

## 2. MATERIALS AND METHODS

### 2.1 Scrapie

#### 2.1.1 TSE strains and animal infection

In this thesis, the TSE strains 263K and ME7 were used. 263K was originally provided by Dr. R. H. Kimberlin but has been serially passaged for more than 20 years in the Robert Koch-Institute. Here, passaging was performed by intracerebral (i.c.) infection of outbred Syrian hamsters with 50 µl aliquots of 1% (w/v) hamster brain homogenates from terminally ill donors in TBS (10 mM Tris-HCl (Sigma), 133 mM NaCl (Merck), pH 7.4). For 263K, hamsters at an age of approximately 8 weeks were fed orally with 1-3 x 10<sup>7</sup> 50% intracerebral lethal doses of scrapie strain 263K as described elsewhere (Baldauf *et al.*, 1997). In short, frozen brain homogenate (10% homogenate in PBS) of either terminally ill (S-homogenate) or healthy animals (N-homogenate) was defrosted and homogenized using an ultrasonic device. Regular food pellets were soaked up with either 100 µl S-homogenate (25 pellets) or 100 µl N-homogenate (15 pellets) and stored until use at -70°C. One day after food deprivation the animals received the prepared pellets. Symptoms of successful infection are most commonly a hyper sensitivity to touch and noise, where the animals often twitch, and difficulties in maintaining balance and rising from a supine position. At the terminal stage, animals show head bobbing, ataxia of gait, generalized tremor and inability to eat and drink. The mean incubation time for 263K scrapie was about 170 days (up to 200 dpi) until the animals reached the terminal stage of the disease.

The other strain used in this thesis, scrapie ME7, was kindly provided by the Institute for Animal Health, Edingburgh, UK. It was generated by Kimberlin after four passages in hamsters (Kimberlin *et al.*, 1989). Unlike the 263K agent, ME7 infected animals were not obviously irritated by touch or noise, frequently and persistently resting and well able to slowly rise from a supine position until very advanced stages of the disease. They showed signs of hind limb paralysis but no tremor, head bobbing or ataxia can be observed. In contrast to 263K, ME7 infected hamster exhibited phlegmatic sluggishness with bradykinesia and kyphosis. Infection was performed i.c. with 50 µl aliquots of 1% (w/v) hamster-brain homogenates in TBS (10 mM Tris-HCl, 133 mM NaCl, pH 7.4) from terminally ill donors (Kimberlin & Walker, 1977).

## 2. Materials and Methods

Incubation times were about 170 - 200 days for hamster scrapie strain 263K and 330 days for ME7. Mock-infected hamsters of the same age were similarly fed or i.c. infected. All animals were regularly observed for clinical symptoms and sacrificed by CO<sub>2</sub> euthanasia at different time points of the disease. To remove the blood of the vessels, the animals were perfused with 30 ml Ringer solution. Brains and spinal cord with attaching dorsal root ganglia were removed, immediately frozen in liquid nitrogen and stored at -70°C.

**Table 2.1:** List of official name, acronym, strain, time point and tissue of the animals used in this thesis

<b>Animal</b>	<b>Acronym</b>	<b>strain</b>	<b>time point</b>	<b>tissue</b>
H100.01 – H100.05	S1 – S5	263K	70 dpi	DMNV
H100.06 – H100.10	S6 – S10	263K	100 dpi	DRG
H100.11, H100.13 – H100.16	S11, S13 – S16	263K	terminal	DRG
H100.17 – H100.20	S17 – S20	263K	fcs	DRG
H100.21 – H100.23	N21- N23	mock	70 dpi	DMNV
H100.24 – H100.26	N24 – N26	mock	100 dpi	DRG
H100.27 – H100.29	N27 – N29	mock	fcs	DRG
H100.30 – H100.32	N30 – N32	mock	terminal	DRG
H100.33 – H100.37	S33 – S37	263K	130 dpi	DRG
H100.38 – H100.40	N38 – N40	mock	130 dpi	DRG
H47.1 and H47.4	-	263K	terminal	DRG
H47.7	-	mock	terminal	DRG
H30.10 and H31.11	-	263K	terminal	DRG
H585.26 and H585.58	585.26 and 585.58	ME7	terminal	DRG
primary name unknown	S70.1 – S70.16	263K	70 dpi	DMNV
primary name unknown	N70.1 – N70.8	mock	70 dpi	DMNV

### 2.1.2 Sample preparation

Using a cryotom (Microm, Germany), sequential sections of each ganglion and brain (10 µm thick) were cut at a temperature of -20°C and mounted on calcium fluoride windows (CaF<sub>2</sub>, 1 mm in thickness) (Korth Kristalle GmbH, Altenholz, Germany), an

IR compatible substrate, and Cr patterned glass slides for x-ray fluorescence microprobe. In addition, adjacent sections were cut and mounted to standard microscope slides for histological immunostaining.

The order of sections was as follows:

1. 12  $\mu\text{m}$  on  $\text{CaF}_2$  for FTIRMS
2. 30  $\mu\text{m}$  on Cr patterned glass slides for XRF microprobe (if applicable)
3. 10  $\mu\text{m}$  on special glass slides for 3F4 immunostaining (if applicable)
4. 10  $\mu\text{m}$  on special glass slides for immunostaining using NMS (if applicable)
5. 10  $\mu\text{m}$  on regular glass slides for cresyl violet or hematoxylin/eosin staining

The  $\text{CaF}_2$  windows were kept in a dry and dark environment until the FTIR microspectroscopic measurements were carried out.

For the XRF Microprobe experiments discussed in this thesis, 20  $\mu\text{m}$  thick sections of thoracic hamster dorsal root ganglia orally infected with the 263K scrapie strain and mock-infected control sections were prepared and mounted on 500 nm thin SiN windows.

## 2.2 Reovirus

### 2.2.1 Reovirus T3C9

A test culture of Reovirus type 3 clone 9 (T3C9) was a kindly gift of Dr. Bernard Fields and Denise Wetzel from the Department of Pediatrics, Vanderbilt Medical School, Nashville, Tennessee 37232.

### 2.2.2 Mouse fibroblasts L929

Mouse fibroblasts L929 were a kindly gift of Gudrun Klein from the Robert Koch-Institute in Berlin, Germany. The cells were kept at a constant level of  $3 \times 10^6$  cells per bottle by performing the following procedure every other day:

- Decant the medium and add 3 ml of EDTA
- Agitate and remove EDTA until 0.5 ml are left in the bottle
- Incubate at 37 °C for 5 minutes

- Add 10 ml medium, agitate and transfer to a falcon tube
- Count cells and calculate the amount of suspension needed to obtain  $3 \times 10^6$  cells
- Transfer calculated amount to a fresh bottle and add fresh medium until reaching a total of 10 ml each bottle.

### 2.2.3 Medium

The medium used for cell cultures is composed of:

500 ml RPMI 1640 (standard cell culture medium, containing inorganic salts, amino acids, vitamins, glucose and other. This medium contains a great deal of phosphate and is formulated for 5% CO<sub>2</sub>)

+ 50 ml fetal calf serum

+ 2.5 ml penicillin

+ 2.5 ml streptomycin

+ 5 ml pyrovate

+ 1 ml mercaptoethanol

### 2.2.4 Cultivation of Reovirus T3C9

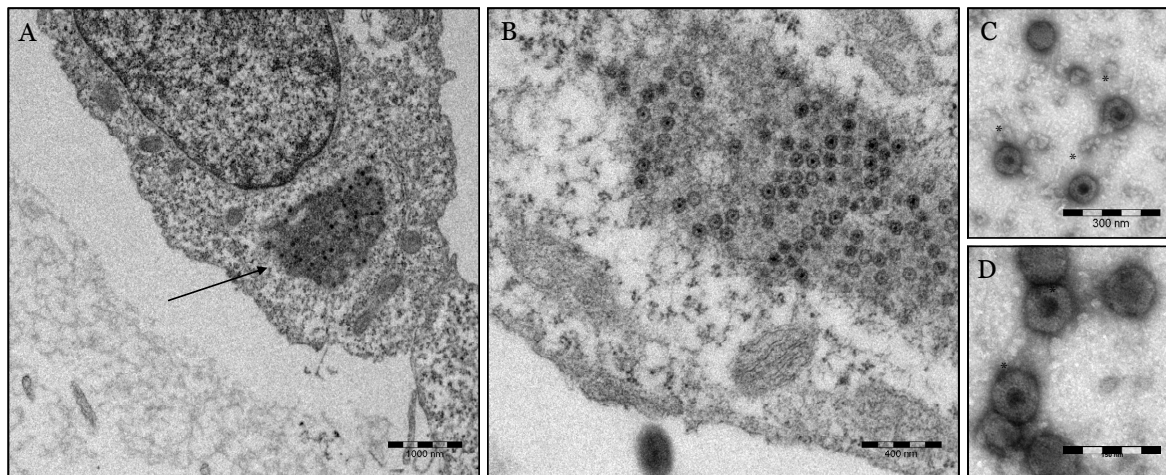
On the first day (day 1),  $1-1.5 \times 10^7$  cells L929 were disseminated in 175 cm<sup>2</sup> culture flasks. The next day (day 2), the supernatant was removed and the fibroblasts were covered with 1 ml of virus suspension and 9 ml medium. Cells were incubated for 1 hour at 37°C and 5% CO<sub>2</sub>. Afterwards, the supernatant was replaced by 30 ml fresh medium. The fibroblasts were then frequently checked for the appearance of the cytopathic effect (CPE). A CPE indicates a succesfull replication of the virus in the host cell that often changes its morphological appearance. Two days after infection, the fibroblasts showed signs of apoptosis, induced by the virus. Therefore, four days after infection, cells were removed from the bottle and centrifuged for 10 min at 2000 rpm. The supernatant was removed but not discarded, the pellet frozen at -75°C and subsequently thawed. Both were then mixed again, vortexed and centrifuged for 10 min at 2000 rpm. 1 ml aliquots of the supernatant were stored at -75°C.

Reovirus T3C9 was plaque purified and passaged in L cells. 200 µl ( $1.2 - 1.5 \times 10^6$  cells/ml) L929 cell suspension was applied onto a 24-wellplate and mixed with 200 µl of 1:10 – 1:10<sup>6</sup> virus dilution each. After incubating for 4-5 hours at 37°C 400 µl 1.6%

CMC was added. The micro dilution plate was then cultivated for 3-4 days at 37°C and 5% CO<sub>2</sub> prior to fixation in 4% formalin and staining with 0.1% Naphthalene black to count plaques for determining the amount of PFU/ml.

### 2.2.5 Transmission Electron Microscopy

To prove successful infection of L929 mouse fibroblasts with Reovirus T3C9, transmission electron microscopy (TEM) was performed. 7 ml of a 30% sucrose solution were covered with 30 ml of virus supernatant and centrifuged for 3 hours at 28000 rpm at 4°C. The pellet was resuspended in 1 ml medium and analyzed by TEM. Additionally, the sucrose solution was studied by TEM as well. TEM images of successful transfection are shown in **Figure 2.2.5**. The photomicrographs were taken at the Robert Koch-Institut in Berlin, Germany and were provided by courtesy of Dr. M. Özel (RKI, Berlin).



**Figure 2.2.5:** TEM photomicrographs of Reovirus T3C9 infected L929 mouse fibroblasts and sucrose gradients. A. Virus particles accumulate in an unidentified compartment of the fibroblast (arrow). B. Close up of the virus filled compartment. C and D. Virus particles in sucrose gradient. Ready assembled particles are marked by an asterisk. By courtesy of Dr. M. Özel (RKI, Berlin, Germany)

### 2.2.6 Animal infection

The animal experiments were carried out at the Bundesinstitut für Risikobewertung (BfR) in Berlin, Germany. After light sedation with Isofluran<sup>®</sup>, 10 one-day-old Syrian hamsters (*Mesocricetus auratus*) were infected perorally using a rounded syringe to avoid injuries in the rodent esophagus by inoculating 30 µl medium or medium containing 4 x 10<sup>10</sup> PFU/ml Reovirus T3C9, respectively. As control, additional 8

animals had been inoculated with medium only. All pups belonging to either the virus or the control group derived from the same dam, respectively. After inoculation the animals were given back to their mothers. At 1 dpi one animal each side was missing, probably killed and eaten by their mothers. 6 days post infection, one of the remaining 9 infected animals showed symptoms of a reovirus induced encephalitis and got sacrificed together with one control animal using an overdose of Isofluran®. Brains were taken and immediately frozen in liquid nitrogen. These brains were used to demonstrate successful infection by staining for activated astrocytes and microglia. At 9 days post infection the remaining 8 infected animals showed symptoms of encephalitis and were euthanized with the remaining 6 control animals as described above.

### **2.2.7 Sample preparation**

Using a cryotom (Microm, Germany), sequential sections of each brain (12 µm thick) were cut at a temperature of -20°C and mounted on calcium fluoride windows (CaF<sub>2</sub>, 1 mm in thickness) (Korth Kristalle GmbH, Altenholz, Germany), an IR compatible substrate. In addition, adjacent sections were cut and mounted to standard microscope slides for histological immunostaining. Brains from pups who were killed 7 days post infection were sliced and mounted on regular glass slides for GFAP and F4/80 staining to prove successful infection. The CaF<sub>2</sub> windows were kept in a dry and dark environment until the FTIR microspectroscopic measurements were carried out.

## **2.3 Histology**

### **2.3.1 Hematoxylin-Eosin staining**

The Hematoxylin-Eosin stain is the most common histological staining. Hematoxylin is a dark purplish dye that will stain the chromatin within the nucleus, leaving it a deep purplish-blue color. Eosin is an orange-pinkish to red dye that stains the cytoplasmic material including connective tissue and collagen, and leaves an orange-pink counterstain. This counterstain acts as a sharp contrast to the purplish-blue nuclear stain and helps identify other entities in the tissues such as cell membranes. The following procedure describes the regressive staining variant.

10 min	Hematoxylin
15 min	wash and blue in tap water differentiate in acetic ethanol (70%) wash out and blue in tap water
4 min	Eosin wash in aqua dest. 70% ethyl alcohol 95% ethyl alcohol 100% ethyl alcohol Xylene I, Xylene II, D.P.X (Sigma-Aldrich) for coverslip

### **2.3.2 Cresyl violet staining**

Cresyl violet stain is used to demonstrate Nissl substance in the tissue. Nissl substance, primarily composed of rough endoplasmic reticulum is present in neurons and gets lost after cell injury and if the axon degenerates. Cresyl violet stains the Nissl substance dark blue to purple.

10 min	Cresyl violet (freshly filtered) rinse in tap water
10 min	blue in tap water ascending alcohol (70%, 95%, absolute ethyl alcohol) Xylene I, Xylene II D.P.X (Sigma-Aldrich) for coverslip

### **2.3.3 GFAP - Staining for Astrocytes**

Astrocytes, also known as astroglia, are characteristic star-shaped glial cells in the brain, expressing glial fibrillary acidic protein (GFAP). Since inflammation up-regulates GFAP, staining for astrocytes can be used as a marker for scrapie or reovirus infection. The reaction product is brown in color and is present in the cytoplasm. To determine astrocytosis, the 6 dpi brain of both an infected and a control animal was divided into halves along the midsagittal line. One half was placed in PLP-fixative (for 100 ml: 0.216 g 0.1M Sodium m-periodate + 1.37 0.075M D L-lysine mono-hydrochloride + 75 ml 0.05 M phosphate buffer (60.75 ml 0.1M Disodium-

## 2. Materials and Methods

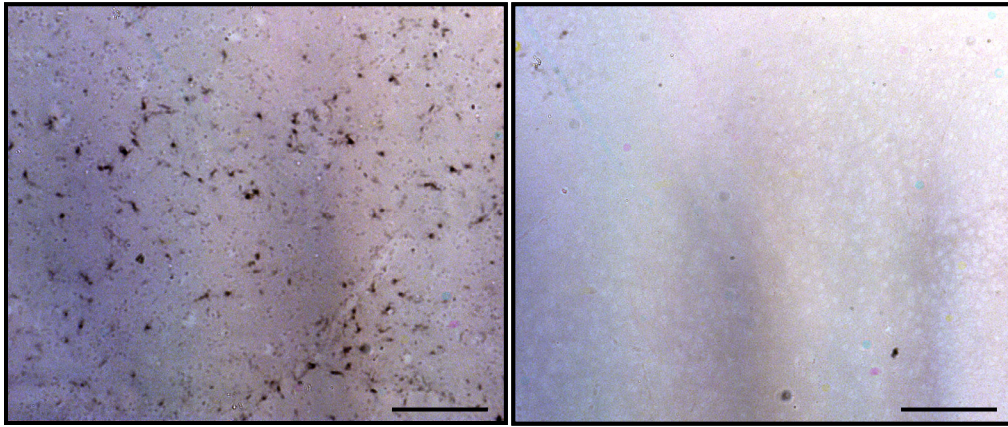
---

hydrogenphosphate + 14.25 ml 0.1M Sodiumdihydrogenphosphate) + 25 ml 8% paraformaldehyde), the other half was frozen in liquid nitrogen. After cutting the brain in 12 µm thick sections the following staining procedure was performed:

Overnight	dry samples at 37°C
4x5 min	rehydrate in Xylene I, Xylene II, Acetone I and Acetone II
2x5 min	wash in PBS
20 min	incubate in 1% H <sub>2</sub> O <sub>2</sub> /Methanol to destroy endogenous peroxidase activity
5 min	wash in PBS
30 min	incubation in 10% fetal calf serum
5 min	wash in PBS
60 min	incubation in 1.AB solution (GFAP 1:1000 in 10% FCS in PBS with 0.2% Triton-X-100)
5 min	wash in PBS
60 min	incubation in 2.AB solution (biotinylated goat anti-mouse IgG, 1:200 in PBS with 1% BSA and 0.2% Triton-X-100)
5 min	wash in PBS
30 min	incubation in Vectastain ABC complex (Vector Lab's, USA)
5 min	wash in PBS
10 min	wash in pre-incubation solution (50 µl 1M Tris solution + 10 µl 1M Imidazol + 10 µl 50 mg/ml DAB solution + 950 µm aqua dest)
3 min	wash in incubation solution (per ml pre-incubation solution + 50 µl H <sub>2</sub> O <sub>2</sub> , 30%, 1:50 in 1000 µl a. bidest)
1 min	wash in A. Bidest
	alcohol (70%, 95%, 100% Ethanol, Ethanol-Xylene, Xylene)
	coverslip

Astrocytosis could be detected in the reovirus infected but not in the control animal (see **Figure 2.3.3**).





**Figure 2.3.3:** GFAP staining of a Reovirus T3C9 (A) and a control hamster (B) 6 days post infection. Astrocytosis can be seen in the reovirus challenged animal as a result of immune response to a successful infection. Scale bars: 100  $\mu\text{m}$

### 2.3.4 F4/80 - Staining for Microglia

Along with astrocytes and oligodendrocytes, microglia, the third type of glial cells, form the macroglia. Since F4/80 intensively stains activated microglia, this staining procedure can be used to detect neuroinflammation.

5 min	Fixation in Ethanol (50%)
5 min	Fixation in Ethanol (100%)
5 min	Fixation in Ethanol (50%) rehydrate in aqua dest.
20 min	block with 1% H <sub>2</sub> O <sub>2</sub> /Methanol
5 min	wash in A. dest
30 min	block with 0,2% I Block-Casein
over night	1. AK F4/80 1:50 in 0,2% I Block-Casein and NMS 1:500, respectively
5 min	wash in 1x PBS
1 h	2. AK 1:100 Goat-Anti-Rat in 0,2% I Block-Casein
5 min	wash in PBS
30 min	Vectastain ABC-Kit 1:100 in 0,2% BSA/PBS
10 min	preincubation solution
3 min	incubations solution
	blue in tapwater
	Alcohol (70%, 95%, 100%)
	Xylol I, Xylol II
	coverslip

### solutions

#### *1% H<sub>2</sub>O<sub>2</sub>/Methanol*

3 ml 30%iges H<sub>2</sub>O<sub>2</sub> + 97 ml Methanol (100%)

#### *0,2% I Block-Casein*

0,2 g I Block-Casein + 100 ml PBS (1x)

#### *preincubation solution + incubation solution*

for 1 ml:

50 µl Tris (1M, pH 7.6)

10 µl Imidazol (1M, pH 7.6 in a. bidest)

10 µl DAB (50 mg/ml in a. bidest)

930 µl a. bidest

add 50 µl H<sub>2</sub>O<sub>2</sub> (0.3%) per ml incubation solution

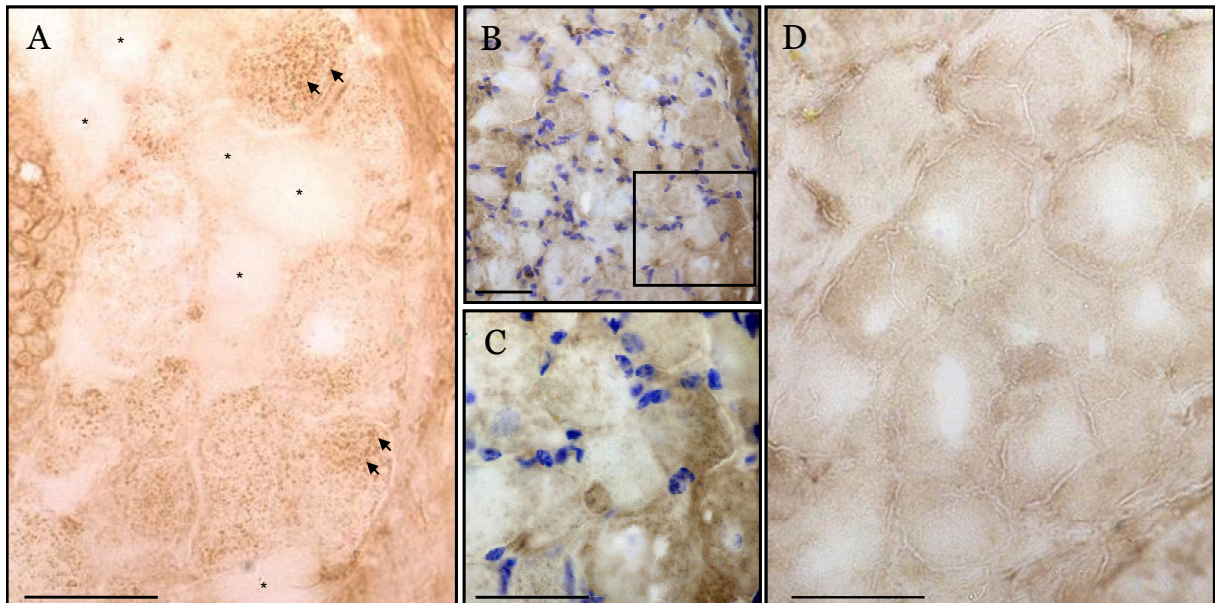
### **2.3.5 Immunohistochemistry**

To determine deposition of PrP<sup>Sc</sup>, the monoclonal antibody 3F4 was used on adjacent slides. Since 3F4 is not PrP specific, it attaches to both PrP<sup>Sc</sup> and PrP<sup>C</sup>, leading to an overall brown color of the tissue section. PrP<sup>Sc</sup> can be distinguished from PrP<sup>C</sup> by its granular deposition (see **Figure 2.3.5**).

5 min	Fixation in 50% Ethanol
5 min	Fixation in 100% Ethanol
5 min	Fixation in 50% Ethanol
	Rehydratisation in dest. water
15 min	10% phosphatbuffered Formalin
	Rinse in PBS
5 min	100 mM NH <sub>4</sub> Cl
	Rinse in PBS
5 min	0.5% Igepal in PBS (pH 7.8)
	Rinse in PBS
10 min	Denaturation in 3M Guanidiniumthiozyanat / 10mM TrisHCl (pH 7.8)
	Rinse in PBS

## 2. Materials and Methods

Over night	Incubation in 10% fetal calf serum in TBST / 0.2% Tween20 at 4°C
	Rinse in PBS
1h 45min	Incubation with Antibody 3F4 (1:100) respectively incubation of the controls with normal mouse serum (Dako) (1:1000) in PBS / 1% bovine serum albumin (BSA) at 37°C
3 x 5 min	wash the slices in PBS / 0.2% BSA
1h	Incubation with biotinylated secondary antibody Goat-anti-Mouse (Dako E 0433) (1:200) in PBS / 1% BSA at room temperature
3 x 5 min	wash the slices in PBS / 0.2% BSA
30 min	Incubation in Vectastain ABC-Kit at room temperature
2 x 5 min	wash the slices in PBS / 1% BSA
10 min	Incubation in 3,3'-Diaminobenzidine (DAB) preincubation solution
3 min	Incubation in 3,3'-Diaminobenzidine (DAB) incubation solution
	wash in dest. water
	alcohol (70%, 95%, 100% Ethanol, Ethanol-Xylol, Xylol)
	coverslip



**Figure 2.3.5:** Sections of dorsal root ganglia from a 263K infected (A), an ME7 infected (B and C) and a control (D) animal stained with the PrP specific antibody 3F4. A. Arrows indicate areas with a high PrP<sup>Sc</sup> deposition. The pathological prion protein can be detected as dark brown dots in most of the cells but not all (see Asterisk marked cells). B and C. 3F4 stained and hematoxylin counterstained section of a thoracic DRG of an ME7 infected terminal diseased hamster. 10x (B) and 40x (C). The rectangular in the left image indicates the area shown in the right in higher magnification. Since 3F4 stains both PrP<sup>Sc</sup> and PrP<sup>C</sup>, D shows a 3F4 stained control slide as an example of the PrP<sup>C</sup> distribution (brown color). (scale bars, 50 µm).

### 2.3.6 Western Blot

Blotanalysis was performed on cervical and thoracic dorsal root ganglia of one 263K infected, one ME7 infected, one BSE-H infected and one control animal to examine the total amount of PrP<sup>Sc</sup> as a function of strain as well as origin (cervical vs. thoracic).

#### *Preparation of blot samples:*

- wash DRG in TBS (3 x each 500 µl)
- digest samples in 100 and 200 µl TBS (+ 25 mg CaCl<sub>2</sub>/100 ml), respectively, + 0.25% collagenase for 90 min at 37°C using a shaker
- heat up to 80°C for 10 min
- treatment with proteinase K: 0.25 µg PK/100 µl [stock solution 1 mg/ml >>> 2.5 µl of a 1:10-dilution]; incubate for 60 min at 37°C
- heat up to 80°C for 10 min
- centrifuge for 40 min at 20.000 rpm and 4°C
- discard supernatant and resuspend pellets in 20 µl 2x LPP
- boil for 5 min
- apply samples on gel

#### *Detection with mAb 3F4 and CDP-Star w/o Nitro Block on PVDF foil:*

- Blot on a 0.45 µm PVDF membrane
- Shake the PVDF membrane for 30 min in 3% (w/v) skim milk powder/TBST (TBS with 0.05% (w/v) Tween20) at room temperature (50 ml each membrane)
- Overnight (~16h) incubation with the monoclonal primary antibody 3F4 (1:2000 in 3% (w/v) skim milk powder TBST) at 4°C (20 ml each membrane)
- Shortly rinse 3x in TBST
- Rinse in 3% (w/v) skim milk powder 3 x 5 min
- Incubation with secondary antibody (AP conjugated anti mouse IgG from goat [Dako, Hamburg, Germany], 1:5000 in 3% (w/v) skim milk powder/TBST for 90 min at room temperature (20 ml each membrane)
- Shortly rinse 3x in TBST
- Shortly rinse 2x in 3% (w/v) skim milk powder/TBST
- Wash in 3% (w/v) skim milk powder/TBST for 1h
- Wash in assay buffer (I) for 2 x 5 min

## 2. Materials and Methods

---

- Remove excessive buffer from membrane by soaking up with filter paper
- Apply 5 ml CDP-Star (diluted 1:50 in assay buffer) to the membrane and incubate for 5 min
- Remove CDP solution with filter paper
- Develop membrane in cassette by letting rest for 1 hour and subsequent exposition for 10 min (film: Kodak XAR-2)
- Result:  $0.6 \times 10^{-7}$  HÄ/10 µl clearly visible

### *Protocol Western-Blot 06.08.03:*

Control ganglia, 263 K ganglia (oral terminal), ME7 ganglia and BSE ganglia (each i. c. terminal) were prepared according to the protocol „E001“ and subsequently blotted. The development of the blot was carried out according to the protocol „CDP-Star ohne Nitroblock 3% Mamipu“.

### *Production of blot samples:*

- Number and weigh samples (04.08.03)

Nr.	Animal	Weight before process	notes
1	N	2.2 mg	N ganglion spiked w. $10^{-5}$ HÄ
2	N	2.1 mg	N ganglion
3	H 31/11 (C 5-8)	2.2 mg	263 K ganglion (cervical)
4	H 31/11 (T 3)	1.1 mg	263 K ganglion (thoracic)
5	585/58 (C 1-4)	1.2 mg	ME7 ganglion (cervical)
6	585/58 (T 5)	0.9 mg	ME7 ganglion (thoracic)
7	590/17 (C 5-8)	1.6 mg	BSE ganglion (cervical)
8	590/14 (T 2)	0.7 mg	BSE ganglion (thoracic)

- Wash samples in 1xTBS (3x each 500 µl)
- Spike the control sample with 10 µl  $10^{-5}$  HÄ (brain equivalent)
- Digest in 500 µl TBS [+ 25 mg  $\text{CaCl}_2$ /100 ml] + 0.25% collagenase for 60 min at 37°C in the shaker; vortex every once in a while (0.25% collagenase: 100 µl collagenase stock solution + 3.9 ml TBS/ $\text{CaCl}_2$ )
- Treat with ultrasound for 10 min at 80°C
- Centrifuge for 3 min at 5000 rpm at 4°C
- Transfer the supernatant into a new vial and discard the pellet

## 2. Materials and Methods

---

- Centrifuge the supernatant for 3 min at 5000 rpm and 4°C
- Transfer the supernatant into a new vial and discard the pellet
- Centrifuge the supernatant for 40 min at 20.000 rpm and 4°C
- Resuspend pellet in 1 ml sarcosyl TBS using ultrasound
- Centrifuge for 2.5 h at 45.000 rpm and 4°C
- Discard supernatant and freeze pellets at -70°C
- Next day, resuspend pellet in 1 ml 0.1% sarcosyl-10% NaCl-TBS with ultrasound
- Add 2.5 µl 1 mg/ml proteinase K
- Centrifuge for 2.5 h at 45.000 rpm and 4°C
- Discard supernatant and resuspend pellet in 20 µl 2x LPP
- Boil for 5 min at 100°C
- Freeze at -20°C

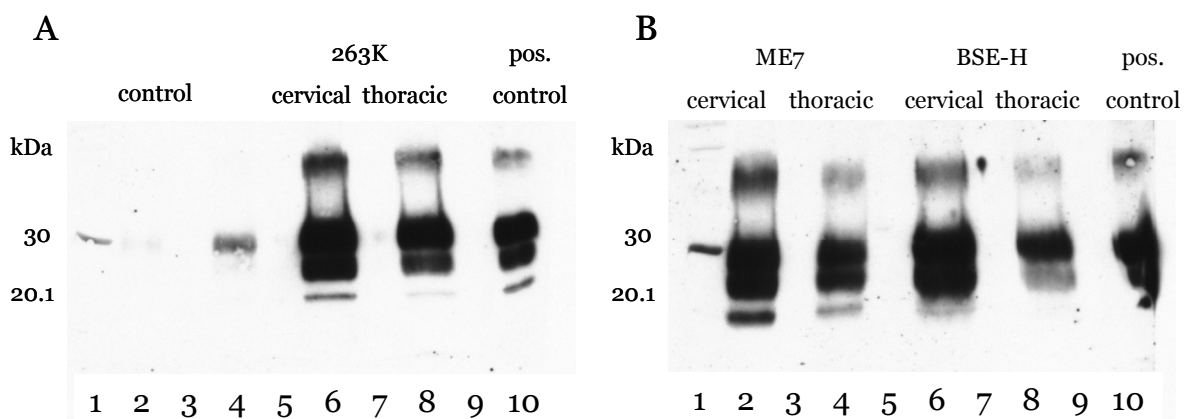
### *Western-Blot (12.5 %)*

<b>Blot Nr.</b>	58 a (A)
<b>Date</b>	06.08.2003
<b>Prim.- AK</b>	3F4 (1:2000); O/N 4°C
<b>Sek.-AK</b>	goat anti-mouse AP (1:5000); 90 min RT
<b>Blocking</b>	3% Mamipu-TBST
<b>Detection</b>	CDP-Star
<b>Exposition</b>	a = 30 min
<b>Lane 1</b>	Pharmacia-Marker [10 µl]
<b>Lane 2</b>	N ganglion [20 µl]
<b>Lane 3</b>	2x LPP [10 µl]
<b>Lane 4</b>	N ganglion spiked with 10 <sup>-5</sup> HÄ [20 µl]
<b>Lane 5</b>	2x LPP [10 µl]
<b>Lane 6</b>	263 K ganglion (cervical) [20 µl]
<b>Lane 7</b>	2x LPP [10 µl]
<b>Lane 8</b>	263 K ganglion (thoracic) [20 µl]
<b>Lane 9</b>	2x LPP [10 µl]
<b>Lane 10</b>	10 <sup>-6</sup> HÄ positive control (brain homogenate) [10 µl]

## 2. Materials and Methods

### Western-Blot (12.5 %)

<b>Blot-Nr.</b>	58 a (B)
<b>Datum</b>	06.08.2003
<b>Prim.- AK</b>	3F4 (1:2000); O/N 4°C
<b>Sek.-AK</b>	goat anti-mouse AP (1:5000); 90 min RT
<b>Blocking</b>	3% Mamipu-TBST
<b>Detektion</b>	CDP-Star
<b>Exposition</b>	a = 30 min
<b>Lane 1</b>	Pharmacia marker [10 µl]
<b>Lane 2</b>	ME7 ganglion (cervical) [20 µl]
<b>Lane 3</b>	2x LPP [10 µl]
<b>Lane 4</b>	ME7 ganglion (thoracic) [20 µl]
<b>Lane 5</b>	2x LPP [10 µl]
<b>Lane 6</b>	BSE ganglion (cervical) [20 µl]
<b>Lane 7</b>	2x LPP [10 µl]
<b>Lane 8</b>	BSE ganglion (thoracic) [20 µl]
<b>Lane 9</b>	2x LPP [10 µl]
<b>Lane 10</b>	10 <sup>-6</sup> HÄ positive control (brain homogenate) [10 µl]



**Figure 2.3.6:** Western Blot Analysis of cervical and thoracic DRG of a terminal 263K infected, an ME7 infected, a BSE-H infected and a control animal

## 2.4 FTIR microspectroscopy

### 2.4.1 DRG of 263K scrapie-infected hamsters

Protein secondary structure in the ganglia was determined using FTIR micro-spectroscopic imaging. The FTIR spectra and images were collected at beamline U10B at the National Synchrotron Light Source, Brookhaven National Laboratory (Upton, NY). A Thermo Nicolet Magna 860 FTIR spectrometer, coupled to a Continuum IR microscope (ThermoNicolet, Madison, WI), was used with synchrotron light as the infrared source. The microscope was equipped with matching 32X Schwarzschild objectives, a motorized x-y mapping stage, an adjustable rectangular aperture, and a mercury cadmium telluride (MCT-A) detector. A pre-defined area including 10-15 single cells was raster scanned with step size of 4  $\mu\text{m}$  using Omnic 7.3 software (ThermoNicolet). At each point, an absorbance spectrum was recorded in transmission mode. Each spectrum was collected in the mid-infrared spectral range (4000 - 800  $\text{cm}^{-1}$ ) with a spectral resolution of 8  $\text{cm}^{-1}$  and 128 scans co-added. The aperture was set to 10 x 10  $\mu\text{m}$ . Happ-Genzel apodization and a zero-filling of level 2 were applied, resulting approximately 1 data point every 4 wavenumbers.

#### Summary:

- Animals:

animal H100.01-20:	pellet + S-homogenate
animal H100.21-32:	pellet + N-homogenate
animal H100.33-37:	pellet + S-homogenate
animal H100.38-40:	pellet + N-homogenate

Each five 263K infected and three control hamsters were sacrificed with  $\text{CO}_2$  after 100 dpi, 130 dpi, first clinical signs (~145 dpi) and in the terminal stage of the disease (~170 dpi). In total, 19 scrapie infected and 12 control hamsters were examined.

- Synchrotron FTIRMS was performed as described.
- Data Analysis:  $\beta$ -sheet content and distribution (2.6.2);  $\alpha$ -helix content and distribution (2.6.3); relative protein content (2.6.4); fingerprint region (2.6.6); lipid composition (2.6.7) and statistical analysis (2.6.9)



### 2.4.2 DRG of terminally diseased 263K and ME7 infected hamsters

Spectra were collected using a Perkin-Elmer Spectrum One/Spotlight 300 Fourier Transform IR micro-spectrometer (Perkin Elmer Corp, Sheldon, Connecticut) in mapping mode. Detection was provided by a 100×100 μm<sup>2</sup> photoconductive HgCdTe (MCT) detector element, operated at liquid nitrogen temperature which covered the entire IR spectral range from 4000 to 650 cm<sup>-1</sup>. At each pixel 16 scans were co-added applying a spectral resolution of 4 cm<sup>-1</sup> and an “aperture” of 6.25 x 6.25 μm<sup>2</sup>. Four terminal diseased 263K infected hamsters, two terminal diseased ME7 infected hamsters and two control hamster were investigated. In addition, 34258 spectra of 2 terminally diseased ME7, 56424 spectra of 3 terminally diseased 263K and 12236 of one mock-infected animal were recorded using an FPA based spectrometer in Dresden, Germany. Here, IR spectroscopic maps were recorded using a FTIR spectrometer IFS66/S (Bruker Optik, Ettlingen, Germany) that was coupled to a Hyperion microscope with 15x Cassegrain objective and with a single-channel MCT detector. The microscope aperture was set to 268 x 268 μm<sup>2</sup>. IR spectra were recorded from 950 to 3900 cm<sup>-1</sup> with a spectral resolution of 8 cm<sup>-1</sup>, co-adding 21 scans per IR pixel spectrum. The so recorded maps consisted of 64 x 64 spectra. Using this device, higher spatial resolutions than with the Perkin Elmer Spotlight could be achieved (Lasch & Naumann, 2006).

#### Summary:

- **Animals:**  
263K: 4 animals (H30.10, H31.11, H47.01 and H47.04) i.o.  
ME7: 2 animals (H585.26 and H585.58) i.c.  
Control: 2 animals (H47.7, N100.32) i.o.

The animals were sacrificed with CO<sub>2</sub> at the terminal stage of the disease (for 263K: ~170 dpi, for ME7: ~280 dpi). The control animal was euthanized at ~170 dpi.

- FTIRMS was performed as described
- Data Analysis: β-sheet / α-helix ratio and distribution (2.6.5); fingerprint region (2.6.6); lipid composition (2.6.7); statistical analysis (2.6.9)

### 2.4.3 DMNV of 263K infected hamsters at 70 dpi

Using a IFS28/B FTIR spectrometer coupled to an IR microscope A590 (both Bruker, Ettlingen, Germany), overview maps of the Medulla oblongata with a 100  $\mu\text{m}$  aperture were recorded. Here, a step size of 90  $\mu\text{m}$  was applied and 4 scans were co-added at each point. A background spectrum on clean substrate co-adding 16 scans was also recorded and automatically subtracted from each sample spectrum. After determining the location of the DMNV, detail maps were recorded, co-adding 256 scans each spectrum (background spectrum: 512 scans) with a globular aperture of 50  $\mu\text{m}$  and a step size of 40  $\mu\text{m}$ , applying a spectral resolution of 8  $\text{cm}^{-1}$ .

Additionally, 5 infected and 3 control animals of the H100 series (H100.01 – H100.05, 70 dpi infected + H100.21 – H100.23, 70 dpi control) were measured using a synchrotron coupled spectrometer. Experiments were performed at beamline U10B, overview maps were generated with an aperture of 80 x 80  $\mu\text{m}$  using a globar, co-adding 4 scans with a resolution of 8  $\text{cm}^{-1}$ . The location of the DMNV was determined by calculating the ratio of the integrated areas 1620-1680  $\text{cm}^{-1}$  (representing the protein content) and 2838-3000  $\text{cm}^{-1}$  (representing the lipid content). In this way the DMNV, which is high in protein could clearly be distinguished by the surrounding lipid rich areas. Subsequent detail measurements were performed with 10 x 10  $\mu\text{m}$  aperture, 8  $\text{cm}^{-1}$  resolution, Happ-Genzel apodization, zero filling level 2, 128 scans co-added and 4  $\mu\text{m}$  steps.

#### Summary:

- Animals:  
16 (termed S70.1-S70.16) + 5 orally 263K infected (H100.01 – H100.05)  
8 (termed N70.1-N70.8) + 3 mock infected controls (H100.21 – H100.23)  
The animals were sacrificed with  $\text{CO}_2$  70 days after inoculation
- FTIRMS was performed as described
- Data Analysis: protein/lipid ratio (2.6.1); cluster analysis (2.6.8);  $\beta$ -sheet content and distribution (2.6.2);  $\alpha$ -helix content and distribution (2.6.3); relative protein content (2.6.4); fingerprint region (2.6.6); lipid composition (2.6.7) and statistical analysis (2.6.9)

### 2.4.4 DMNV of Reovirus T3C9 infected hamsters

The Reovirus measurements were carried out at the Robert Koch-Institute using a Bruker IFS28/B spectrometer coupled to an IR microscope A590 (both Bruker, Ettlingen, Germany) and a globar as light source, the same device as described in 2.4.3. Overview maps with an aperture of 100  $\mu\text{m}$  in diameter were recorded prior to detailed maps. Here, 4 scans with a resolution of 4  $\text{cm}^{-1}$  each spectrum were collected. The protein/lipid distribution was plotted, the coordinates of the DMNV were determined and a second map applying an aperture of 50  $\mu\text{m}$  was performed to achieve higher spatial resolution. Here, an area of 500 x 500  $\mu\text{m}$ , co-adding 256 scans per pixel and a step size of 40  $\mu\text{m}$  were chosen, resulting in 13 x 13 spectra per detail map.

#### Summary:

- **Animals:**  
9 orally Reovirus T3C9 infected newborn hamsters, seven of them included in the FTIR study (R1-R7) + 7 mock infected age matched controls, six of them included in the FTIR study (N1-N6)  
The animals were sacrificed with Isofluran at 6 and 9 days after infection, respectively.
- FTIRMS was performed as described
- Data Analysis: protein/lipid ratio (2.6.1); cluster analysis (2.6.8); relative protein content (2.6.4); fingerprint region (2.6.6); lipid composition (2.6.7); statistical analysis (2.7.10)

### 2.5 XRF Microprobe

The concentration and distribution of trace metals in ganglion tissues were measured with x-ray fluorescence microprobe. The experiments were carried out at beamline 2-IDE at the Advanced Photon Source (APS), Argonne National Laboratory (Chicago, IL). At this beamline, synchrotron light from a bending magnet passes through a silicon [111] monochromator and the undulator-generated x-rays of 10 keV incident energy were focused to a measured spot size of 0.3 x 0.5  $\mu\text{m}$  using Fresnel zone plate

optics (X-radia, Concord, CA). Cells were raster scanned in steps of 5  $\mu\text{m}$  and fluorescence spectra were collected for 1 sec dwell time by using a three-element UltraLE Ge-detector (Canberra, Meridien, CT). Based on this so-called coarse scan, cells of interest were selected and coordinates were determined. Subsequent fine scans were performed with 0.5  $\mu\text{m}$  step size, 1 sec dwell time, including 1 to about 4 single neuron cells each map. In this way, single neuron cells from three terminal scrapie diseased animals (H100.11, H100.14 and H100.16) and one age-matched control (H100.32) were recorded.

### Summary:

- **Animals:**

This experiment was performed on 20  $\mu\text{m}$  thick serial sections of infected and uninfected hamster DRG on SiN windows

at 70 dpi: H100.03 + H100.04 and H100.23, respectively;

at 100 dpi: H100.08 + H100.10 and H100.26, respectively;

at fcs: H100.18 + H100.19 + H100.20 and H100.29, respectively and

at the terminal stage: H100.11, H100.14 + H100.16 and H100.32, respectively

- XRF Microprobe was performed as described
- Data Analysis: see 2.6.11

## **2.6 Data Analysis**

### **2.6.1 protein/lipid ratio**

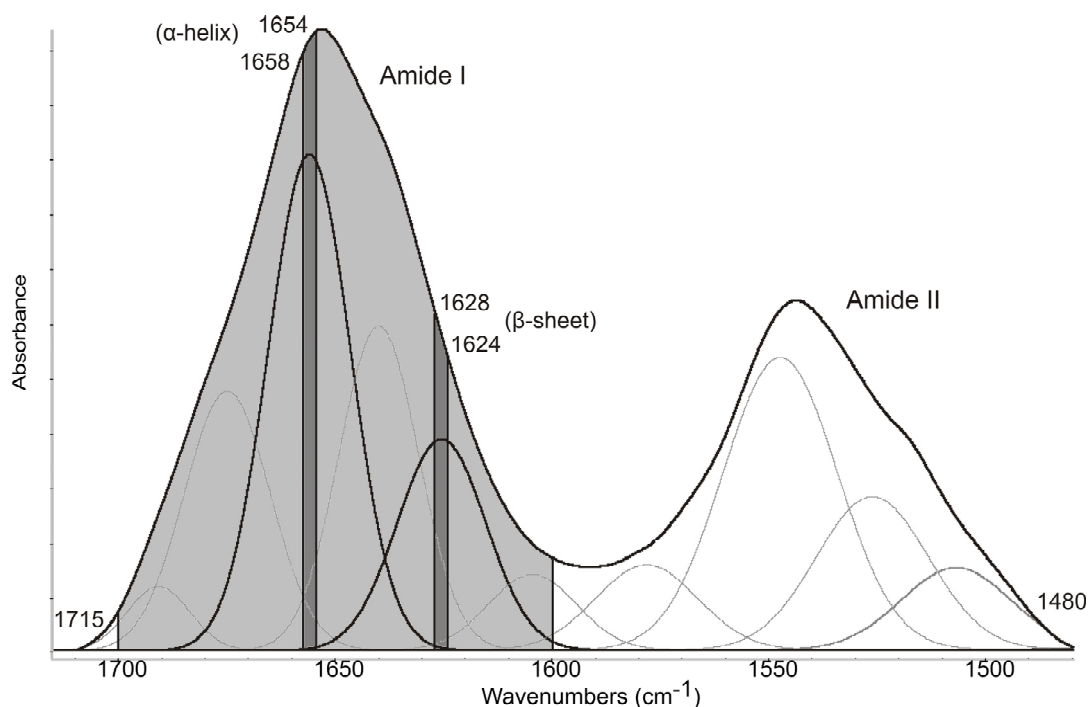
The protein/lipid ratio is useful to distinguish spectra of the nuclei of the cranial nerves from the surrounding tissue (high protein content of the nuclei and high lipid content of the adjacent areas) in the medulla oblongata. Therefore, ratios were calculated using the Cytospec™ software version 1.3.01 (Lasch, 2001-2007) determining the integrated area between 1620 – 1680  $\text{cm}^{-1}$  (for protein content, baseline 1480 – 1715  $\text{cm}^{-1}$ ) divided by the integrated area from 2838 – 3000  $\text{cm}^{-1}$  (for lipid content, baseline 2800 – 3000  $\text{cm}^{-1}$ ).

### 2.6.2 $\beta$ -sheet content and distribution

To examine the amount of  $\beta$ -sheet in the tissue, the absorbance spectrum at each pixel was integrated from 1624 - 1628  $\text{cm}^{-1}$ . The spectral ranges were chosen by the result of a curve fitting which was performed using Grams/32 software version 5.1 (Galactic Industries Corporation, USA) (see **Figure 2.6.2**). To normalize for potential differences in tissue thickness among samples, the  $\beta$ -sheet area was divided by the area under the amide I band (1600  $\text{cm}^{-1}$  – 1700  $\text{cm}^{-1}$ ), applying the spectral region of 1480-1715  $\text{cm}^{-1}$  as baseline.

### 2.6.3 $\alpha$ -helix content and distribution

To examine the amount of  $\alpha$ -helix in the tissue, the absorbance spectrum at each pixel was integrated from 1654 - 1658  $\text{cm}^{-1}$ . The spectral ranges were chosen by the result of a curve fitting which was performed using Grams/32 software version 5.1 (Galactic Industries Corporation, USA) (see **Figure 2.6.2**). To normalize for potential differences in tissue thickness among samples, the  $\beta$ -sheet area was divided by the area under the amide I band (1600  $\text{cm}^{-1}$  – 1700  $\text{cm}^{-1}$ ), applying the spectral region of 1480-1715  $\text{cm}^{-1}$  as the baseline.



**Figure 2.6.2:** Curve fitting result of a spectrum derived from a terminally infected animal. The integrated area between 1624 and 1628  $\text{cm}^{-1}$  for  $\beta$ -sheet content and 1654 and 1658  $\text{cm}^{-1}$  for  $\alpha$ -helical content (dark grey), divided by the area under the amide I band (1600  $\text{cm}^{-1}$  – 1700  $\text{cm}^{-1}$ ), applying the spectral region of 1480-1715  $\text{cm}^{-1}$  as the baseline (light grey) was used for the calculation of secondary structure content.

### 2.6.4 relative protein content

To determine the relative amount of protein at each pixel, the protein content (integrated area: 1600 – 1700  $\text{cm}^{-1}$ ; baseline: 1480 – 1715  $\text{cm}^{-1}$ ) was divided by the integrated area: 2700 – 3700  $\text{cm}^{-1}$ ; baseline: 2700 – 3700  $\text{cm}^{-1}$ . Since the region between 2700 – 3700  $\text{cm}^{-1}$  includes the vibrational modes of C-H, N-H and C-O bonds from all major biomolecules, it was termed as ‘total biomass’. However, this calculation can only give a rough estimation of the relative amount of protein in the tissue.

### 2.6.5 $\beta$ -sheet / $\alpha$ -helix ratio and distribution

The  $\beta$ -sheet to  $\alpha$ -helix ratio was calculated as ratio of the peak intensity at 1637  $\text{cm}^{-1}$  (amide I of  $\beta$ -pleated sheet structures) and 1657  $\text{cm}^{-1}$  (amide I of  $\alpha$ -helical structures) in original spectra, applying a baseline from 1715 – 1480  $\text{cm}^{-1}$ , and plotted as a function of pixel location. The higher the value the relatively higher is the amount of  $\beta$ -sheet/lower the amount of  $\alpha$ -helix and vice versa.

### 2.6.6 ‘fingerprint’ (predominantly phosphates and carbohydrates)

The quantification of phosphate metabolites, which are composed of energy yielding molecules (AMP, ADP and ATP) and nucleic acids, provide a clue about the various states of the cell (Yang *et al.*, 1995). Changes in the phosphate region were calculated by the integrated area from 980 – 1149  $\text{cm}^{-1}$  (baseline: 980 – 1149  $\text{cm}^{-1}$ , symmetric P=O stretching in  $\text{PO}_2^-$ ) divided by the integrated area from 1151 – 1350  $\text{cm}^{-1}$  (baseline: 1151 – 1350  $\text{cm}^{-1}$ , asymmetric P=O stretching in  $\text{PO}_2^-$ ) from original spectra as described elsewhere (Salman *et al.*, 2003). Since these regions of the spectrum also include vibrational modes of other compounds such as carbohydrates, it only provides a rough estimation of changes in phosphates (phospholipids and nucleic acids) and carbohydrates.

### 2.6.7 lipid composition

Spectral indicators for changes in lipid composition were analyzed by calculating the ratio of the peak intensities at 2957  $\text{cm}^{-1}$  ( $\text{CH}_3$  stretching vibrations of methylene groups in lipids) and 2925  $\text{cm}^{-1}$  ( $\text{CH}_2$  stretching vibrations of methylene groups in lipids) of original spectra, using a baseline in the range 2838 – 3000  $\text{cm}^{-1}$ . It applies

that the higher the ratio the shorter (or higher branched) the fatty acid chain and vice versa.

### **2.6.8 Cluster Analysis**

Hierarchical cluster analysis was performed to differentiate between histological regions in the brain using Opus5 software (Bruker, Ettlingen, Germany). Therefore, vector normalized original or derivative spectra in various regions were analyzed using Opus' method "standard". Pearson's correlation coefficient method was applied to calculate the distance matrix and Ward's algorithm was used for hierarchical clustering.

### **2.6.9 Statistical Analysis**

An unpaired two-sided t-test (Student's t-test) was performed to test for significant differences ( $p < 0.05$ ) between scrapie and control at each time point, and differences between time points.

### **2.6.10 Derivative spectra**

Derivative spectra (1<sup>st</sup> and 2<sup>nd</sup> derivatives) were calculated in Opus5 using the Savitzky-Golay algorithm applying 9 smoothing points, to reduce the noise generated by the derivatization.

### **2.6.11 XRF microprobe**

Quantitation and image-processing was performed using MAPS software (Vogt, 2003) (Vogt *et al.*, 2003), and standardization to convert the fluorescence signal to a two-dimensional concentration in  $\mu\text{g per cm}^2$  was achieved by fitting the spectra against the signal derived from thin-film standards NBS-1832 and NBS-1833 (National Bureau of Standards, Gaithersburg, MD). These values can be calculated as  $\text{mM l}^{-1}$  or  $\mu\text{M l}^{-1}$  by using the molar mass of the respective elements and the thickness of the tissue section, i.e. 20  $\mu\text{m}$ .

The elements analyzed were copper, zinc, iron, calcium, manganese, potassium and phosphor. In the coarse scans, regions outside the area of neurons, i.e. outside or at the edge of the ganglion, and other non-neuronal regions, for example bubbles in the

tissue, were manually excluded from the analysis of elemental content. For terminal stage, 6 maps were recorded and averaged for the control and 1 map each infected animal.

In the fine scans, areas containing the nucleus, cytoplasm and extracellular matrix were defined separately and stored as so-called regions of interest. In this way it was possible to analyze these subcellular structures individually.