Brain Pathology 8: 615-623(1998)

RESEARCH ARTICLE

Severe, Early and Selective Loss of a Subpopulation of GABAergic Inhibitory Neurons in Experimental Transmissible Spongiform Encephalopathies

Marin Guentchev MD[1], Martin H. Groschup DVM[2], Radzislaw Kordek MD[3], Pawel P. Liberski MD[3], Herbert Budka MD[1]

[1] Austrian Reference Center for Human Prion Diseases and Institute of Neurology, University of Vienna, A-1097 Vienna, Austria
[2] Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany
[3] Laboratory of Tumor Biology, Chair of Oncology, Medical Academy Lodz, Lodz, Poland.

Little is known about the pathogenetic basis of characteristic symptoms in transmissible spongiform encephalopathies (TSEs) such as myoclonus and characteristic EEG hyperactivity. We investigated the GABAergic system and its subpopulations in mice inoculated with experimental scrapie (ME7, RML, 22A strains) and Creutzfeldt-Jakob disease (CJD; Fujisaki strain), to study damage to inhibitory neurons. Since recent studies have shown electrophysiological changes in prion protein (PrP) knockout mice, we also studied mice lacking or overexpressing the PrP gene. Antibodies against glutamic acid decarboxylase (GAD), parvalbumin (PV), calbindin (CB), and calretinin (CR) were used to stain GABAergic neurons, and isolectin-B_4 to stain perineuronal nets around PV+ neurons. In scrapie infected mice, cortical PV+ neurons were severely reduced while CB+ and CR+ neurons were well preserved. In CJD inoculated mice, loss of PV+ neurons was severe and occurred very early after inoculation. $PrP^{-/-}$ and tg20 mice showed normal appearance of PV, CB, CR, GAD+ neurons and their neuropil, and of isolectin-B_4+ perineuronal nets. The early, severe and selective loss of cortical PV+ neurons in experimental scrapie and CJD suggest selective loss of PV+ GABAergic neurons as important event during disease development, possibly as one basis of excitatory symptoms in TSEs.

Corresponding author:
Dr. H. Budka, Institute of Neurology, University of Vienna, AKH 04J, Währinger Gürtel 18-20, POB 48, A-1097 Wien, Austria; Phone +43-1-40400-5501 or -5573; FAX +43-1-40400-5511 or -5573; E-mail: h.budka@akh-wien.ac.at

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious, inherited, sporadic or iatrogenic neurodegenerative disorders [for a review see (11, 25)]. Human TSEs include Creutzfeldt-Jakob disease (CJD) (3), Gerstmann-Sträussler-Scheinker disease (22), kuru (23) and fatal familial insomnia (19). Animal prion diseases include most notably scrapie and bovine spongiform encephalopathy or "mad cow" disease (35). Spongiform change, astrogliosis and neuronal loss are the classical neuropathological triad of tissue lesioning in TSEs (3); deposition of an abnormal isoform, the scrapie isoform (PrP^{Sc}), of the host-encoded glycoprotein PrP^{c} in the CNS is another hallmark (34). The infectious agent of TSEs, frequently called prion, seems to differ from both virioids and viruses (31). The biological basis of disease manifestation is yet unknown; possibilities include loss of functionally relevant cellular prion protein (PrP^{c}) or neurotoxicity of accumulated PrP^{Sc}. Despite the obvious importance of PrP^{Sc} in prion disease, the normal cellular function of PrP^{c} remains unknown. Mice devoid of PrP^{c} ($PrP^{-/-}$) appeared normal and showed no detectable neuroanatomical changes (4). However, later studies showed subtle physiological, morphological and behavioral abnormalities (9, 33).

Little is known about the pathogenetic basis of characteristic symptoms in TSEs such as myoclonus and the characteristic EEG pattern. Recent studies showed aberrant neuropeptide Y (NPY) mRNA induction (12) and a decrease of NPY Y2 receptor binding sites in the hippocampus of scrapie infected mice (13). Those results suggested that loss of NPY receptor binding is a candidate factor to cause clinical symptoms in scrapie (13). However, the symptoms might be explained by loss of neuronal inhibition; preferential alteration of the inhibitory GABAergic system in TSEs was demonstrated (14-16, 20, 32). Parvalbumin (PV), calbindin-D28K (CB) and calretinin (CR) are calcium binding proteins which demarcate not-overlapping subpopulations of GABAergic inhibitory neurons in the cortex (1, 6, 18).

Recent studies showed diffuse and severe loss of PV positive (+) neurons in CJD (16, 21) and topographical correlation of tissue lesioning in CJD with the density of PV+ neurons in controls (21). Thus it was suggested that loss of PV+ neurons might form the pathogenetic basis for typical symptoms in CJD. However, some questions remain open: Is there real loss of PV+ neurons, or do the observations reflect changed immunocharacteristics of PV e.g. by interaction with another protein or posttranslational modifications? If this selective neuronal loss is real, at which stage of the disease starts the demise of these neurons?

Electrophysiological changes in the CA1 region of the hippocampus in $PrP^{-/-}$ mice suggested a function of PrP^{C} for forming and maintaining of GABAergic synapses (9); thus neurodegeneration in prion disease might be, at least in part, due to loss of function of PrP^{c}. In contrast, later investigations (24, 29) support the view that synaptic transmission is unimpaired in $PrP^{-/-}$ mice. These findings were neither confirmed nor disproved by morphological studies. Nevertheless, late loss of Purkinje cells and GABAergic synapses in the cerebellum in another line of $PrP^{-/-}$ mice suggested that postsynaptic PrP^{c} might be necessary for inhibitory synapses to be fully functional, and for the long-time survival of Purkinje cells (33).

The aim of this study is to investigate in experimental TSEs whether there is loss of PV+ neurons and when it occurs during disease development. Moreover, we investigated mice overexpressing or lacking PrP, in order to check for changes in the morphology and density of the GABAergic system under the influence of PrP.

Materials and Methods

Twelve diseased mice which had been intracerebrally inoculated with scrapie (five ME7, two RML, five 22A strains) (5) and 8 age-matched controls were investigated. All scrapie mice and corresponding controls were C57BL/6 except two 22A were VM-mice. Nine mice carrying multiple copies of the PrP gene (tg20) (17), 9 $PrP^{-/-}$ mice (4) and 9 age-matched controls from two age groups, 38-39 weeks and 52 weeks, were analyzed. Every age group was composed by 4 or 5 animals. We also studied 25 NIH-Swiss mice inoculated with the Fujisaki CJD strain from a series reported previously (28) and 3 controls. These Fujisaki CJD animals were euthanized weekly starting at the second week post intracerebral inoculation. At week 22 all remaining mice were euthanized. This strain is characterized by an incubation period in mice of approximately 16-18 weeks post intracerebral inoculation. First clinical symptoms occur at week 14 post inoculation. First pathology in subcortical regions appears at week 10 post inoculation and in the cortex at week 13. All mice brains were fixed by immersion in formol and embedded in paraffin. The CJD-inoculated mouse brains and the corresponding control brains went through an intermediate step in concentrated formic acid before embedding in paraffin in order to reduce infectivity. The Fujisaki mice were divided into 5 groups: controls (3 mice), 2-4 weeks post intracerebral inoculation (3 mice), 5-9 weeks post inoculation (5 mice), 10-13 weeks post inoculation (5 mice), 14-18 weeks post inoculation (6 mice) and six terminally diseased mice. Due to the fact that this material has already been used for other studies, material for ILB_4 staining was not available in 8 mice: 3, 13, 14, 17, 18 weeks post inoculation and three terminal stage brains, respectively.

We visualized glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, as a marker for the whole GABAergic system (27), PV, CB, CR to demarcate GABAergic subpopulations (2), and isolectin-B_4 (ILB_4) to decorate perineuronal nets around PV+ neurons (7, 8). Sections for GAD and CR staining were cut at 4μm, for PV and CB at 10μm. Monoclonal antibodies against CB-D28K (CL-300, 1:200, Sigma, St. Louis), and PV (PA-235, 1:5000, Sigma, St. Louis), and polyclonal antibodies against CR (1:100, Chemicon, Temecula, CA) and GAD (1:100, Chemicon, Temecula, CA) were used. Sections for PV labeling were boiled 12 min. in Target Retrieval Solution (High pH) (Dako, Glostrup, Denmark). We used the ChemMate™ Detection Kit as secondary system (Dako, Glostrup, Denmark). Lectin staining (ILB_4 -peroxidase labeled from vicia villosa, 10μg/ml TBS, Sigma, St. Louis) was performed on 4μm thick sections. All sections were developed with diaminobenzidine. Hematoxylin counterstaining was performed on all but PV, CR and CB stained sections.

PV, GAD+ neurons and ILB_4 + perineuronal nets were quantified in the retrosplenial granular cortex (=medial frontal cortex) of control, scrapie and CJD mice in a diagnosis-blinded fashion by counting nucleated cells (GAD), immunostained cell bodies (CB, PV) or cells surrounded by positive perineuronal nets (ILB_4+) in several fields (3-4) with a x60 objective (21). The average cell number per field was entered into statistical evaluation. In control brains, usually a total of about 37 (min=25; max=55) cells were seen in the fields entering quantitative assessment. Due to the fact that in

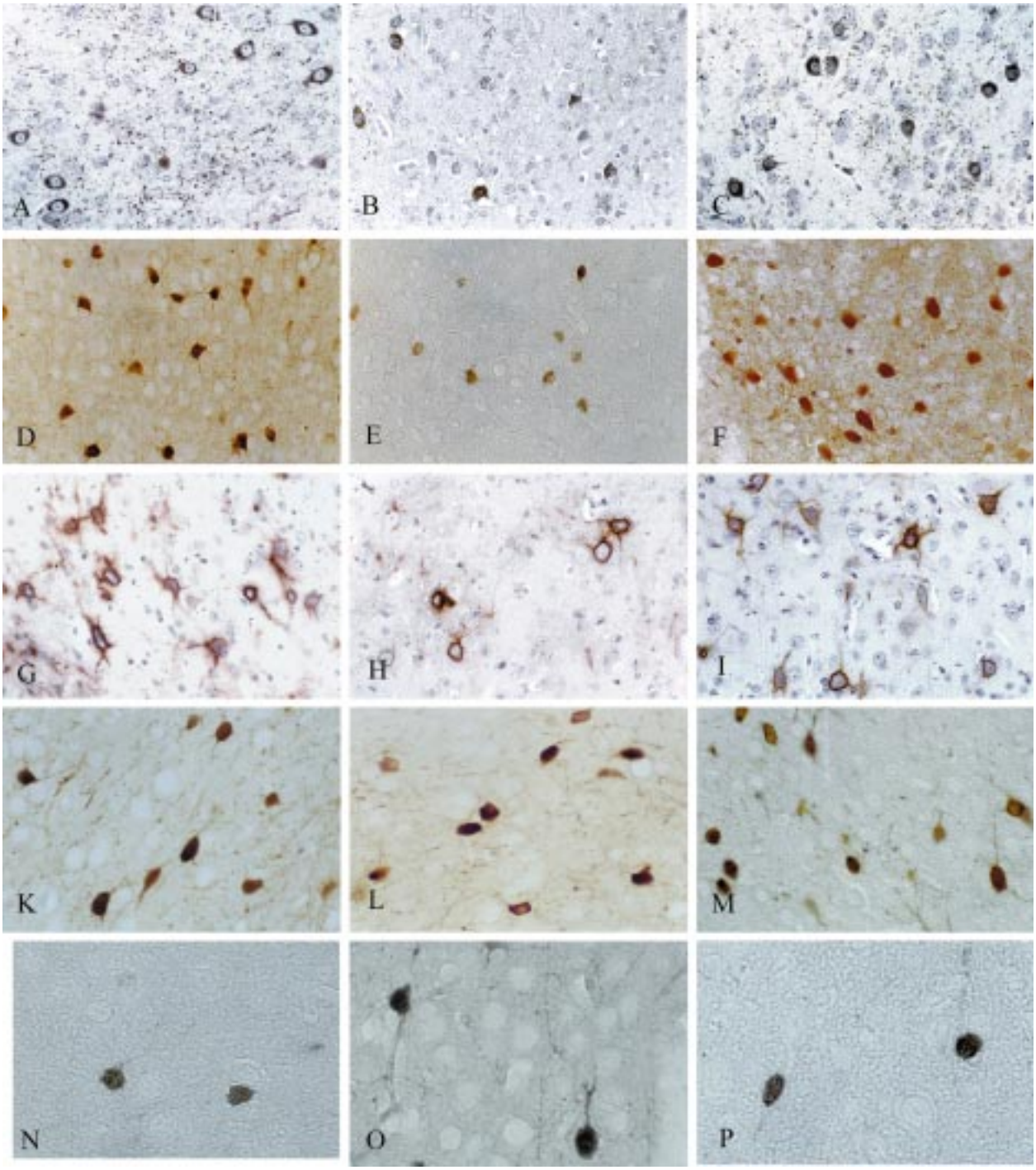

Figure 1. Glutamic acid decarboxylase (GAD) (**A, B, C**), parvalbumin (PV) (**D, E, F**), isolectin-B_4 (ILB_4) perineuronal nets (PNN) (**G, H, I**), calbindin (CB) (**K, L, M**) and calretinin (CR) (**N, O, P**) immunostains of the cortex in a control (**A, D, G, K, N**), scrapie (**B, E, H, L, O**) and $PrP^{-/-}$ mouse (**C, F, I, M, P**) brain. There is severe loss of PNN (**H**), GAD (**B**) and PV+ neurons and neuropil staining (**E**) in the scrapie brain; CB+ and CR+ cells are well preserved (**K, L, N, O**). The GABAergic system in $PrP^{-/-}$ mice appears normal (**C, F, I, M, P**). PV, GAD, ILB4 micrographs were taken from the retrosplenial granular cortex, CB micrographs from the piriform cortex, CR micrographs from the neocortical convexity. Original magnification PV, GAD, ILB_4 and CB: x256; CR : x364

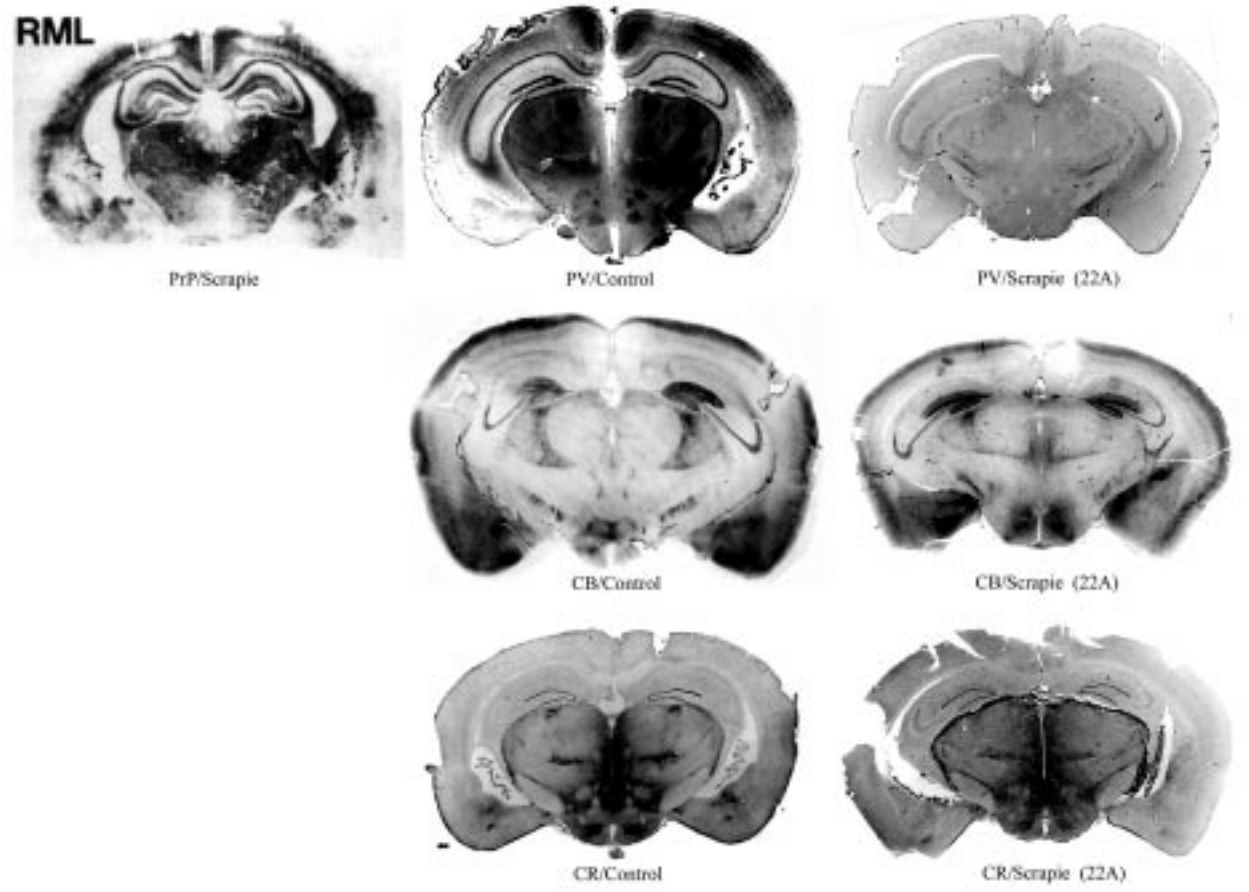

Figure 2. Upper left, PrP^{sc} histoblot of a scrapie brain (from Fig.4, upper part, of ref. 5 with permission, copyright 1996 by Springer-Verlag). Center and right column, PV, CB and CR immunostains of control and scrapie brains. The white arrowhead indicates the CA1 region of the hippocampus, the black arrowhead indicates the retrosplenial granular cortex. Note the striking similarity between the distribution of PV immunoreactivity in the control brain and the PrP^{sc} deposition in the scrapie brain.

the cortex calcium binding proteins occur in almost entirely separate subpopulations of GABAergic neurons (2), we focused on cortical GABAergic neurons. We counted PV, GAD+ neurons and ILB_4 + perineuronal nets in the retrosplenial granular cortex, because almost no CB and CR+ positive cells were present in this area.

The difference of densities of PV and GAD+ neurons and ILB_4 + perineuronal nets in experimental scrapie, CJD and controls was tested by means of a two-tailed unpaired t-test.

Results

Wild type, PrP$^{-/-}$ and tg20 mice. PV+ neurons were present in all parts of the cerebral cortex, especially in the retrosplenial granular cortex (Fig. 1) and as all cells of the reticular thalamic nucleus. PV immunoreactive neuropil was seen in the thalamic nuclei and all parts of the hippocampus (Fig. 2). All Purkinje cells were positive for PV (Fig. 3). CB+ neurons and neuropil were present in all parts of the neocortex (Fig. 1) and as Purkinje cells (Fig. 3). The highest cortical density of CB+ neurons was found in the piriform cortex (Fig. 2). Additionally CB+ neuropil was present in the CA4-2 areas of the hippocampus and gyrus dentatus. There was no CB immunoreactive neuropil in CA1 (Fig. 2). Almost no CB+ neurons or neuropil were found in the retrosplenial granular cortex (Fig. 2). CR+ neurons and neuropil were restricted to the cortex, CA4 region of the hippocampus, gyrus dentatus and several thalamic nuclei but not reticular thalamic nucleus (Figs. 1 and 2). Neurons surrounded by ILB_4 + perineuronal nets had a similar density and distribution as PV+ neurons. They were present in all parts of the neocortex (especially in

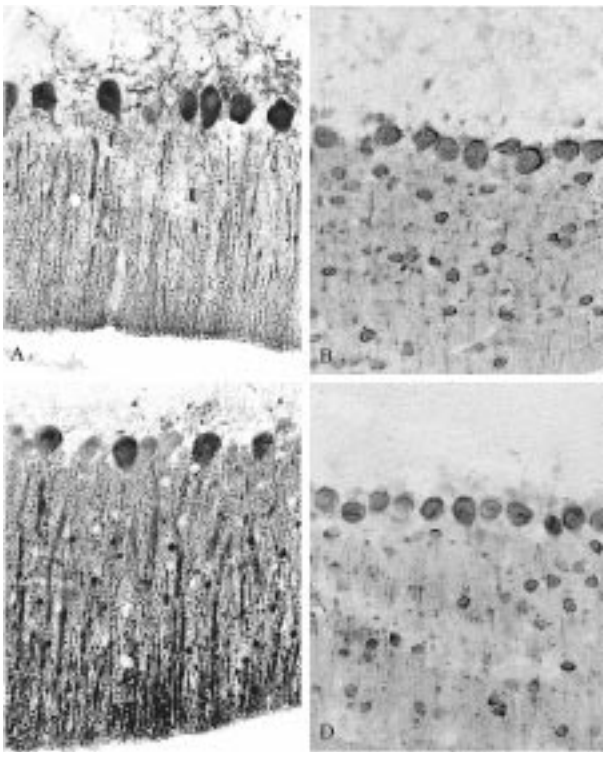

Figure 3. CB (**A, C**) and PV (**B, D**) immunostains of the cerebellar cortex in a scrapie (**C, D**) and control (**A, B**) brain. In Purkinje cells, both calcium binding proteins are present and are not affected in scrapie. Original magnification: x256

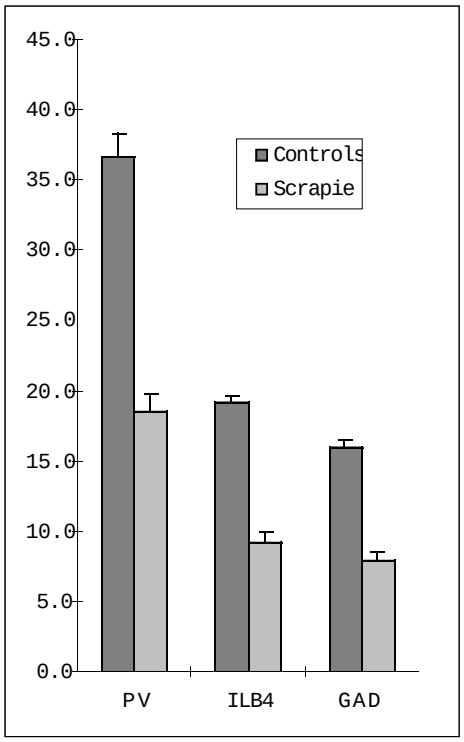

Figure 4. Graphic representation of the cortical (retrosplenial granular cortex) density of GAD and PV+ neurons and ILB_4+ perineuronal nets (PNN) in controls and scrapie-inoculated mice. The columns depict the average numbers of GAD and PV+ neurons and of ILB_4+ PNN per field in controls and scrapie (bar = SEM); for counting of GAD+ neurons and ILB_4+ PNN, 4 mm thick sections were used, for PV+ neurons 10 mm thick sections. There is severe loss of GAD, PV+ neurons and ILB_4+ in scrapie (in all p<0,001).

the retrosplenial granular cortex) (Fig. 1), in the cerebellum (Purkinje cells) and reticular thalamic nucleus. GAD+ neurons and their neuropil were present in all parts of the neocortex (Fig. 1), in the cerebellum (Purkinje cells) and reticular thalamic nucleus. $PrP^{-/-}$ and tg20 mice showed normal distribution and morphology of PV, CB, CR, GAD+ neurons, synaptic buttons and neuropil, and ILB_4+ perineuronal nets in cerebral cortex (Fig. 1), thalamic nuclei and cerebellum. There was no difference in the density, morphology and general appearance of PV, CB, CR, GAD+ neurons and ILB_4+ perineuronal nets between two age groups: 38/39 weeks and 52 week old wild type mice (data not shown).

Experimental scrapie. All scrapie brains showed a statistically significant neuronal loss of PV, GAD+ neurons and ILB_4+ perineuronal nets. Cortical PV+ neurons were severely reduced (49.3%, p<0,001) (Figs. 1, 4), while cerebellar and thalamic PV+ cells were well preserved (Fig 3). Cortical and thalamic PV+ neuropil was almost completely lost (Fig. 2). Neurons surrounded by ILB_4+ perineuronal nets also were reduced (51.9% in the cortex, p<0,001) (Figs. 1, 4) in all investigated regions, even in the reticular thalamic nucleus. In regions with a high degree of pathologic changes, many microglial cells showed ILB_4 staining of the cytoplasm. Cortical GAD+ neurons and neuropil were decreased especially in regions with a high density of PV+ neurons such as in retrosplenial granular cortex (Figs. 1, 4) (50.6% loss in the cortex, p<0,001). Cerebellar GAD+ neurons and neuropil staining appeared normal. Loss of PV+/GAD+ synaptic buttons and neuropil appeared to be more severe than the loss of the corresponding neuronal somata. The CB+ neurons and neuropil were well preserved (Figs. 1 and 2). Purkinje cells in scrapie showed more intense CB immunostaining in the cell body and less intense in the neuropil (Fig. 3). CR+ neurons and neuropil were also well preserved (Fig 1 and 2). We found that the distribution of PV (but not CB or CR) in the normal mouse brain correlates well with the

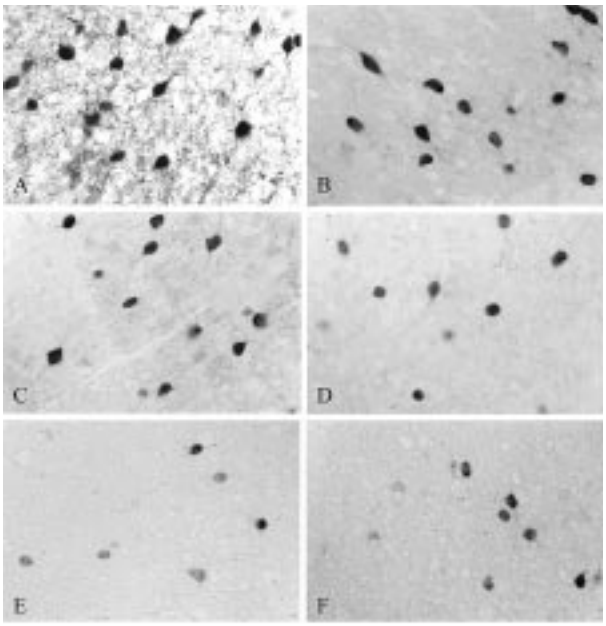

Figure 5. PV immunostains of the retrosplenial granular cortex in a control (**A**) and CJD brains at 4 (**B**), 6 (**C**), 11 (**D**), and 15 (**E**) weeks post intracerebral inoculation and at the terminal stadium (**F**). There is severe and early loss of PV+ neurons in experimental CJD. Original magnification: x256.

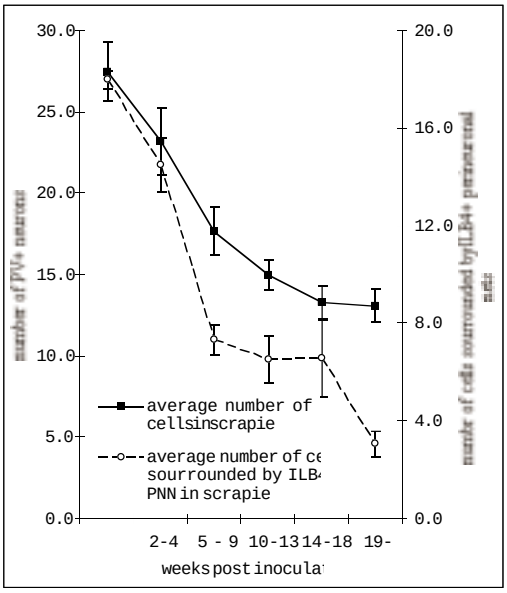

Figure 6. Graphic representation of the cortical density of PV+ neurons and ILB_4+ perineuronal nets (PNN) in controls and CJD inoculated mice. The lines depict the average numbers of PV+ neurons and ILB_4+ PNN in controls and CJD mice (bar = SEM). Note the severe and early loss of PV+ neurons and ILB_4+ PNN in experimental CJD.

distribution of PrP^{Sc} in the RML strain (Fig. 2) and roughly with the distribution of PrP^{Sc} in the strain 22A.

Experimental CJD. Mice inoculated with the Fujisaki CJD strain showed a similar pattern of neuronal loss as scrapie inoculated brains. After weeks 5-8 post intracerebral inoculation, PV+ neurons were significantly decreased in the cerebral cortex (35%, $p<0,03$) (Figs. 5, 6). At the terminal stadium (after week 18), cortical PV+ neurons were severely reduced (52.3%, $p<0.001$), while the density and morphology of cerebellar and thalamic PV+ neurons appeared normal. Loss of PV+ synaptic buttons and neuropil occurred earlier and was more severe than the loss of PV+ neurons. After weeks 5-8 post intracerebral inoculation, ILB_4+ perineuronal nets were significantly decreased in the cerebral cortex (59%, $p<0,001$) (Fig. 6). It seemed that decrease of the density of ILB_4+ perineuronal nets appeared earlier and was more severe (82% at the end stage of the disease, $p<0,001$) than loss of PV+ neurons (Fig. 6). Pretreatment with formic acid did not seem to affect the immunoreactivity for PV and the staining characteristics for ILB_4. However, immunostaining for CB, CR, and GAD was not consistently achieved.

Discussion

The initial report that $PrP^{-/-}$ mice are normal was consistent with a pathogenetic model of accumulation of neurotoxic PrP^{Sc} causing damage in prion disease. Electrophysiological changes in $PrP^{-/-}$ mice, however, suggested that neurodegeneration in prion disease might be due, at least in part, to loss of function of PrP^{c} in the adult nervous system (9, 36). Nevertheless, later investigations (24, 29) found unimpaired synaptic transmission in $PrP^{-/-}$ mice. At the light microscopical level, we didn't find any morphological substrate supporting the hypothesis that PrP^{c} has a role in the formation and maintenance of GABAergic synapses (36). Our results show that overexpression or lack of PrP^{c} does not alter the morphology and general appearance of inhibitory synapses and neurons; thus PrP^{c} does not appear to be essential for development or maintenance of GABAergic neurons and synaptic buttons in different brain regions.

Previous studies showed a decrease (-30%, at the clinical stage of the disease) of GAD activity in scrapie-infected hamsters (14, 15) and mice (10). This correlates well with the results observed by us. However, another group showed increased GABA-like immunoreactivity (30) in scrapie hamsters. It is conceivable that such increased GABA-like immunoreactivity reflects a com-

pensatory upregulation of GABA in surviving GABAergic neurons.

Recent studies have shown severe neuronal loss (37), early mRNA GFAP upregulation (13) and altered membrane and synaptic properties of pyramidal neurons (26) in the CA1 hippocampal region of scrapie mice. The selective involvement of this distinct hippocampal region becomes more interesting due to the fact that CA1 is the only hippocampal region showing exclusively PV+ (but not CB or CR+) neuropil and synaptic buttons. The early loss of PV+ neuropil and synaptic buttons shown in our study may be compensated by inhibition from non-PV+ neurons in the regions CA2-4 and gyrus dentatus. This might explain the selective vulnerability of CA1 in scrapie.

This report shows an early and selective loss of a distinct subpopulation of GABAergic neurons in experimental scrapie and experimental CJD, as previously shown for human CJD (16, 21). Loss of PV+ neuropil and neurons appears very early (statistically significant after week 5-8 post intracerebral inoculation) in incubation, weeks before the appearance of PrP deposition, spongiform change, and clinical signs, and is thus the first detectable neuropathologic change in scrapie. The loss of PV+ neurons in experimental CJD is very similar to the loss observed in the scrapie-infected mice. The decrease of a second marker for the PV subpopulation of GABAergic neurons, ILB_4, and the loss of GAD+ neurons (in a region with almost no other subpopulations of GABAergic neurons) confirm the loss of this neuronal subset, distinct from downregulation, interaction with another protein, or posttranslational modification. The loss of PV+ neuropil and synaptic buttons appeared earlier than the loss of PV+ neurons, suggesting a peripheral lesion of these neurons as early event in disease. Interestingly, PV+ neurons in the cerebellum (Purkinje cells) and reticular thalamic nucleus are well preserved. Apparently only cortical PV+ neurons are vulnerable in TSEs. This suggests another factor modulating vulnerability, e.g. protective coexpression of another calcium binding protein (Purkinje cells contain also CB). Indeed, CB+ and CR+ neurons (another subset of GABAergic neurons) were well preserved, seemingly resistant to damage.

While we found the demise of a subset of inhibitory neurons to start early in preclinical disease, signs of increased neuronal electrical activity are usually a late hallmark of TSEs. This might argue against the significance of our findings for bioelectrical manifestation. However, the cortical EEG might undergo structural modifications as long as inhibitory neurons degenerate. An early neuronal loss might still be functionally compensated, or signs of increased electrical activity might finally manifest only after demise of additional neuronal populations.

We conclude that the early, severe and selective loss of cortical PV+ neurons in experimental TSEs and the correlation between PrP^{Sc} deposition and distribution of PV in the normal brain confirms an important role of this neuronal subpopulation in the pathogenesis of prion disease. However, it remains to be established why PV+ neurons are selectively vulnerable in TSEs.

Acknowledgments

Special thanks are due to Marco Celio, Fribourg, Switzerland, for helpful suggestions, and Adriano Aguzzi and Charles Weissmann, Zurich, Switzerland, for making PrP knockout and tg20 mice available to us. Mrs. H. Flicker and Ms. E. Schibich are kindly acknowledged for excellent technical assistance. This work was supported by a European Union Biomed-2 Shared Cost Action “Molecular biology of prion diseases” (Project Leader: J. Collinge, London) and is part of the European Union Biomed-2 Concerted Action “Human transmissible spongiform encephalopathies (prion diseases): neuropathology and phenotypic variation” (Project Leader: H. Budka). We are grateful to George A. Carlson and Springer-Verlag for permission to use their Fig.4, upper part, of ref.5 as our Fig. 2, upper left; their figure was originally slightly modified from Fig.2 E in Carlson GA et al, Proc Natl Acad Sci USA 1994;91:5690-5694, copyright (1994) National Academy of Sciences, USA.

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