether the decrease is due to the disease or based on individual differences. PrP is a copper binding protein (Brown *et al.*, 1997; Hornshaw *et al.*, 1995; Viles *et al.*, 1999), binding up to 4 atoms per molecule (Thackray *et al.*, 2002b), and it has been shown that copper binding can trigger a conformational change from α-helix to β-sheet in prion proteins, which leads to misfolding (Miura *et al.*, 1996; Stockel *et al.*, 1998). More recent evidence indicates that copper binds to the so-called octarepeat domain of N-terminal of PrP, with the normal prion protein possibly playing a significant role in the metabolism and transfer of copper and also participates in scavenging and detoxification of O_2^- (Burns *et al.*, 2003; Aronoff-Spencer *et al.*, 2000; Millhauser, 2004; Burns *et al.*, 2002; Brown *et al.*, 2001; Brown, 2003; Brown *et al.*, 1997; Cereghetti *et al.*, 2001). Binding of copper to the prion protein has been suggested to promote the formation of a defined structure by stabilizing the protein (McKenzie *et al.*, 1998). High intracellular levels of copper were also found in neurons of the brain from Parkinson's disease patients and may contribute to radical formation leading to oxidative damage, with disruption of lipid membrane, alteration of protein function or DNA damage that finally cause cell death (Linder, 2001). Additionally, the prion protein was discussed to play a role in copper homeostasis (Sakudo *et al.*, 2004), so changes in content or function of PrP^C might result in alterations of copper content and/or distribution as well. Finally, it was also shown that copper content varies in healthy brain of different ages (Brown, 2003), supporting the detected changes in copper content in the controls studied here.

The detected decrease in calcium at pre-clinical time points might possibly be due to its release from intracellular stores such as the mitochondria and the endoplasmic reticulum as an early apoptotic event or due to perturbations in the calcium channels

in the cell membrane (Wong *et al.*, 1996; Whatley *et al.*, 1995; Thellung *et al.*, 2000; Krebs *et al.*, 2007; Korte *et al.*, 2003; Kawahara *et al.*, 2000; Florio *et al.*, 1998; Florio *et al.*, 1996; Brini *et al.*, 2005) (**Figure 3.6.1D**). Moreover, the loss of PrP^{C}, as for example a result of its conversion to PrP^{Sc}, was shown to alter the intracellular calcium homeostasis of cultured cerebellar granule cells (Herms *et al.*, 2000). However, in that study, a decrease of free intracellular calcium was reported, the opposite of what was found in this study.

While control animals showed strongly decreased zinc levels with age, scrapie-infected animals at the terminal stage exhibited increased concentrations (**Figure 3.6.1B**). Zinc plays a crucial role in the metabolism of the brain and has been linked to other neurodegenerative disorders besides prion diseases (Brown & Harris, 2003; Jobling *et al.*, 2001; Watt & Hooper, 2003) such as Alzheimers disease (Cuajungco & Lees, 1997; Ide-Ektessabi & Rabionet, 2005; Strausak *et al.*, 2001), amyotrophic lateral sclerosis (Aoki *et al.*, 1994; Pamphlett & Kum-Jew, 2003; Valentine *et al.*, 1999) and Huntington disease (Albano *et al.*, 1984) as well as apoptosis (Levenson, 2005), where it was found to occur in abnormal levels (Leach *et al.*, 2006).

In this study, iron was found to slightly increase in terminally diseased scrapie infected animals (**Figure 3.6.1C**), as is supported by the literature reporting on the influence of iron content in scrapie infected neuroblastoma cells (Fernaeus *et al.*, 2005a; Fernaeus & Land, 2005; Fernaeus *et al.*, 2005b; Gudmundsdottir *et al.*, 2006; Kim *et al.*, 2000). It was noted in the literature that cyclic amplification of misfolded prion protein in normal hamster brain homogenates treated with manganese, copper and iron lead to conversion of PrP^{C} to PrP^{Sc}, indicating the involvement of these trace elements in scrapie disease (Kim *et al.*, 2005).

Although most of the elements were found to increase in neurodegenerative diseases and apoptosis, phosphorus levels increased in controls and stayed constant in infected animals, leading to lower concentrations in diseased hamsters at the terminal stage. This decrease could possibly be due to a decrease in nucleic acids or cell activity, as has been shown for apoptotic cells (Bushell *et al.*, 2004).

4.5.2 Subcellular distribution and content of Cu, Zn, Fe, Ca, P and Mn

The study of subcellular metal concentrations and distribution can give further insights than comparing ”bulk measurements” averaged over the whole tissue section

since it was demonstrated by cultured lymphoblasts, increased intracellular levels of zinc content was not due to excess zinc uptake from the medium in early apoptosis but rather to relocation of already existing amounts of zinc (Zalewski *et al.*, 1994).

Based on the distribution of copper in the control cell (**Figure 3.6.3A**), which showed the highest concentration of Cu in the cytoplasm and less in the nucleus and ecm, the numbers of cells exhibiting relatively high amounts of copper seemed to increased during scrapie pathogenesis. In mouse fibroblast cells, copper was shown to colocalize with the Golgi and mitochondria (Yang *et al.*, 2005), both organelles of the cytosol which explains its location in the cytoplasm. However, subcellular distribution of copper did not alter during pathogenesis.

Zinc was shown to be increased in the nucleus and cytoplasm of scrapie-infected neurons. The finding of highest concentration in the nucleus is consistent with reports from the literature (Chandler *et al.*, 1977a; Chandler *et al.*, 1977b). Zinc is involved in cell division (Chesters, 1978; Fujii, 1954; Prasad *et al.*, 1971) and protein synthesis (Prasad & Oberleas, 1970) and has been correlated to the conversion of the cellular into the misfolded form of the prion protein (Jobling *et al.*, 2001). Scrapie is a disease associated with alterations of cell division and protein synthesis, i.e. a shutoff of those events, alterations in zinc content seem to be likely. Since zinc is a component of the Cu-Zn superoxide dismutase, an important factor of antioxidant defenses (Bush, 2000), the observed increased level of Zn may indicate intensified reactions against superoxide radicals. This suggestion can be supported by the observed higher accumulation of also copper.

Iron levels were found to be increased, but no relocations in subcellular distribution could be detected. However, **Figure 3.6.3C** indicates slightly higher intracellular iron levels in infected neurons. Numerous studies have suggested that redox-active iron might play a role in neurodegenerative diseases such as Alzheimer's (Thompson *et al.*, 2001; Loske *et al.*, 2000; Huang *et al.*, 2004). Analytical techniques such as X-ray absorption near edge structure (XANES) could be used to adress possible differences in oxidation state of iron, which are not detectable with the method used here (Kwiatek *et al.*, 2001).

Highest concentrations of calcium were detected in the extracellular matrix, independent from the infectious status. At 70 dpi (see **Figure 3.6.2**), both intra- and extracellular calcium levels were found to be decreased compared to control and then increased during scrapie pathogenesis. Calcium plays a significant role in apoptosis (Pinton *et al.*, 2002; Rizzuto *et al.*, 2003; Demaurex & Distelhorst, 2003). Because of its toxicity, calcium levels must be kept at low levels and most of the cellular calcium is stored in the endoplasmic reticulum (Demaurex & Distelhorst, 2003). Released from the ER, it synchronizes the mass exodus of cytochrome *c* from the mitochondria (Hetz *et al.*, 2003; Mattson & Chan, 2003). Failure of the mechanisms implicated in the regulation of its intracellular concentration (Ca(2+)-channels, calcium binding proteins), leads to a cascade of events which causes cytotoxicity and neuronal death. Scrapie is known to induce apoptosis in neuronal tissue, which in turn, is characterized by the release of calcium from the endoplasmic reticulum into the cytosol as early and pivoting event (Beaver & Waring, 1994; Grebinyk *et al.*, 2004; Scoltock *et al.*, 2000; Yoshino *et al.*, 1996; Florio *et al.*, 1996; Florio *et al.*, 1998; Kawahara *et al.*, 2000; Krebs *et al.*, 2007; Kristensson *et al.*, 1993; Whatley *et al.*, 1995). In scrapie infected cells exhibiting relatively low amounts of β-sheet (as was seen for the terminal infected animal H100.11), increased levels of calcium in the cytoplasm together with the low amount of β-sheet could argue for an earlier stage in the disease process for that specific animal, i.e. an early apoptotic level. The other two terminally infected animals (H100.14 and H100.16) exhibited high amounts of β-sheet (not shown), arguing for late stages of the disease together with low levels of calcium, due to their late apoptotic stage.

Decreased phosphorus levels, as seen in the scrapie-infected cell shown in **Figure 3.6.4E** could indicate a different metabolic state of the affected neuron. Finally, scrapie infected animals exhibited gradually increased levels of manganese. On a subcellular level, the increase was mainly detected in strongly elevated manganese levels in the cytoplasm of infected neurons. Its involvement in prion diseases has been demonstrated in numerous studies (Brown *et al.*, 2000; Gaggelli *et al.*, 2005; Levin *et al.*, 2005; Treiber *et al.*, 2006). Manganese has been shown to exhibit increased levels in 263K hamster brain (Kim *et al.*, 2005). Other studies have proposed that Mn2+ replace Cu2+, initializing the pathological transformation of the prion protein (Purdey, 1996b; Purdey, 1996a). Although concentrations of manganese are generally very low, the technique used in this thesis provided sufficient sensitivity to detect even

low levels of this element. In this pilot study of orally 263K infected hamster DRG discussed here, manganese levels were found to be (strongly) increased in terminally diseased animals compared to control, supporting the existent hypothesis of an influence of manganese in prion diseases.

4.6 Limitations and Possible error sources

Although XRF microprobe and FTIR microspectroscopy have been shown to be powerful tools for studying the subcellular distribution and concentration of trace elements and the biomolecular profiling of cells, possible sources of error exist and need to be considered.

4.6.1 Experimental design

Possible sources of error can originate from the chosen experimental design. Firstly, differences in all measured parameters could be related to the investigated gender. Male and female individuals can differ in their abilities of metal uptake and storage (Bristow-Craig *et al.*, 1994) as well as for example in lipid metabolism, as was shown in Syrian hamster (Morise *et al.*, 2006). Indeed, CH metabolism was more affected by gender than by diet (Morise *et al.*, 2006). The investigated animals in this thesis were both males and females which could cause some of the differences in the studied trace elements and biomolecules. Physiological levels of trace metals and physiologically relevant macromolecules are also individually different. “Normal” levels are therefore given in ranges rather than fixed numbers. Not only differences between animals of the same group were seen for all the studied aspects but also in different cells of the same animal. The copper level, for example, was found to be different in two neighboring cells of the control animal (compare **Figure 3.6.3A**), and also e.g. the protein and lipid composition was shown not to be distributed equally in the tissue. Additionally, for the time points fcs and terminal, animals were not exactly the same age. For example, the terminal control animal (H100.32) was 236 days old, the same age than S14 (H100.14), whereas S11 was 225 days and S16 only 201 days old. This makes a difference of 35 days between S16 and S14. Due to the exponential increase in misfolded prion protein and other molecules and the increase in metal content in most of the elements from fcs to terminal (**Figure 3.6.1**), these “missing” 35 days could partly be the reason for differences in animals at fcs and the terminal stage. Furthermore, the thickness of the tissue sections also influences the detected

variations: the thicker the sample, the higher is the detected concentration /absorbance at a given wavenumber and vice versa. Since modern cryotomes are very accurate, differences based on different thicknesses are considered to be negligible. For XRF microprobe, normalization to the Si peak and for FTIR experiments, calculating ratios rather than single peak intensities can compensate this effect. The low number of investigated animals, partly due to limited assigned beamtime, is another possible source of error, pointing out the necessity of either larger sample numbers and/or faster data acquisition in future experiments. Although current synchrotron FTIRMS provides the highest possible spatial resolution, i.e. only diffraction limited, because of the single element detector, data collection is very time consuming. By coupling an FPA detection system to a synchrotron ring, data acquisition can be accelerated. Furthermore, mathematical correction for diffraction is possible by deconvolution methods, possibly extending the resolution limit down to about 1 μm (Miller & Dumas, 20006).

4.6.2 Analysis

In the longitudinal FTIRM study on DRG it was seen that not only in infected but also in control cells, areas where the tissue was ruptured, as frequently observed during sample preparation at the cells membrane, exhibited spectra with occasionally sloped baselines and downshifted amide I bands. Those spectra were not detected on intact tissue and can not be the reason for the appearance of misfolded prion protein signals quite simply because of its non-presence in control samples. Therefore, the detection of increased β-sheet at the cell membrane could be caused by other effects than the accumulation of misfolded prion protein. A look at the corresponding high resolution photomicrograph (not shown) revealed that the tissue is often broken at those locations leaving a gap which might have caused the so-called dispersion artifact in the spectra. This effect occurs at the edges of cells and tissue in high numerical aperture measurements, as has recently been reported by Diem and colleagues, not only in spectra recorded in reflection mode (Mohlenhoff *et al.*, 2005). Since a large number of cells has been measured by FTIRM per animal, it was assumed that this "measurement artefacts" affected control and scrapie infected animals to an equal extent, the detected alterations in protein secondary structure are considered to be caused by the disease.

Finally, analysis of the biochemical composition of the tissue samples, as performed according to the description in 2.6.1 – 2.6.7, does not provide exact quantitative results and allows only estimations about e.g. the lipid composition or the total protein content.