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TERHI PIRTTILÄ

Expression and Functions of Cystatin C in Epileptogenesis and Epilepsy

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium, Microteknia building, University of Kuopio, on Saturday 21st January 2006, at 12 noon

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ABSTRACT

Among the epilepsies, which affect approximately 1% of the population, temporal lobe epilepsy (TLE) is the most common type in adults. In most subjects, epilepsy develops as a consequence of a prior brain damaging insult such as head trauma, stroke, or status epilepticus (SE) that leads to epileptogenesis and eventually to the appearance of spontaneous seizures. Epileptogenesis thus provides an attractive target for the development of therapeutic interventions to prevent the development of epilepsy. The molecular mechanisms underlying epileptogenesis, however, are unknown. cDNA microarray technology provides an unbiased method to screen for molecular alterations, such as changes in gene expression during epileptogenesis, and has led to the finding that several genes have altered expression following SE, many of which have not been previously linked to epilepsy or seizures. One example of these is cystatin C, a cysteine proteinase inhibitor whose expression is increased during SE-induced epileptogenesis.

The aim of the present study was to investigate the expression of cystatin C protein in the hippocampus in normal rat, mouse, and human brain, as well as during epileptogenesis and epilepsy. We also elucidated the functional role of cystatin C in common neuropathological alterations that occur during epileptogenesis, such as neuronal cell death and neurogenesis.

The main results can be summarized as follows: 1) In normal rat, mouse, and human hippocampus, cystatin C is present at low levels, mainly in microglia and astrocytes. 2) Cystatin C expression is robustly increased in activated glial cells following an epileptogenic brain insult such as SE or photothrombotic stroke. The upregulation occurs in a delayed manner (*i.e.,* 4-7 d after the insult). 3) Cystatin C upregulation persists in the chronic phase of epilepsy both in a rat model of TLE and in human patients with TLE. 4) Cystatin C deficiency is associated with reduced neurodegeneration after SE and impaired proliferation and migration of newborn neurons in the dentate gyrus, suggesting that cystatin C functions to promote neuronal death and/or as a migratory cue for newborn neurons. Also, in human patients with TLE, increased cystatin C expression is associated with the abnormal migration of newborn neurons to the molecular layer and granule cell dispersion, suggesting that cystatin C participates in the development of granule cell dispersion.

In conclusion, cystatin C upregulation is a common brain injury-related response that occurs in the glial cells of the hippocampus. The present results suggest that cystatin C participates in the neuropathological alterations that occur during epileptogenesis, such as neuronal cell death and neurogenesis, but whether these alterations lead to the lowered seizure threshold and subsequently to epilepsy is unknown. The finding that cystatin C upregulation occurs also at the chronic phase of epilepsy indicates that reorganization is an ongoing process that continues beyond the appearance of spontaneous seizures.

National Library of Medicine Classification: WL 385, WL 203, QU 136, QU 475
Medical Subject Headings: epilepsy, temporal lobe; seizures; status epilepticus; brain/pathology; disease models, animal; kainic acid; electric stimulation; electroencephalography; cerebrovascular accident; hippocampus; dentate gyrus; cystatins; cysteine proteinase inhibitors; up-regulation; astrocytes; microglia; neurons; cell death; regeneration; immunohistochemistry; humans; mice, knockout; rat

In beloving memory

of my father

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ABBREVIATIONS

A	Alanine
Aβ	Amyloid beta protein
AED	Antiepileptic drugs
AD	Alzheimer's disease
BrdU	5-bromo-3'deoxyuridine
CA1	Cornu Ammonis 1 region of the hippocampus proper
CA2	Cornu Ammonis 2 region of the hippocampus proper
CA3	Cornu Ammonis 3 region of the hippocampus proper
cDNA	Complementary deoxyribonucleic acid
CSF	Cerebrospinal fluid
EEG	Electroencephalography
FGF-2	Fibroblast growth factor-2
G	Glycine
HAFD	High amplitude and frequency discharge
HCAA	Hereditary cystatin C amyloid angiopathy
I	Isoleucine
IgG	Immunoglobulin G
i.p.	Intraperitoneal
MW	Molecular weight
P	Proline
PSA-NCAM	Polysialylated neural cell adhesion molecule
Q	Glutamine
SD	Standard deviation
SE	Status epilepticus
SGZ	Subgranule zone of the dentate gyrus
SVZ	Subventricular zone
TGF-β	Transforming growth factor- beta
TLE	Temporal lobe epilepsy
V	Valine
W	Tryptophan

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their corresponding Roman numerals:

I Lukasiuk K., **Pirttilä T.J.**, and Pitkänen A. (2002) Upregulation of cystatin C expression in the rat hippocampus during epileptogenesis in the amygdala stimulation model of temporal lobe epilepsy. *Epilepsia*, 43 (Suppl. 5): 137-145.

II **Pirttilä T.J.**, Lukasiuk K., Håkansson K., Grubb A., Abrahamson M., and Pitkänen A. (2005) Cystatin C modulates neurodegeneration and neurogenesis following status epilepticus in mouse. *Neurobiology of Disease*, 20 (2): 241-253.

III **Pirttilä T.J.**, Manninen A., Jutila L., Nissinen J., Kälviäinen R., Vapalahti M., Immonen A., Paljärvi L., Karkola K., Alafuzoff I., Mervaala E., and Pitkänen A. (2005) Cystatin C expression is associated with granule cell dispersion in epilepsy. *Annals of Neurology*, 58 (2): 211-223.

IV **Pirttilä T.J.** and Pitkänen A. (2005) Cystatin C expression is increased in the hippocampus following photothrombotic stroke in rat. *Neuroscience Letters*, in press.

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1. INTRODUCTION

Epilepsy is a common neurological disease characterized by epileptic seizures, which are transient disturbances in brain function arising from overexcitation of neuronal networks. Among the epilepsies, which affect approximately 1% of the population, temporal lobe epilepsy (TLE) is the most common type in adults (Engel, 1996). It typically develops following a brain damaging insult such as head trauma, cerebrovascular disease, tumor, infection, or status epilepticus (SE) (Mathern et al., 1996). The initial insult initiates several neurobiological alterations that eventually lead to the appearance of spontaneous recurrent seizures, which are the hallmark of epilepsy. The period between the initial insult and the emergence of spontaneous seizures is called epileptogenesis, and it is associated with neuronal death, neurogenesis, gliosis, axonal and dendritic plasticity, and changes in the extracellular matrix (reviewed by Jutila et al., 2002).

The current treatment of epilepsy is purely symptomatic; that is, antiepileptic drugs (AEDs) only alleviate the symptoms, but do not cure the disease. A subgroup of TLE patients does not become seizure-free with the current medication and they often develop resistance to all available AEDs. Only a fraction of these patients are suitable for epilepsy surgery. Therefore, the design of treatments to either prevent the development of epilepsy in patients at risk or modify the course of disease so that it is easier to treat is in high demand. The design of antiepileptogenic treatments, however, has been compromised by the lack of information about the molecular changes underlying epileptogenesis.

cDNA microarray technology, which allows the simultaneous study of thousands of genes (Harrington et al., 2000), has provided a new method to study the molecular changes underlying the development of epilepsy (reviewed by Lukasiuk and Pitkanen, 2004). cDNA microarray screening is an unbiased technique to search for new research targets and to develop new ideas for possible therapeutic interventions. cDNA microarray studies have revealed that hundreds of genes in several functional classes have altered expression after SE, many of which were not previously linked to epilepsy or seizures (Hendriksen et al., 2001; Becker et al., 2003; Lukasiuk et al., 2003). One example of these candidate epileptogenesis-related genes is a cysteine proteinase inhibitor, cystatin C, whose gene expression is increased in the rat hippocampus during SE-induced epileptogenesis (Hendriksen et al., 2001; Lukasiuk et al., 2003). A review of the literature indicates that cystatin C is linked to several human neuropathological conditions and its expression is increased after cerebral ischemia in rat and gerbil (Palm et al., 1995; Ishimaru et al., 1996), suggesting that cystatin C could be an underlying factor in the neuropathological changes also

during epileptogenesis and thus merits further investigation. Furthermore, cystatin B, a protein belonging to the same cystatin superfamily as cystatin C, is linked to myoclonus epilepsy and its expression is similarly increased after seizures (Amato et al., 2000; Lehesjoki, 2003). Based on these observations, we hypothesized that cystatin C participates in the cellular alterations underlying network reorganization during epileptogenesis.

The aim of the present study was to investigate the expression of cystatin C protein in the hippocampus in normal rat, mouse, and human brain, as well as during epileptogenesis and epilepsy. The hippocampus was selected as an area of interest because it is a well-defined structure in the medial temporal lobe that undergoes several neurobiological alterations during epileptogenesis. The majority of previous investigations of cystatin C focused on non-neuronal systems and therefore the precise function of cystatin C in the brain is unknown. Therefore, our aim was also to elucidate the functional role of cystatin C in common neuropathological alterations that occur during epileptogenesis, such as neuronal cell death and neurogenesis.

2. REVIEW OF LITERATURE

2.1 Epilepsy

The word *epilepsy* comes from the Greek word *epilambanien*, which means "to be seized or overwhelmed by surprise". Epilepsy has been surrounded by great deal of myth and prejudice some of which, unfortunately, still prevail today. During ancient times, epilepsy was considered a sacred disease and was attributed to possession by evil spirits or gods. The divine character of epilepsy was rejected by Hippocrates (400 BC), who gave the first natural explanations for the causes of epilepsy. For many years, however, the religious doctrine was that people with epilepsy were possessed with evil spirits, which affected society's attitudes towards the patients with epilepsy. Epilepsy patients were believed to be contagious and unclean and were therefore isolated from others. During the 18th century, the belief of supernatural causes of epilepsy was slowly discarded and more natural explanations started to gain ground in public thinking. Despite the increased knowledge and clinical progress in epilepsy treatment, the stigma of epilepsy still prevailed in the 20th century. In some cases, people with epilepsy were not allowed to marry or have children or even to enter restaurants, theatres, and other public places. Nonetheless, many famous people have accomplished significant achievements in their lives despite epilepsy and prevailing prejudice: Socrates, Alexander the Great, Peter the Great, Julius Caesar, Joan of Arc, Napoleon Bonaparte, Vincent van Gogh, Isaac Newton, Alfred Nobel, Leo Tolstoi, and Feodor Dostoevsky. (Engel, 1989; EUCARE, 2003)

2.1.1 Definitions and classification

Epilepsy is not a single disease, but rather comprises a group of diverse disorders with recurring epileptic seizures. By current definition according to the International League Against Epilepsy (ILAE), epilepsy is *a disorder of the brain that is characterized by predisposition to generate epileptic seizures*, which are transient symptoms arising from abnormal neuronal activity (Fisher et al., 2005). Epilepsy is also *associated with neurobiological, cognitive, psychological and social disturbances* (Fisher et al., 2005).

ILAE has put forth great effort to launch classification and terminology schemes for epileptic seizures and syndromes that currently form the basis of epilepsy diagnosis (Commission on Classification and Terminology of the ILAE, 1981; 1989; Engel, 2001). Based on the present classification, seizures can be divided as *partial* (focal) or *generalised*, depending on whether the seizures start in only one hemisphere or in both hemispheres simultaneously. Partial seizures might further evolve to secondarily generalized seizures when the seizure activity spreads from the

original focus to other parts of the brain. The behavioural manifestations of partial seizures can be associated with motor, sensory, psychic, or autonomic signs depending on the location of the seizure focus. Generalized seizures may involve a sudden onset of unresponsiveness (absence seizures), muscle contractions (myoclonic), increased muscle tone (tonic), loss of muscle tone (atonic), bilateral jerks (clonic), or tonic-clonic (Grand Mal) manifestations (Engel, 1989; Browne and Holmes, 2000). Further, the seizures are described as *simple* when consciousness is not impaired and *complex* if consciousness is impaired. Epilepsy syndromes are classified based on the underlying etiology. *Idiopathic* epilepsies comprise syndromes that involve only epilepsy with no structural brain lesions or other neurologic symptoms. They are usually genetically transmitted and age-dependent (Engel, 1996). *Symptomatic* epilepsies are syndromes due to one or more structural lesions of the brain, and they represent the most common type of epilepsy in adults. A large proportion of symptomatic epilepsies have an unknown etiology and are thus called *probably symptomatic* (previously called cryptogenic; Commission on Classification and Terminology of ILAE, 1981; 1989; Engel, 1989; Engel, 2001).

2.1.2 Temporal lobe epilepsy and the concept of epileptogenesis

TLE is the most common localization-related symptomatic epilepsy, in which the seizures typically start from temporal lobe structures such as the hippocampus or amygdala (Engel, 1996). Temporal lobe seizures manifest as simple or complex partial seizures and can lead to secondary generalization. A typical temporal lobe seizure starts with an aura that commonly involves abdominal sensations (butterflies in the stomach), fear, anxiety, and feelings of déjà vu (Kotagal, 1991; Engel, 1996). The seizure then evolves to staring, arrest of motion, altered responsiveness, and stereotyped automatisms such as lip smacking and picking at clothing, tonic posturing, and head version to one side (Kotagal, 1991; Engel, 1996). The seizure is typically followed by transient amnesia and disorientation (Engel, 1996).

Most patients with TLE have a history of prior brain damaging insult such as birth trauma, febrile seizures in childhood, meningitis, head trauma, status epilepticus (SE), or stroke (Mathern et al., 1996; Blümcke et al., 2002). The initial insult is commonly followed by a latent period, called epileptogenesis, before the spontaneous recurrent seizures occur. During epileptogenesis, several cellular and molecular alterations take place, making the brain more susceptible to seizure generation (Jutila et al., 2002). Recurrent epileptic seizures begin to occur within months to years following the initial insult and the epilepsy lasts for the lifetime of the patient. The process of epileptogenesis is illustrated in Figure 1.

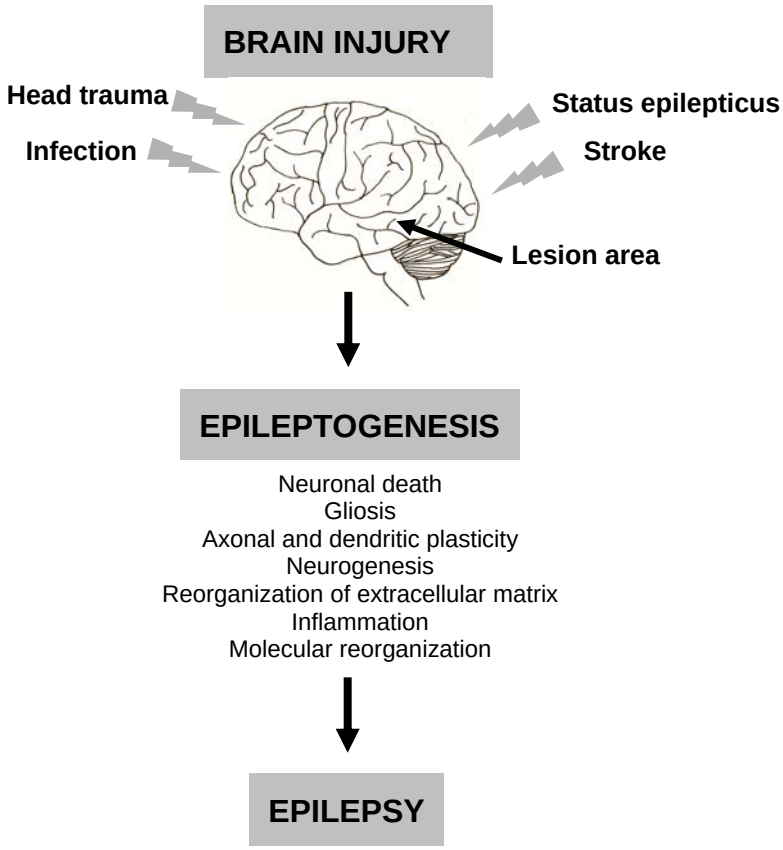

Figure 1. The process of epileptogenesis in symptomatic temporal lobe epilepsy. The initial brain damaging injury (head trauma, infection, status epilepticus, or stroke) initiates epileptogenesis characterized by several cellular and molecular alterations, leading ultimately to the reorganization of neuronal networks and the appearance of spontaneous recurrent seizures, *i.e.*, epilepsy. (Modified from Pitkänen and Sutula, 2002)

Currently, there is no cure for TLE and the treatment is purely symptomatic, directed towards alleviating the seizures. Some patients become seizure-free with the currently available AEDs, but a large group of patients (30%) develop intractable seizures that are difficult to treat (Hauser and Hesdorffer, 2001). A subgroup of these patients obtain relief from surgical resection of the epileptogenic focus, but the majority of drug-refractory patients with TLE continue to suffer from recurring seizures, which ultimately leads to the worsening of cognitive function (Pitkänen and Sutula, 2002). The period of epileptogenesis provides an attractive target for novel therapeutic interventions to prevent the development of epilepsy in patients at risk. Therefore, it is important to recognize and understand the events that occur during epileptogenesis. Epileptogenesis has been

under vigorous investigation during the past several decades, and a great deal of what we know today has been gained from experiments using animal models of epilepsy.

2.1.3 Animal models of temporal lobe epilepsy

The best model for studying the human condition would be human itself, but for obvious reasons the use of patients for basic science is not tenable. Therefore, during the past few decades attempts have been made to create animal models that mimic the human condition: demonstrate spontaneous seizures, have a latent period following an initial insult, similar neuropathology as humans, and display persistent chronic hyperexcitability (reviewed by Sarkisian, 2001; Coulter et al., 2002; White, 2002; Morimoto et al., 2004). In an ideal situation, the model would also have similar etiology, reflect a similar age as humans, and have seizure characteristics similar to those of humans (Sarkisian, 2001). Unfortunately, none of the current animal models replicate all aspects of human epilepsy. They do, however, provide a valuable tool to investigate the process of epileptogenesis from the initial insult to the latent period and the emergence of epileptic seizures. This is not possible in human samples obtained solely at the chronic stage of epilepsy.

The different animal models of epilepsy can be broadly separated into two categories: seizure models and epilepsy models (Sarkisian, 2001; White, 2002). Models with SE as an initial insult currently best mimic human epilepsy by demonstrating spontaneous recurrent seizures following the latent period and neuropathological alterations similar to those of patients with TLE (Morimoto et al., 2004). SE can be induced by systemic or local administration of a chemoconvulsant (*e.g.*, kainic acid or pilocarpine) or by electrical stimulation of the temporal lobe structures (Turski et al., 1983; Ben-Ari, 1985; Nissinen et al., 2000). These models, however, only model epilepsy that has developed as a consequence of SE. Models for posttraumatic and post-stroke epilepsy have only recently been developed, and their characterization is still in its infancy (Kelly et al., 2001; Pitkänen et al., 2005).

2.1.3.1 Kainic acid model

Kainic acid (or kainate) is a glutamate agonist that was originally extracted and purified from a red marine algae, *Digenea simplex*, and used as an antihelminthic drug in Japan (Nadler, 1979). The neurotoxic action of kainate was first demonstrated by Olney and coworkers (Olney et al., 1974). Since then several researchers have described the actions of kainate on neuronal populations, and its ability to induce seizures and epilepsy in rodents, especially rats (Ben-Ari et al., 1980; Nadler et al., 1980; Schwob et al., 1980; Sperk et al., 1983; Ben-Ari, 1985; Tanaka et al., 1992; Sperk, 1994). Kainate can be applied either systemically (*i.e.,* intraperitoneally, intravenously,

subcutaneously) or intracerebrally (*i.e.,* intraventricularly or locally to the brain parenchyma, such as hippocampus or amygdala) (Nadler et al., 1980; Sperk et al., 1983; Ben-Ari, 1985). Systemic or intraventricular kainate injection results in bilateral excitation of the hippocampus, whereas locally administered kainate elicits focal seizures originating from the injection site (Tanaka et al., 1992). The behavioural and neuropathological changes, however, are quite similar between these two application routes. Systemic kainate induces various behavioural signs such as immobility and staring, "wet-dog shakes", and generalized convulsions characterized by masticatory and facial movements, forepaw tremor, rearing, and loss of postural control (Schwob et al., 1980; Ben-Ari, 1985; Sperk, 1994; Tuunanen et al., 1999). The kainate-induced seizures then become more complex and animals develop SE attributed to continuous convulsions lasting for several hours. The behavioural signs are associated with localized discharges in the hippocampus, seizure spread to the limbic system, and generalization to other brain structures (Ben-Ari, 1985). SE induces epileptogenesis in affected animals, leading to the appearance of recurrent spontaneous seizures within weeks to months following SE (Tanaka et al., 1992; Kemppainen and Pitkänen, 2004; Narkilahti and Pitkänen, 2005).

The kainate model of TLE is not as well characterized in mice as it is in rats. There are reports of systemic, intrahippocampal, or intraamygdaloid injections of kainate in mice (Hu et al., 1998; Bouilleret et al., 1999; Araki et al., 2002; Riban et al., 2002). There are, however, several differences between mouse and rat, such as the higher mortality, more severe seizures, and lower number of animals developing chronic epilepsy in mice (Lähteinen et al., 2003). Also, the different susceptibility of various mouse strains (DBA/2 vs. C57BL/6) to kainate-induced seizures and to subsequent neuronal death makes the application of the kainate model difficult in mice (Ferraro et al., 1995; Schauwecker and Steward, 1997; McKhann et al., 2003). The careful characterization of the kainate model in mouse is also important because of the increased use of transgenic mice in current research strategies.

2.1.3.2 Amygdala stimulation model

SE and subsequent development of epilepsy with the emergence of spontaneous seizures can also be elicited by electrical stimulation of the temporal lobe structures, such as the amygdala (Nissinen et al., 2000). Stimulation of the lateral nucleus of the amygdala induces self-sustained SE in rats that is characterized by high amplitude and frequency discharges (HAFDs) observed in electroencephalography (EEG) and which are associated with behavioural seizures. Following SE, the rats undergo epileptogenesis and begin to experience spontaneous recurrent seizures within approximately one month after stimulation (Nissinen et al., 2000). The recurrent seizures are

secondarily generalized, but evolve later to partial seizures, similar to human TLE (Tinuper et al., 1996). Once the seizures occur, there is no remission. The great advantage of the electrical stimulation model compared to the kainate model is that direct neurotoxicity by the chemoconvulsant can be avoided. Also, the severity of SE can be more easily controlled and the effects of seizure severity on epileptogenesis can be investigated. The surgery and implantation of electrodes with subsequent stimulation paradigms, however, makes it laborious to perform.

2.1.3.3. Photothrombotic stroke model

Stroke is the single most common cause of TLE in the elderly population (Beghi, 2004). For a long time, there were no animal models for post-stroke epileptogenesis or epilepsy. There is a great need for such models because the increasing average age of the population has increased the contribution of stroke to the number of cases of symptomatic epilepsy. The widely used animal model of ischemic stroke, middle cerebral artery occlusion, is not suitable for epilepsy research because while seizures occur in ischemic animals within 2 weeks of surgery, the animals do not develop epilepsy (Karhunen et al., 2003). A photosensitive dye, Rose Bengal, can be used to produce focal cortical lesions of highly reproducible size and location (Watson et al., 1985). Intravenously injected Rose Bengal is transcranially activated with a light beam, leading to the generation of oxygen radicals and subsequent damage to the endothelial cells, platelet aggregation, and eventually arterial blockage (Dietrich et al., 1987b). Stroke induced by photothrombosis results in epilepsy in approximately 20% to 30% of rats (Kelly et al., 2001; Bezvenyuk et al., 2003; Kharlamov et al., 2003). Recurrent spontaneous seizures begin to occur within 2 months after stroke (Kelly et al., 2001; Kharlamov et al., 2003).

2.2 Neuropathology of the temporal lobe epilepsy

2.2.1 Anatomy of the hippocampus

The hippocampal formation is a well-defined structure in the temporal lobe that is essential for long-term memory, and is implicated to have a central role in TLE. The anatomical features of the hippocampus are evolutionarily rather conserved; that is, there is a high degree of structural similarity between human and rat as illustrated in Figure 2. The hippocampal formation is comprised of several cytoarchitectonically distinct regions that are interconnected: the dentate gyrus, hippocampus proper (also known as Ammon's horn), subicular complex, and entorhinal cortex (Amaral and Insausti, 1990; Amaral and Witter, 1995). The dentate gyrus is further divided into three layers: the granule cell layer, molecular layer, and hilus (or polymorphic layer) (Fig. 2). The granule cell layer contains the principal cells of the dentate gyrus. The molecular layer

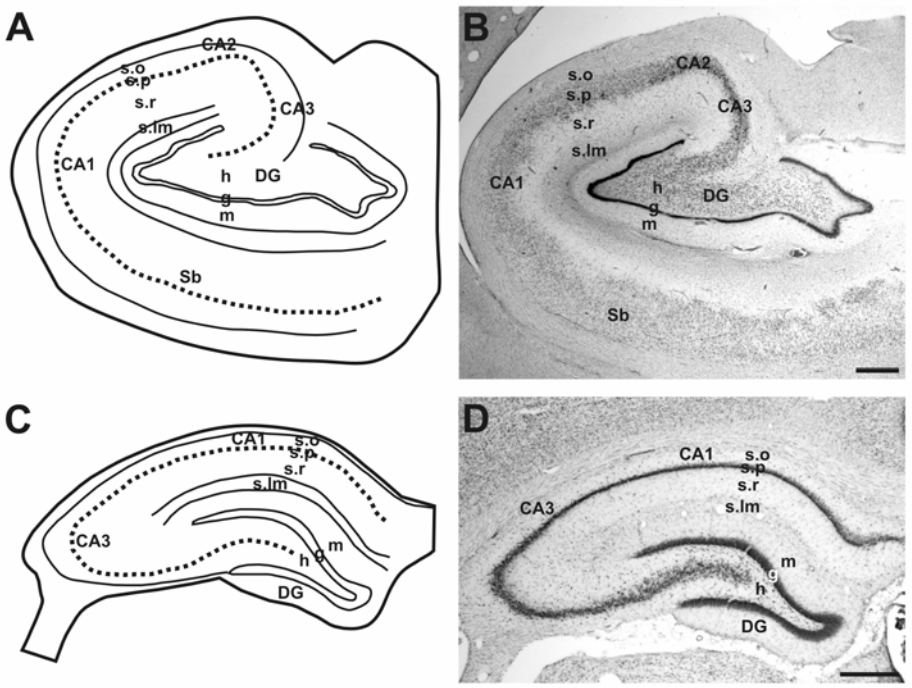

Figure 2. Illustration of the hippocampal anatomy. (A, C) Schematic line drawings of human (A) and rat (C) hippocampal formation demonstrating principal subregions and layers. (B, D) Digital photomicrographs of Nissl-stained sections illustrating the human (B) and rat (C) hippocampus. Abbreviations: s.o, *stratum oriens*; s.p, *stratum pyramidale*; s.r, *stratum radiatum*; s.lm, *stratum lacunosum-moleculare*; Sb, Subiculum; m, molecular layer; h, hilus; g, granule cell layer of the dentate gyrus. Scale bars: (B) 1 mm, (D) 500 µm.

harbours the apical dendrites of the granule cells, scattered interneurons, and synaptic terminals of the perforant path. The hilus includes several different cell types such as mossy cells and interneurons, hence the name polymorphic layer (Scharfman, 1999). The hippocampus proper is separated into CA1 (Cornu Ammonis 1), CA2, and CA3 regions, which are further laminated into the alveus, *stratum oriens*, *stratum pyramidale*, *stratum radiatum*, and *stratum lacunosum-moleculare*, or *stratum lucidum* (only in CA3) (Amaral and Insausti, 1990; Amaral and Witter, 1995). The principal cells of the hippocampus proper, the pyramidal cells, are located in the *stratum pyramidale* with their axons in the alveus and dendrites in the *stratum oriens* and *stratum radiatum*. In this thesis, the term hippocampus is used to describe hippocampus proper together with the dentate gyrus if not stated otherwise.

Classically, the information flow within the hippocampus is described as a trisynaptic-loop, but current knowledge about intrinsic hippocampal connections suggests that it is actually more

complicated (Amaral, 1993). The principles of intrinsic hippocampal connections are illustrated in Figure 3. The major input to the dentate gyrus and hippocampus proper comes from the entorhinal cortex where the two perforant path projections arise. One projection runs from layer II of the entorhinal cortex to the dentate gyrus and CA3, and the other from layer III to the CA1 and subiculum (Amaral, 1993; Amaral and Witter, 1995). From the dentate gyrus, the information flows to the CA3 via mossy fibers, the granule cell axons, which run in the *stratum lucidum*. The CA3 region is further connected to CA1 by Schaffer collaterals that synapse with CA1 pyramidal cells in the *stratum radiatum*. CA1 thus receives information from the entorhinal cortex via monosynaptic, disynaptic, and trisynaptic connections (*i.e.,* straight from the entorhinal cortex and via CA3 and dentate gyrus, respectively). The processed information returns to the deep layers of the entorhinal cortex (IV-VI) either straight from the CA1 or via the subiculum (Amaral, 1993; Amaral and Witter, 1995).

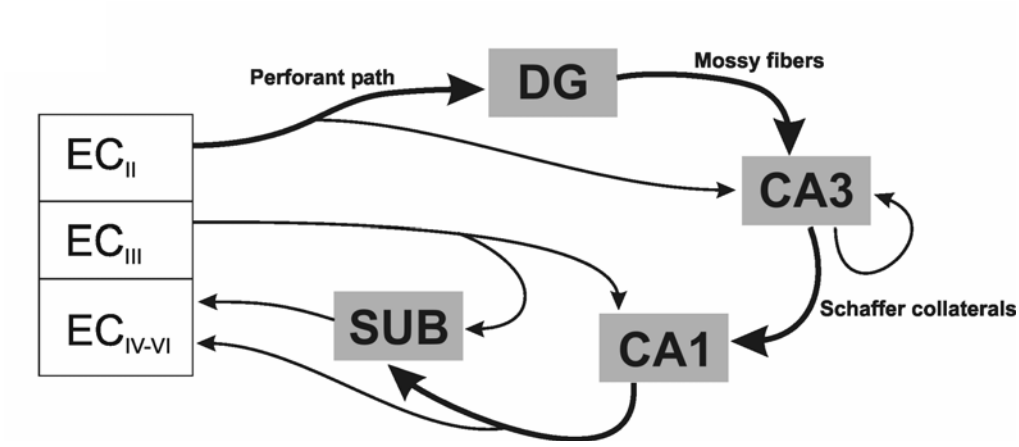

Figure 3. Schematic representation of the principal intrahippocampal connections. The major input to the dentate gyrus (DG) and hippocampus proper (CA3 and CA1) originates from layers II and III of the entorhinal cortex (EC_{II}, EC_{III}). The information then flows to the CA3 via mossy fibers and further to the CA1 via the Schaffer collaterals. The processed information returns back to the deep layers of the entorhinal cortex (EC_{IV-VI}) either straight from the CA1 or via the subiculum (SUB). Thick arrows depict the classical tri-synaptic loop. (Modified from Amaral and Witter, 1995)

2.2.2 Hippocampal sclerosis

2.2.2.1 Hippocampal sclerosis in human epileptic patients

Hippocampal sclerosis is a term used to describe a specific pattern of neuronal loss and subsequent gliosis detected in the hippocampus in 45% to 70% of patients with TLE (Armstrong, 1993; Babb and Pretorius, 1996; Blümcke et al., 2002). Hippocampal sclerosis was first described in autopsied epileptic brain already at the beginning of the 19th century (1825) by Bouchet and Cazuvieilh (cited from Gloor, 1991). Their definition was based merely on the macroscopic evaluation of shrunken and hardened hippocampal tissue. The first histological description of hippocampal sclerosis was provided by Sommer in 1880, who defined the neuronal damage in a region that was later known as Sommer´s sector (CA1). The findings of Sommer were confirmed by Bratz (1899) who described all the microscopic features of hippocampal sclerosis that are known today. The first link between hippocampal sclerosis and TLE originated from the studies of Stauder in 1936 and was later proven by Margerison and Corsellis in 1966 (cited from Gloor, 1991). Samples obtained from epilepsy surgery also confirmed the presence of hippocampal sclerosis in patients with intractable TLE (Falconer et al., 1964; Mathern et al., 1996). The presence of hippocampal sclerosis both in autopsy and surgery samples from epileptic patients is clearly described.

Hippocampal sclerosis (also called Ammon's horn sclerosis or mesial temporal sclerosis) is defined as segmental pyramidal cell loss and subsequent gliosis in the CA1, CA3, and hilus, which leads to shrinkage and hardening of the hippocampus (Margerison and Corsellis, 1966; Meencke and Veith, 1991; Blümcke et al., 2002). The CA2 neurons and granule cells of the dentate gyrus are more resistant to hippocampal damage but may also degenerate. Disorganization of dentate granule cells, *i.e.,* granule cell dispersion, is a common histopathologically distinguished phenomenon that occurs in hippocampal sclerosis (Houser, 1990). Neuronal loss in the hippocampus is also frequently accompanied by robust reorganization of the granule cell axons, which is called mossy fiber sprouting (Sutula et al., 1989; Houser et al., 1990). The axons of the granule cells make new connections with the proximal portions of the apical dendrites of the granule cells that are located in the molecular layer of the dentate gyrus (Sutula et al., 1989). Many other cellular and molecular alterations have been linked to hippocampal sclerosis, such as changes in neurotransmitters and their receptors, neuropeptides, and ion channels (Glass and Dragunow, 1995; Blümcke et al., 1999).

2.2.2.2 Hippocampal sclerosis in experimental animal models

The neuropathological changes in animal models with SE as the initial insult mimic those in human TLE, but there are differences in the degree and location of the damage between various models. In the kainate model, the pattern of neuronal damage is dependent on the application route of the kainate, the most sensitive areas being hippocampal CA3 and hilus. Systemic kainate injection induces widespread damage in the olfactory cortex, amygdala, and thalamus, but most prominently in the hippocampal CA3 and hilar regions (Schwob et al., 1980; Ben-Ari, 1985). The CA1 region is also damaged, but to a lesser extent. Intracerebral injection of kainate induces damage to the injection site as well as in regions that are connected to the injection site. For example, after intraamygdaloid application of kainate there is also damage in the CA3 area of the hippocampus (Henshall et al., 2000). In contrast to rat models, intrahippocampal injection of kainate in Swiss mice results in extensive neuronal loss in the CA1 region (Bouilleret et al., 1999; Riban et al., 2002). Mossy fiber sprouting is also associated with neuronal loss in kainate-treated animals, similar to human TLE (Sutula et al., 1998).

In mice, the conspicuous feature of kainate-induced neuronal death is that it is largely dependent on the genetic background of the mouse strain (Schauwecker and Steward, 1997; Schauwecker, 2002). Several commonly used mouse strains (*e.g.*, C57BL/6 and BALB/c) are resistant to kainate-induced neuronal death whereas other strains such as DBA/2J, FVB/N, and 129/SvEMS are highly vulnerable to excitotoxic damage induced by systemic kainate injection (Schauwecker and Steward, 1997; Araki et al., 2002; Schauwecker, 2002; McKhann et al., 2003). A recent study, however, demonstrated that by using sensitive indicators of neuronal death (such as cupric-silver and Fluoro-Jade B [FJB] stainings), cell death can be detected in C56BL/6 mice following kainate-induced seizures (Benkovic et al., 2004). The resistance to kainate-induced cell death might also be dependent on the application route of the kainate; accordingly Araki and colleagues (2002) demonstrated that intraamygdaloid injection of kainate in C56BL/6 mice produces robust neurodegeneration in the hippocampus.

Electrical stimulation of the amygdala leads to widespread damage in the amygdala as well as the surrounding cortex and hippocampus (Nissinen et al., 2000). Neuronal death in the hippocampus is located in the hilus, CA1, CA3, and subicular regions and is accompanied by robust mossy fiber sprouting similar to the kainate model and human TLE (Sutula et al., 1989; Nissinen et al., 2000). The damage induced by SE continues for weeks and months following stimulation, indicating the progressive features of neuronal death during epileptogenesis (Pitkänen et al., 2002).

2.2.3 Granule cell dispersion

2.2.3.1 Granule cell dispersion in human epileptic patients

Granule cells of the hippocampal dentate gyrus are resistant to neurodegeneration in epilepsy as compared to other regions in the hippocampus (Bruton, 1988). Hippocampal sclerosis is, however, typically associated with the dispersion of the granule cell layer, in which the normal organization of the granule cell layer is impaired (Houser, 1990; Houser et al., 1992; Lurton et al., 1997; Mathern et al., 1997; El Bahh et al., 1999). Originally, Houser (1990) described granule cell dispersion as a widening of the granule cell layer, in which the granule cells are no longer tightly positioned next to each other, and the clear border between the granule cell layer and the molecular layer of the dentate gyrus becomes irregular. In some cases, the dispersion is even more prominent and develops a bilaminar organization (Houser, 1990).

Granule cell dispersion occurs in 45% to 73% of patients with TLE (Houser, 1990; Lurton et al., 1997; Mathern et al., 1997; Lurton et al., 1998; El Bahh et al., 1999). It is typically associated with a risk factor, an early life seizure-insult such as febrile convulsions or seizures due to meningitis or encephalitis (Houser, 1990; O'Connor et al., 1996; Lurton et al., 1998; El Bahh et al., 1999). Also, granule cell dispersion is directly linked to the degree of hilar cell death in the hippocampus (Houser, 1990; Lurton et al., 1997; Mathern et al., 1997), and is thus one type of morphological change associated with hippocampal sclerosis.

2.2.3.2 Granule cell dispersion in experimental animal models

In contrast to human studies, there are only a few reports of granule cell dispersion in experimental animal models of TLE. Mello and coworkers (1992) reported widening of the granule cell layer 9 days following pilocarpine-induced SE in rats. The widening increased over time and became constant within 100 days after SE (Mello et al., 1992). Prominent granule cell dispersion was also observed 15 days after intrahippocampal injection of kainic acid in mice (Bouilleret et al., 1999). In this model, the dispersion reached maximal levels by 30 days. Oubain, a Na^+-K^+-ATPase blocker, induces a rapid dispersion of the granule cell layer when injected into the dentate gyrus (Omar et al., 2000). In contrast, granule cell dispersion has not been reported after systemic injection of kainate.

2.2.3.3 Mechanisms underlying granule cell dispersion

Several hypotheses are proposed for the mechanisms underlying the development of granule cell dispersion. Houser (1990) first proposed that granule cell dispersion is due to defective migration

of granule cells that occurs in the aftermath of an early epileptic event such as febrile seizures. This hypothesis was later replaced by the suggestion that granule cell dispersion is a form of seizure-induced structural plasticity, because it also occurs in adult animals after seizure activity (Mello et al., 1992; Bouilleret et al., 1999). In support of this idea, it was proposed that high levels of neuronal activation are a prerequisite for the development of granule cell dispersion because repression of glutamatergic neurotransmission or enhancement of GABAergic inhibition reduces granule cell dispersion induced by intrahippocampal kainate (Suzuki et al., 2005). On the other hand, dispersion might arise from enhanced neurogenesis and abnormal migration of the newborn granule cells following seizures (Haas et al., 2002; Thom et al., 2005). The expression of reelin is decreased in epileptic patients with granule cell dispersion (Haas et al., 2002; Frotscher et al., 2003). Reelin is proposed to act as a stop signal for migrating neurons during development (Frotscher, 1998). The hypothesis of abnormal neurogenesis is further supported by the increased number of newborn granule cells in the molecular layer of the dentate gyrus in epileptic patients and in especially those with granule cell dispersion (Thom et al., 2005). Despite previous work and proposed hypotheses the significance of granule cell dispersion in epilepsy remains disputable. It has, however, been hypothesized that granule cell dispersion leads to the formation of abnormal connections in the dentate gyrus and thus contributes to the altered hippocampal network in epileptic tissue (Houser, 1990).

2.2.4 Neurogenesis

The old dogma that the generation of new neurons, *i.e.,* neurogenesis, occurs only during development and not in adult brain has been disproved during the past decades. Forty years ago, Altman and Das demonstrated that new neurons continue to be born in the postnatal rat hippocampus (Altman and Das, 1965), but the significance of this finding was not acknowledged until later (Gross, 2000). There is now clear evidence that in the adult mammalian brain there are two regions where neurogenesis persists throughout life: the subgranule zone of hippocampal dentate gyrus (SGZ) and the subventricular zone in the walls of the lateral ventricles (SVZ) (reviewed by Abrous et al., 2005). The process of neurogenesis is a series of distinct steps, including proliferation, survival, and migration of neuronal progenitor cells, and their differentiation and maturation into functional neurons (Gage et al., 1998). In the dentate gyrus, the neural progenitor cells reside and proliferate in the SGZ located between the granule cell layer and hilus of the dentate gyrus (Kuhn et al., 1996). From there they migrate to the granule cell layer (Kuhn et al., 1996), differentiate into neurons (Cameron et al., 1993), and develop the morphological and electrophysiological characteristics of functional granule cells, such as

dendritic spines, axons projecting into CA3 region, action potentials, and synaptic inputs (Hastings and Gould, 1999;van Praag et al., 2002). Only a minority of neural progenitor cells differentiate into glial cells (Cameron et al., 1993). The integration of newborn neurons into existing neuronal networks suggests that neurogenesis is not just a remnant of the developmental period with no functional significance, but has unknown effects on hippocampal function.

Neurogenesis occurs in the rodent brain at a basal rate, approximately 9000 new cells per day (Cameron and McKay, 2001), throughout life, but declines with increasing age (Seki and Arai, 1995; Kuhn et al., 1996). Also, many other factors decrease the neurogenesis in adult mammalian brain (Gage et al., 1998; Abrous et al., 2005), including aging, stress, corticosteroids, opiates, and NMDA receptor activation (Gould et al., 1992; Cameron et al., 1995; Gould and Tanapat, 1999; Eisch et al., 2000). In contrast, physical exercise, enriched environment, learning, growth factors, estrogen, and several neurotransmitters increase the rate of neurogenesis (Kempermann et al., 1997b; Gould et al., 1999; Tanapat et al., 1999; van Praag et al., 1999). Furthermore, there is increased proliferation of neural progenitors following different types of brain injury such as mechanical lesions, excitotoxicity, ischemia, traumatic brain injury, and seizures (Gould and Tanapat, 1997; Parent et al., 1997; Dash et al., 2001; Kee et al., 2001).

2.2.4.1 Acute seizure–induced neurogenesis in experimental animal models

Increased proliferation of neural progenitor cells is one of the neuroplastic changes that occur in the dentate gyrus following seizures (reviewed by Ribak and Dashtipour, 2002). The effects of different types of seizures on neurogenesis have been widely studied in rodent models of acute seizures and epileptogenesis (see Table 1). There is increased neurogenesis in the SGZ of the dentate gyrus following both chemically (e.g., pilocarpine, kainate, and pentylenetetrazol) and electrically (e.g., hippocampus or perforant path stimulation) induced seizures (Bengzon et al., 1997; Parent et al., 1997; Parent et al., 1998; Scott et al., 1998; Covolan et al., 2000; Nakagawa et al., 2000; Ferland et al., 2002; Jiang et al., 2003; Zhu et al., 2005). Even a single afterdischarge produces an increased rate of neurogenesis in the dentate gyrus (Bengzon et al., 1997). Chemoconvulsants induce a 3 to 8-fold increase in the proliferation of neural stem/progenitor cells, which peaks 3 to 7 days after SE and returns to baseline within 28 days (Parent et al., 1997; Gray and Sundstrom, 1998). Electrically-induced kindling produces a slightly lower increase in proliferation (2 to 3-fold) (Bengzon et al., 1997; Parent et al., 1998; Nakagawa et al., 2000).

TABLE 1. Experimental studies assessing neurogenesis in the subgranule zone of the dentate gyrus (SGZ) following seizures and status epilepticus.

MODEL	RODENT	METHOD	RESULTS	REFERENCE
Pilocarpine				
320-350 mg/kg i.p.	Rat (SD)	BrdU (50 mg/kg i.p.)	A 6-8-fold increase in the number of BrdU+ cells at 3, 6, and 13 d after SE, back to baseline by 27 d	Parent et al., 1997
		PCNA	Increase in PCNA+ cells comparable to BrdU	
		TOAD-64	Increased number of TOAD-64+ cells 28 d after SE, ectopic locations	
320-350 mg/kg i.p.	Rat (Wistar)	BrdU (50 mg/kg i.p.)	A 3-7-fold increase in the number of BrdU+ cells	Covolan et al., 2000
340 mg/kg i.p.	Mice (♀ C57BL/6)	BrdU (50 mg/kg i.p.)	A 2-fold increase in the number of BrdU+ cells at 7 d and 14 d post-SE	Couillard-Despres et al., 2005
		Doublecortin (DCX)	Numbers of DCX+ cells doubled after seizures	
60 mg/kg s.c.	Rat (SD) P21	BrdU (50 mg/kg i.p.)	A modest increase in the number of BrdU+ cells after recurrent seizures	Cha et al., 2004
Kainic acid				
i.c.v.	Rat (Wistar)	BrdU (50 mg/kg i.v.)	A 6-fold increase in the number of BrdU+ cells bilaterally 1 wk after kainate	Gray and Sundstrom, 1998
10 mg/kg i.p.	Rat (Wistar)	BrdU (50 mg/kg i.p.)	An increase in the number of BrdU+ cells, higher than in pilocarpine-treated animals	Covolan et al., 2000
	Rat (SD)	RNR and BrdU (50 mg/kg i.p.)	A 5-fold increase in the number of RNR-IR cells at 7d and 28d after SE	Zhu et al., 2005
12 mg/kg i.p.	Rat (SD)	BrdU (100 mg/kg i.p.)	An increase in the number of BrdU+ cells peaked at 5 d and returned to baseline by 10 d	Nakagawa et al., 2000
Pentylenetetrazol (PTZ)				
60 mg/kg i.p.	Rat (SD)	BrdU (50 mg/kg i.p.)	A 6-fold increase in the number of BrdU+ cells at 3,7 and 14 d after PTZ, back to baseline by 28 d	Jiang et al., 2003

Abbreviations: BrdU, 5-Bromo-3′-deoxyuridine; GCL, granule cell layer; i.c.v., intracerebroventricularly; i.p., intraperitoneally; i.v., intravenously; PCNA, proliferating cell nuclear antigen; RNR, ribonucleotide reductase; s.c., subcutaneously; SD, Sprague-Dawley; SE, status epilepticus; sz, seizure; TOAD-64, Turned on after division-64.

TABLE 1. Experimental studies assessing neurogenesis in the subgranule zone of the dentate gyrus (SGZ) following seizures and status epilepticus.

MODEL	RODENT	METHOD	RESULTS	REFERENCE
Kindling				
Hippocampus	Rat (SD)	BrdU (37.5 mg/kg i.p.)	Increased number of BrdU+ mature and immature neurons after a single stimulation, further increased after 40 stimulations	Bengzon et al., 1997
Perforant path	Rat (SD)	BrdU (50 mg/kg i.p.)	An increase in the number of BrdU+ cells at 6 d after 6 h stimulation	Parent et al., 1997
	Rat (SD)	BrdU (100 mg/kg i.p.)	An increase in the number of BrdU+ cells after 5 stage I motor seizures	Nakagawa et al., 2000
Amygdala	Rat (SD)	BrdU (50 mg/kg i.p.)	A 2-3-fold increase in the number of BrdU+ cells in animals receiving $\geq$9 class 4/5 sz	Parent et al., 1998
		TOAD-64	An increase in TOAD-64+ immature neurons in the hilar portion of GCL	
	Rat (Wistar)	BrdU (100 mg/kg i.p.)	An increase in the number of BrdU+ cells following secondary generalized motor seizures	Scott et al., 1998
Fluorothyl kindling				
1 or 8 sz	Mice (C57BL/6J)	BrdU (50 mg/kg i.p.)	Increased number of BrdU+ cells at 1 and 3 d after single sz, and at 1,3,7 d after 8 sz	Ferland et al., 2002
Electroconvulsive seizures				
Earbar electrodes	Rat (Wistar)	BrdU (37.5 mg/kg i.p.)	A single ECS doubled the number of BrdU+ cells at 3 d returned to baseline by 7 d 1 mo after a single ECS, a 3-fold increase in the number of BrdU+ cells	Madsen et al., 2000
Corneal electrodes	Rat (Wistar)	BrdU (50 mg/kg i.p.)	ECS-treatment doubled the number of BrdU+ cells at 1 mo	Scott et al., 2000

The majority of newborn cells differentiate into neurons and migrate into the granule cell layer within 4 weeks. Some newborn cells, however, migrate to abnormal locations in the hilus, hilar/CA3 border, and inner molecular layer of the dentate gyrus (Parent et al., 1997; Scharfman et

al., 2000). Scharfman and colleagues also demonstrated that newborn neurons in the hilar/CA3 border develop electrophysiological characteristics resembling those of mature granule cells, suggesting that they are functional neurons and have integrated into existing networks (Scharfman et al., 2000; Scharfman et al., 2002).

2.2.4.2 Neurogenesis in chronic epilepsy – evidence from human studies

The impact of spontaneous recurrent seizures and chronic epilepsy on neurogenesis has been studied much less than the acute effects of induced seizures. In rodents, Cha and colleagues (2004) demonstrated that recurrent seizures occurring 2 months after pilocarpine-induced SE resulted in a modest increase in neurogenesis compared with non-seizing animals or controls. In contrast, chronically epileptic rats, 5 months after kainate-induced SE, demonstrated a severe decline in neurogenesis as assessed using the immature neuronal marker doublecortin (Hattiangady et al., 2004). The decline was more prominent when seizure frequency was higher, suggesting that a greater number of seizures is detrimental to neurogenesis (Hattiangady et al., 2004). Therefore, a similar situation might occur in human patients with chronic epilepsy. The data regarding neurogenesis in human epilepsy, however, remain controversial.

The study of neurogenesis in human tissue has been hampered by the inability to use BrdU as a direct marker for dividing cells, as used in rodents. Instead, a wide variety of indirect markers of neurogenesis that label either dividing cells or immature neurons have been used (see Table 2), thus making the data interpretation more difficult. Mikkonen and coworkers (1998) demonstrated a 2-fold increase in newborn cells in the SGZ in adult patients with drug-refractory TLE using polysialylated neural adhesion molecule (PSA-NCAM). If the hippocampal damage was severe, however, there were only occasional PSA-NCAM-positive cells in the SGZ (Mikkonen et al., 1998). The same marker revealed decreased neurogenesis in hippocampal specimens from pediatric patients with extratemporal epilepsy (Mathern et al., 2002). Moreover, in pediatric patients with the epileptic focus in the temporal lobe, Blümcke and colleagues (2001) described an increased number of immature, nestin-positive neurons in patients younger than 2 years of age. In adult patients with TLE, there is an increased number of dividing cells in the granule cell layer observed using Mcm2 (mini chromosome maintenance protein 2), $p27^{Kip1}$, PCNA (proliferating cell nuclear antigen), and Musashi-1 as markers for neurogenesis (Crespel et al., 2005; Thom et al., 2005). These data, in spite of the inconsistent findings, suggest that proliferation of neural progenitor cells is increased in chronic epileptic hippocampus but their survival, differentiation, and maturation is impaired, because PSA-NCAM is expressed later in the course of neurogenesis than are the division markers.

TABLE 2. Cellular markers for the study of neurogenesis.

MARKER	TARGET	REFERENCE
Proliferating cells		
BrdU *(5-bromo-3´-deoxyuridine)*	Incorporates into DNA of dividing cells during the S-phase of the cell cycle, permanent incorporation enables study of long-term fate of neural stem cells	Miller and Nowakowski, 1988 Parent et al., 1997
Mcm2 *(Mini chromosome maintenance protein 2)*	A protein part of the DNA replication complex, expressed in all cell cycle phases, expressed in neural stem cells and progenitor cells, down-regulated in mature neurons	Maslov et al., 2004 Thom et al., 2005
PCNA *(Proliferating cell nuclear antigen)*	A nuclear protein expressed mainly during the G_1-phase of the cell cycle	Rankin et al., 2004 Crespel et al., 2005
$P27^{kip1}$	A G_1-phase cyclin-dependent kinase inhibitor	Polyak et al., 1994 Crespel et al., 2005
RNR *(ribonucleotide reductase)*	A cytoplasmic enzyme required for DNA synthesis, catalyzes the reduction of ribonucleotides to deoxyribo-nucleotides, expressed in all phases of the cell cycle	Zhu et al., 2003 Zhu et al., 2005
Ki-64	A nuclear protein expressed in all phases of the cell cycle	Scholzen and Gerdes, 2000 Kee et al., 2002
Progenitor cells		
Musashi-1 *(Msh-1)*	An RNA binding protein highly expressed in the nervous system, expressed in dividing and post-mitotic cells	Okano et al., 2002 Crespel et al., 2005
Nestin	An intermediate filament protein expressed at the time of early neural differentiation	Lendahl et al., 1990 Blümcke et al., 2001
Immature neurons		
Doublecortin	A microtubule-associated phospho-protein required for neuronal migration and differentiation, expressed in proliferating progenitor cells and neuroblasts	Brown et al., 2003 Rao and Shetty, 2004 Couillard-Despres et al., 2005
PSA-NCAM *(Polysialylated neural cell adhesion molecule)*	A glycoprotein involved in neuronal migration, extension of dendrites and pathfinding, transiently expressed in post-mitotic cells, also in mature cells	Seki and Arai, 1993 Mathern et al., 2002 Seki, 2002
Tuc-4 *(Collapsin response mediated protein-4, Turned-on after division-64)*	A protein associated with growth cone formation, expressed by post-mitotic neurons as they begin their migration, not in progenitor cells	Minturn et al., 1995 Parent et al., 1997 Nacher et al., 2000

2.2.4.3 Mechanisms for seizure-induced neurogenesis

The underlying mechanisms that result in seizure-induced increase of neurogenesis remain largely obscure, but several mechanisms have, however, been proposed. The triggering factors might arise directly from electrical activation of the precursor cells or indirectly from seizure-induced neuronal death, alterations in levels of growth factors, neurotransmitters, neurotrophic factors, extracellular matrix proteins, or adrenal hormones (Parent, 2002). Accordingly, it was demonstrated that increased neurogenesis occurs concomitantly with increased expression of neurotrophic factors after pilocarpine-induced seizures (Hagihara et al., 2005).

The observation that neurogenesis declines severely in epileptic rats (Hattiangady et al., 2004) raises questions about the possible mechanisms that lead to attenuation of progenitor cell proliferation after acute induction. One possibility is that spontaneous seizures increase death of newborn neurons similarly to the way they induce cell death of mature neurons. Also, inflammation is detrimental for neurogenesis (Ekdahl et al., 2003a; Monje et al., 2003). Because inflammation occurs in epileptic tissue both in rodent models and epileptic patients, it might contribute to the reduced survival of newborn neurons (Crespel et al., 2002; Voutsinos-Porche et al., 2004). Furthermore, the concentrations of cell proliferation factors (fibroblast growth factor-2 [FGF-2] and insulin growth factor-1 [IGF-1]) are decreased in epileptic rats at 5 -months following kainate administration, thus suggesting that the growth factor levels affect the rate of proliferation (Hattiangady et al., 2004). Finally, the differentiation of newly generated cells into mature granule cells is impaired in the hippocampus of human patients with chronic epilepsy, leading to a decline in neurogenesis (Crespel et al., 2005). These results suggest that the microenvironment of epileptic hippocampus (the "neurogenic niche") controls not only the proliferation state, but also the migration and differentiation of newborn cells.

2.2.4.4 Significance of seizure-induced neurogenesis

Originally the birth of new neurons in the adult brain was considered to be a potential mechanism for neuronal repair and thereby beneficial for the brain. Currently, there is a growing body of evidence that altered neurogenesis might be harmful, and lead to hyperexcitability and promotion of the seizure state (Parent, 2002; Scharfman, 2004; Shapiro and Ribak, 2005). There are several possible effects of altered neurogenesis on epilepsy. First, the newborn neurons in the dentate gyrus might be responsible for the abnormal axonal reorganization of the dentate granule cells, *i.e.*, mossy fiber sprouting, which occurs in the rodent as well as in human epilepsy (Sutula et al., 1989; Sutula et al., 1998; Nissinen et al., 2000). This is supported by the finding that axons of newly-born neurons project to aberrant locations in the inner molecular layer of the dentate gyrus where

mossy fiber sprouting typically occurs (Parent et al., 1997). This hypothesis was, however, disproved by the demonstration that mossy fiber sprouting was not abolished by preventing neurogenesis (Parent et al., 1999). Second, ectopically located (*i.e.*, not within the granule cell layer) newborn neurons might underlie the hyperexcitability state leading to seizure generation (Parent et al., 1997; Scharfman et al., 2000). Ectopic hilar granule neurons exhibit many of the electrophysiologic characteristics of normal granule cells, but display hyperexcitability that occurs synchronously with CA3 pyramidal cell firing (Scharfman *et al.*, 2000). In addition, newly generated granule cells exhibit enhanced synaptic plasticity (Schmidt-Hieber et al., 2004), which also supports the hypothesis of newborn neurons underlying hyperexcitability. Furthermore, hyperexcitability might also result from impaired differentiation of newborn neurons into mature neurons. A number of newborn cells differentiate into GABAergic inhibitory interneurons (Liu et al., 2003) and if this fails, decreased inhibition and increased excitability in the hippocampus will prevail. Also, immature cells with glial-like properties can contribute to hyperexcitability. Third, newborn granule cells might underlie the development of granule cell dispersion, which is a common finding in hippocampal sclerosis in human epileptic patients (Houser, 1990). This is supported by the observation that newborn neurons expressing immature neuronal markers are found in aberrant locations in the hilus and molecular layer of the dentate gyrus following pilocarpine-induced SE (Parent et al., 1997). Furthermore, increased proliferation of neural progenitors in epileptic patients is associated with severe granule cell dispersion (Thom et al., 2005). Finally, reduced neurogenesis following seizures might cause memory disturbances, which are commonly associated with chronic epilepsy. The current hypothesis for the role of neurogenesis in hippocampal function is that hippocampus-dependent learning requires the integration of newborn neurons into existing circuits in the dentate gyrus (Schinder and Gage, 2004).

The effect of an increased number of new neurons on hippocampal function might be compromised because only a minority of newborn cells survive beyond 4 weeks and become fully mature neurons (Ekdahl et al., 2001). Following pilocarpine- or electrically-induced SE, more than 80% of newborn cells die within 4 weeks (Ekdahl et al., 2001; Ekdahl et al., 2003b) and thus are not available for long-term network alterations. Regardless of the speculation about the significance of neurogenesis in epilepsy, the exact role of newborn neurons remains to be elucidated.

2.2.5 Changes in gene expression

It is hypothesized that the alterations in gene expression due to seizure activity underlie the morphological changes that occur during epileptogenesis (Lukasiuk et al., 2003). The screening of these genetic modifications would help in finding new targets for AED design and offer new ways to control the seizure-associated pathology.

In recent years, the use of cDNA microarray technology has revolutionized the study of gene expression, allowing simultaneous investigation of a large number of genes (Harrington et al., 2000; Luo and Geschwind, 2001). The technology also provides an unbiased way to screen for the molecular changes underlying epileptogenesis. Seizure-induced changes in gene expression have been studied acutely following pentylenetetrazole-induced seizures (Sandberg et al., 2000), after kainate-induced SE (Tang et al., 2002), and amygdala kindled seizures (Gu et al., 2004), as well as later during epileptogenesis following pilocarpine- or electrically-induced SE (Hendriksen et al., 2001; Becker et al., 2003; Lukasiuk et al., 2003). Although there have been several studies, there is surprisingly little overlap in the genes detected between different studies (reviewed by Lukasiuk and Pitkänen, 2004). The functional groups of differentially expressed genes are more similar, which is perhaps more relevant because it is unlikely that any single gene is responsible for the wide variety of morphologic changes that occur during epileptogenesis. Genes that are differentially regulated acutely after seizures or early during epileptogenesis are associated with transcriptional regulation, stress-response, cellular damage, immune response, proteases, and cytoskeletal proteins (Tang et al., 2002; Becker et al., 2003; Lukasiuk et al., 2003; Gu et al., 2004; Lukasiuk and Pitkänen, 2004). Later, during epileptogenesis there is altered expression of genes associated with synaptic, axonal, and cytoskeletal plasticity, protein processing, gliosis, cell adhesion, cellular metabolism and proteases and their inhibitors (Hendriksen et al., 2001; Becker et al., 2003; Elliott et al., 2003; Lukasiuk et al., 2003). These data further support the idea that epileptogenesis is regulated by a large number of genes affecting many functional processes. The cysteine proteinase inhibitor cystatin C gene is one of such candidate epileptogenesis-related genes whose expression is increased during the SE-induced epileptogenesis.

2.3 Cystatin C

Cystatin C is a member of the cystatin superfamily, which consists of a group of evolutionarily related proteins that inhibit lysosomal cysteine proteinases, *i.e.*, cathepsins. The cystatin superfamily is divided into four major families according to their sequence similarities (Barrett et al., 1984; Abrahamson et al., 2003; Mussap and Plebani, 2004). Type 1 cystatins, also called stefins, are small (MW~11 kDa) intracellular cytoplasmic proteins. Human cystatin A and cystatin B, as well as their homologues in rat, mouse, cow, and pig, are representatives of this family. Cystatin A is found mainly in epithelial cells and polymorphonuclear leukocytes, whereas cystatin B is widely distributed in cells and tissues. Cystatin B gene mutations are linked to Unverricht-Lundborg type progressive myoclonus epilepsy (Lehesjoki, 2003). Type 2 cystatins are mainly extracellular proteins. Cystatin C, together with chicken egg-white cystatin and salivary cystatins, cystatin D, S, SA, and SN belong to this family (Turk and Bode, 1991; Bobek and Levine, 1992; Abrahamson et al., 2003; Mussap and Plebani, 2004). The third family is the kininogen family, which contains three high-molecular mass cystatins, H- (high molecular weight), L- (low molecular weight), and T-kininogen. They are intravascular proteins that are expressed in the liver and function as precursors for vasoactive peptides, the kinins. The recently discovered type 4 cystatins (fetuins) are expressed mainly in the liver and function in the blood and brain during fetal life (Mussap and Plebani, 2004). Many other proteins with cystatin-related sequences have also been discovered (reviewed by Abrahamson et al., 2003; Mussap and Plebani, 2004).

Cystatin C and chicken egg-white cystatin are the most studied members of the cystatin superfamily. Cystatin C was first discovered in 1961 from human cerebrospinal fluid (CSF) and urine and was called γ-CSF or post-γ-protein (Mussap and Plebani, 2004). It was also called human γ-trace and post-γ-globulin before the name cystatin C was assigned by Barrett in 1984 (Barrett et al., 1984).

2.3.1 Cystatin C gene

The human cystatin C (CST3) gene is located in a gene cluster on chromosome 20p11.2 together with six other family 2 cystatin members (Abrahamson et al., 1989; Schnittger et al., 1993). Genes encoding mouse and rat cystatin C are located on chromosomes 2 and 3, respectively (Huh et al., 1995; Gauguier et al., 1999). The overall size of the human cystatin C gene is approximately 4.3 kb and consists of three exons interrupted by two introns (Abrahamson et al., 1990). The promoter region (5' flanking region) has several properties that are typical of housekeeping genes, and their presence might explain the ubiquitous and non-tissue specific expression of the cystatin C gene (Abrahamson et al., 1990). The promoter region does not contain a sequence for a CAAT-box, and

has a high GC-nucleotide content and a large number of CpG dinucleotides (Levy et al., 1989; Abrahamson et al., 1990), which might be related to its constant transcription (Bird, 1986). In addition, the promoter region contains a TATA-motif, ATAAA, (-119), and two binding sites for the transcription factor Sp1 (-730 and -146) (Levy et al., 1989; Abrahamson et al., 1990). In mouse, the sequence for the androgen responsive element and for pituitary transcription factor -1 are present in the cystatin C promoter (Huh et al., 1995). Recently, a regulatory site for interferon regulatory factor-8 was located in the human cystatin C promoter (Tamura et al., 2005) but, other than that, detailed descriptions of regulatory sites within the promoter region are lacking. The overall structure and organization of the cystatin C gene in mouse is similar to that in human, although there are some differences in the promoter region (Huh et al., 1995).

The human cystatin C gene has three polymorphic *Ksp*I restriction sites located in the 5' flanking region at positions -157 and -72, and in exon 1 at position +73 relative to the translation starting point (Balbin and Abrahamson, 1991; Balbin et al., 1993). These polymorphisms result in two human haplotypes: CST3 A has sequences GAG and CST3 B has sequence CCA at these positions (Finckh et al., 2000). The polymorphism in the coding region is located at the penultimate position of the signal peptide and results in a threonine for alanine substitution (Balbin et al., 1993). This polymorphism is associated with Alzheimer´s disease (AD) as well as with age-dependent macular degeneration (Crawford et al., 2000; Finckh et al., 2000; Zurdel et al., 2002). A single base substitution in exon 2 (CAG instead of CTG) is the primary cause of hereditary cystatin C amyloid angiopathy (HCAA) (Jensson et al., 1987; Palsdottir et al., 1988; Levy et al., 1989).

2.3.2 Cystatin C protein

Cystatin C protein is translated from the cystatin C gene as a proprotein containing a hydrophobic signal sequence of 26 amino acid residues, which is removed upon maturation (Grubb and Löfberg, 1982; Abrahamson et al., 1987; Abrahamson et al., 1990; Esnard et al., 1990). The active cystatin C protein is composed of 120 amino acids in a single polypeptide chain with two disulfide bonds in the C-terminal region. The presence of two disulfide bonds is characteristic of all family 2 cystatins (Rawlings and Barrett, 1990). The molecular weight of cystatin C protein is approximately 13 kDa. The highly conserved protein sequences for human, monkey, mouse, and rat cystatin C are shown in Figure 4. There is a 71% homology in the cystatin C protein sequence between human and mouse and 73% between human and rat (Håkansson et al., 1996). The protein sequences of mouse and rat are 88% identical (Håkansson et al., 1996). Human cystatin C is a non-glycosylated protein, but in mouse and rat up to 20% of cystatin C can be glycosylated (Esnard et

al., 1990). There is an N-glycosylation site at position 79 in the rat cystatin C sequence (Esnard et al., 1990), but other glycosylated forms have also been demonstrated (Dahl et al., 2004).

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HUMAN MAGPLRAPLLLLAILAVALAVSPAAGSSPGKPPRLVGGPMDASVEEEGVRRALDFAVGEY
MACMU MAGPLRAPLLLLAILAVALAVSPAAGASPGKPPRLVGGPMDASVEEEGVRRALDFAVSEY
SAISC MAGPLRAPLLLLAILAVALALSPAAGASPGRTPRLLGGPMDASVEEEGVRRALDFAVSEY
MOUSE MASPLRSLLFLLAVLAVAWAATPKQ------GPRMLGAPEEADANEEGVRRALDFAVSEY
RAT   -------------VLAVAWAGTSRP------PPRLLGAPQEADASEEGVQRALDFAVSEY
                  **** *           **  * *  *   **** ******* **

HUMAN NKASNDMYHSRALQVVRARKQIVAGVNYFLDVELGRTTCTKTQPNLDNCPFHDQPHLKRK
MACMU NKASNDMYHSRALQVVRARKQIVAGVNYFLDVELGRTTCTKTQPNLDNCPFHEQPHLKRK
SAISC NKASNDMYHSRALQVVRARKQIVAGVNYFLDVEMGRTTCTKNQPNLDNCPFHEQPHLKRK
MOUSE NKGSNDAYHSRAIQVVRARKQLVAGVNYFLDVEMGRTTCTKSQTNLTDCPFHDQPHLMRK
RAT   NKGSNDAYHSRAIQVVRARKQLVAGINYYLDVEMGRTTCTKSQTNLTNCPFHDQPHLMRK
      ** *** ***** ******** *** ** **** ******* * **  **** **** **

HUMAN AFCSFQIYAVPWQGTMTLSKSTCQDA 146[1]
MACMU AFCSFQIYTVPWQGTMTLSKSTCQDA 146[2]
SAISC AFCSFQIYSVPWQGIMTLSKSTCQDA 146[2]
MOUSE ALCSFQIYSVPWKGTHSLTKFSCKNA 140[3]
RAT   ALCSFQIYSVPWKGTHTLTKSSCKNA 127[4]
      * ****** *** *   * *  *  *
```

Figure 4. Multiple sequence alignment for cystatin C protein sequences of human, monkey (Macaca mulatta [MACMU] and Saimiri sciureus [SAISC]), mouse [Mus musculus], and rat [Rattus norvegigus]. Sequence alignment was obtained using SWISSPROT. The homologous amino acids are marked with a star (*), and the sequences for the signal peptide are underlined. [1]Grubb and Löfberg, 1982, [2]Wei et al., 1996, [3]Huh *et al.*, 1995, [4]Esnard *et al.*, 1990.

The secondary structure of cystatin C is similar to that of chicken egg-white cystatin (see Figure 5), and is composed of a five-stranded antiparallel β-sheet wrapped around an α-helix (Bode et al., 1988; Ekiel and Abrahamson, 1996; Ekiel et al., 1997). The first and second β-strands are separated by the α-helix and the others by two hairpin loops. There are three regions in the cystatin C protein that are conserved within the cystatin superfamily and these form the cysteine proteinase inhibitory site (Mason et al., 1998). These include amino acids 9 to 11 in the N-terminal region, the Q^{55}-I-V-A-G^{59} sequence in the first hairpin loop, and the C-terminal $P^{105}W^{106}$ dipeptide in the second hairpin loop (Mason et al., 1998). In the three-dimensional structure, these three regions are close to each other and form a wedge-shaped structure that fits into the active site of the protease. The N-terminal segment is required for binding and inhibition of cysteine proteinases (Abrahamson et al., 1991b). Cystatin C exists normally as a monomer but can form dimeric structures via three-dimensional domain swapping (Ekiel and Abrahamson, 1996; Janowski et al.,

2001). This is a phenomenon where a part of a protein is replaced with an identical element from another protein (Bennett et al., 1994). In cystatin C, the exchanged domain includes the α-helix and the two adjacent β-strands (Janowski et al., 2001; Jaskolski, 2001; Nilsson et al., 2004). The dimerization leads to the complete loss of cathepsin inhibitory function (Ekiel and Abrahamson, 1996). The inhibition of mammalian legumain proteases is, however, not affected by the dimerization (Alvarez-Fernandez et al., 1999). In physiological conditions, dimer formation is rare but occurs in pathological conditions such as HCAA. A cystatin C variant with a single point mutation dimerizes more easily than normal cystatin C, and it forms tetramers via three-dimensional domain swapping (Wei et al., 1998; Sanders et al., 2004).

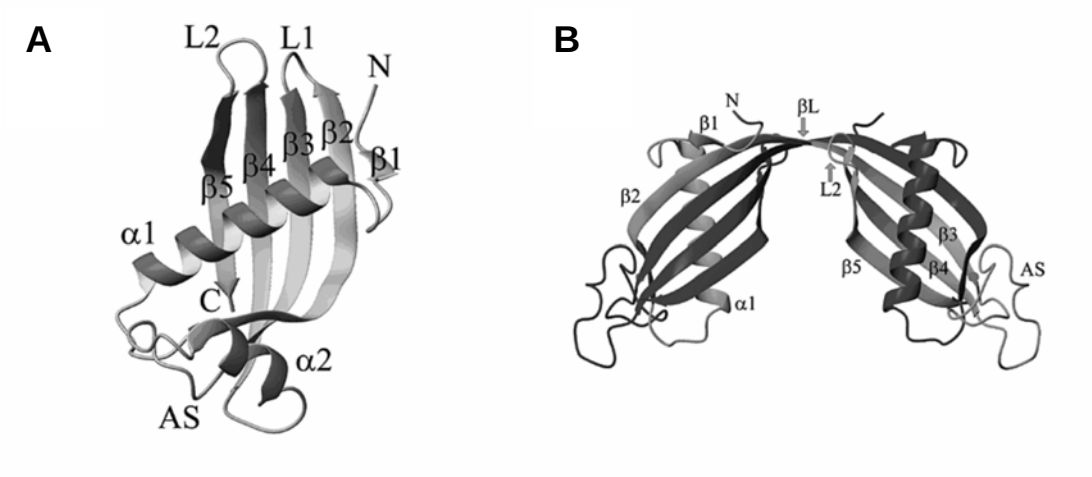

Figure 5. (A) **T**he secondary structure of chicken egg-white cystatin illustrating the common secondary structure of cystatin family proteins. The secondary structure of human cystatin C is similar to that of chicken egg-white cystatin. (B) The secondary structure of the cystatin C dimer. (Modified from Janowski *et al.*, 2001, reprinted with permission from the © Nature Publishing Group.)

Cystatin C is mainly an extracellular protein, but it is also found intracellularly, locating in the endosome/lysosome compartments or endoplasmic reticulum (ER)(Merz et al., 1997). The 26-amino acid signal sequence of cystatin C is needed for proper trafficking of cystatin C inside the cell. Before cystatin C is secreted, it forms transient dimeric structures in the ER (Merz et al., 1997). These dimers dissociate into monomeric cystatin C before or upon secretion, and monomeric cystatin C is secreted out of the cell (Merz et al., 1997). The loss of the leader sequence or amino acid substitution(s) that change the properties of the signal sequence can thus lead to impaired secretion of cystatin C and its accumulation inside the cell. Cystatin C without the

leader sequence is not processed through the secretory pathway (Paraoan et al., 2003). Further, the cystatin C variant harbouring the point mutation in exon 2 and lacking the 10 most N-terminal amino acids accumulates intracellularly in the ER (Bjarnadottir et al., 1998). Also, the polymorphic substitution of an amino acid that is located near the cleavage site of the signal peptide leads to impaired cystatin C secretion (Benussi et al., 2003; Paraoan et al., 2004). Secreted cystatin C is taken up by the neighbouring cells and degraded in the lysosomes. Cystatin C that is present in body fluids and eventually in the blood plasma is reabsorbed by tubular cells in the kidney, transported to the lysosomes, and degraded (Jacobsson et al., 1995). In normal physiological conditions, urine contains hardly any cystatin C, but with tubular failure, the concentration of cystatin C in the plasma rises, which makes it a suitable marker for renal filtration rate (Mussap and Plebani, 2004).

A serine proteinase, leukocyte elastase, cleaves cystatin C *in vitro* between amino acids 10 and 11, leading to the truncation of the protein and decreased inhibitory potential for cathepsin B and L (Abrahamson et al., 1991a). The cleavage is specific for leukocyte elastase and is not observed with other serine proteinases (Abrahamson et al., 1991a). Leukocyte elastase could thus function as a regulator of extracellular cystatin C activity at sites of inflammation. Transforming growth factor- β (TGF- β) regulates cystatin C expression (Solem et al., 1990; Shi et al., 1999; Afonso et al., 2002; Sokol and Schiemann, 2004), and increases cystatin C secretion (Sokol and Schiemann, 2004).

Cystatin C is the most abundant member of the cystatin superfamily and is present at moderate levels in all human tissues and body fluids (Abrahamson et al., 1987; Abrahamson et al., 1990; Abrahamson, 1994; Dickinson et al., 2002). It is expressed in tissues such as kidney, liver, pancreas, intestine, stomach, lung, placenta, seminal vesicles, and brain (Abrahamson et al., 1990). The highest levels of cystatin C are in the CSF and semen, the CSF levels being 5.5 times higher than in plasma. Similarly, in mouse and rat, cystatin C is expressed in all tissues that have been examined so far, including stomach, intestine, liver, muscle, spleen, heart, kidney, lung, pancreas, testis, seminal vesicles, uterus, and ovary (Cole et al., 1989; Tavera et al., 1990; Huh et al., 1995; Håkansson et al., 1996). The brain has particularly high levels of cystatin C, 15-fold that of liver (Håkansson et al., 1996). Overall, cystatin C has an apparent non-tissue specific expression, and the pattern and distribution are very similar in human, rat, and mouse.

Cystatin C was first isolated from the CSF during the 1960's, but cystatin C expression in the brain was not recognized until later. One of the first reports on cystatin C in the brain was an

immunohistochemical study demonstrating cystatin C in post-mortem human hypothalamus, particularly in the nuclei supraopticus, paraventricularis, and arcuatus (Bernstein et al., 1988), suggesting that cystatin C in the CSF comes from the hypothalamic neurons that project directly to the third ventricle (Bernstein et al., 1988). Later, high and conserved expression of cystatin C was demonstrated in the choroid plexus epithelial cells (Tu et al., 1992). The first detailed description of cystatin C in the brain was provided by Yasuhara, who demonstrated its expression in human, monkey, and rat brain (Yasuhara et al., 1993). Yasuhara and colleagues described cystatin C in astrocytes and some neuronal cells in the cortex, thalamus, hypothalamus, and striatum in all species (Yasuhara et al., 1993). Overall, cystatin C expression in the brain has been demonstrated in the neurons and glial cells of striatum, cerebral cortex, cerebellum, hypothalamus, thalamus, and hippocampus (Tu et al., 1992; Lignelid et al., 1997; Olsson et al., 2000; Deng et al., 2001; Ying et al., 2002). It is also expressed in the choroid plexus epithelial cells, macrophage-like cells of the arachnoid membrane, and neuroendocrine cells of the anterior pituitary lobe (Tu et al., 1992; Lignelid et al., 1997; Olsson et al., 2000). Cystatin C expression in the brain is altered following a brain damaging insult (Table 3). Cystatin C levels are increased following focal ischemia, axotomy, and hypophysectomy, as well as SE (Palm et al., 1995; Ishimaru et al., 1996; Miyake et al., 1996; Katakai et al., 1997; Aronica et al., 2001).

2.3.3 Physiological functions of cystatin C

2.3.3.1 Inhibitor of cathepsins

Cystatin C is a tight-binding and reversible inhibitor of cathepsins B, H, L, and S (Abrahamson, 1994). Cathepsins, the lysosomal cysteine proteinases, are part of the papain family of cysteine proteinases, and consist of 11 members, of which cathepsin B and L are the most abundant in human (Turk et al., 2000). They are involved in the normal degradation of cellular proteins, processing of prohormones, antigen presentation, collagen breakdown, and bone resorption (reviewed by Turk et al., 2000; Berdowska, 2004). When they are secreted outside the cells, they can be extremely harmful to the surrounding environment leading to pathological conditions. For instance, cathepsins are related to rheumatoid arthritis, cancer, apoptosis, neurological disorders, osteoporosis, and lysosomal storage diseases (reviewed by Chapman et al., 1997).

The main function of cystatin C is to regulate the activity of cathepsins and thereby modulate and control extracellular proteolysis. The balance between cystatin C and cysteine peptidases is important for maintaining normal physiological conditions, and if that fails, pathological symptoms follow. For example, in atherosclerotic vessel walls, the upregulation of cathepsins K

and S in vascular wall smooth muscle cells is coincident with low cystatin C expression (Shi et al., 1999). Also, low serum levels of cystatin C correlate with the progression of abdominal aortic aneurysms (Lindholt et al., 2001). Further, increased activity of cathepsin K, which is responsible for bone resorption, can lead to osteoporosis (Yasuda *et al.*, 2005). Excessive cathepsin activity can also lead to degradation of the extracellular matrix, thus enabling cancer growth and invasion (Buck et al., 1992). Reduced cystatin C expression concomitant with increased cathepsin B expression has been described in human breast cancer and glioma cell lines (Yano et al., 2001;Konduri et al., 2002). The reduction corresponds with increasing malignancy (Konduri et al., 2002). Further, overexpression of cystatin C inhibits the motility and invasion of B16 melanoma cells, glioma cells, and ovarian cancer cells (Sexton and Cox, 1997; Konduri et al., 2002; Nishikawa et al., 2004). Overexpression of cystatin C is also reported to inhibit of B16 melanoma cell metastasis (Cox et al., 1999); however, this finding was contradicted by the finding that metastatic spread of B16 melanoma cells is reduced in cystatin C-deficient mice (Huh et al., 1999). These data, apart from the discrepancies, highlight the importance of the proper expression of cystatin C in the control of physiological tissue integrity.

2.3.3.2 Modulator of inflammation and antiviral actions

Cystatin C is synthesized and secreted by immune system cells such as monocytes and macrophages, and its release is down-regulated when macrophages are stimulated by lipopolysaccharides or interferon–γ (IFN-γ) (Warfel et al., 1987). There are several lines of evidence indicating that cystatin C also regulates neutrophil behaviour and motility. Incubation of neutrophils with cystatin C leads to decreased superoxide release and phagocytosis, which are characteristics of neutrophil activation (Leung-Tack et al., 1990a). Cystatin C also modulates neutrophil chemotaxis (Leung-Tack et al., 1990b). The role of cystatin C in controlling antigen presentation in peripheral dendritic cells has been investigated. Cystatin C is suggested to control the activity of cathepsin S, which cleaves the invariant chain from the major histocompatibility complex II-molecules enabling them to be transported to the plasma membrane and to present antigens to the immune system cells (Pierre and Mellman, 1998). Recent studies have, however, contradicted this idea by demonstrating that although cystatin C is highly abundant in certain dendritic cells, it is not necessary for controlling major histocompatibility complex II antigen presentation (El-Sukkari et al., 2003). Cystatin C is differentially expressed during maturation of the dendritic cells (Zavasnik-Bergant et al., 2005).

TABLE 3. Review of alterations in cystatin C in various neuropathological conditions.

PATHOLOGY	FINDING	REFERENCE
Hereditary cystatin C Amyloid angiopathy (HCAA)	Mutation in cystatin C leads to deposition of cystatin C in arterial walls, levels of CSF cystatin C ↓	Ghiso et al., 1986 Palsdottir et al., 1988 Levy et al., 1989
Sporadic amyloid angiopathy	Mutation in *cystatin C*	Graffagnino et al., 1995
Alzheimer's disease (AD)	CSTB haplotype is a risk factor for late-onset AD Cystatin is codeposited with amyloid beta-protein Cystatin C is expressed in vulnerable neurons in AD brain	Finckh et al., 2000 Crawford et al., 2000 Levy et al., 2001 Deng et al., 2001
Amyotrophic lateral sclerosis	Cystatin C in skin ↑	Ono et al., 2000
Ischemia	Cystatin C levels in degenerating CA1 neurons and astrocytes ↑	Palm et al., 1995 Ishimaru et al., 1996
Axotomy	Cystatin C levels in microglia in facial nucleus ↑	Miyake et al., 1996
Hypophysectomy	Cystatin C levels in magnocellular neurons of hypothalamus ↑	Katakai et al., 1997
Perforant path transection	Cystatin C expression in the hippocampus ↑	Ying et al., 2002
Status epilepticus (SE)	*Cystatin C* expression after SE ↑ Cystatin C levels in the hippocampus ↑	Hendriksen et al., 2001 Aronica et al., 2001
Persistent noxious state induced by carrageenan	Cystatin C expression in the rat dorsal horn of spinal cord ↑	Yang et al., 2001
Autoimmune diseases	Cystatin C in CSF ↑	Brzin et al., 1984
Multiple sclerosis	Cystatin C in CSF ↓	Bollengier, 1987 Nagai et al., 2000
Guillan-Barré Syndrome	Cystatin C in CSF ↓	Nagai et al., 2000
Chronic inflammatory demyelinating polyneuropathy	Cystatin C in CSF ↓	Nagai et al., 2000

Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; SE, status epilepticus.

Cystatin C is also suggested to regulate cytokine production. It induces the synthesis of tumor necrosis factor–α and interleukin (IL) -10 in IFN-γ activated macrophages and further upregulates nitric oxide release from macrophages (Verdot et al., 1996; Verdot et al., 1999). Also, cystatin C upregulates the production of IL-6 and IL-8 in murine splenocytes and human gingival fibroblasts

(Kato et al., 2000). In addition, cystatin C has antiviral properties against certain viruses and inhibits the replication of *Herpes simplex* virus and *Coronavirus* (Bjorck et al., 1990). The cytokine regulatory activity and modulation of the immune system are unrelated to its function as a cysteine proteinase inhibitor.

2.3.3.3 Growth-promoting functions

Cystatin C is suggested to have a role in cellular proliferation and differentiation. Sun (1989) first demonstrated that cystatin C induces proliferation of mouse 3T3-fibroblasts in culture. This was later confirmed by Tavera and colleagues (1992) who demonstrated that cystatin C increases the proliferation of rat glomerular mesangial cells. They also suggested that its growth-promoting function is related to the N-terminal tetrapeptide, because the tetrapeptide stimulates proliferation as effectively as native cystatin C (Tavera et al., 1992). One of the most interesting reports on cystatin C function in the brain was provided by Taupin and colleagues (2000) who demonstrated that the N-glycosylated form of cystatin C is a cofactor for FGF - 2 and is required for neurogenesis in vitro and in vivo. Cystatin C was purified from cell culture as a factor that was secreted by adult hippocampal progenitor cells to the cell culture medium (Taupin et al., 2000). Also, the progenitor cells in vitro and in vivo in the SGZ are cystatin C-immunopositive, indicating that those cells produce and secrete cystatin C (Taupin et al., 2000). Cystatin C is also suggested to have a role in the development of astrocytes, especially in their differentiation. Kumada and colleagues (2004) reported that in mouse, cystatin C upregulates glial fibrillary acidic protein (GFAP), which is present in mature astrocytes. Further, they demonstrated that cystatin C expression starts when gliogenesis begins during development, and precedes that of GFAP. Cai and colleagues (2004) further demonstrated that cystatin C is expressed in glia-restricted precursor cells, indicating a role in astrocyte differentiation. In addition to proteinase inhibitory function, cystatin C is also involved in cell growth, proliferation, and differentiation.

2.3.3.4 Modulator of brain injury response – beneficial or detrimental?

Many studies demonstrate that cystatin C is upregulated in response to brain injury (summarized in Table 3). The function of cystatin C in the brain is unknown and therefore the significance of cystatin C upregulation remains a puzzle. Several reports suggest a protective role for cystatin C. As a secretable protein locating mainly in the glial cells, it might prevent excessive extracellular proteolysis after injury and function as an endogenous neuroprotectant (Miyake et al., 1996). The cystatin C expression time course in degenerating cerebral cortex neurons after transient cerebral ischemia coincides with the time course of increased immunoreactivity for cathepsins B, H, and L (Palm et al., 1995). Ying and coworkers (2002) demonstrated increased cystatin C expression

following perforant path transection in the region innervated by the entorhinal cortex. They suggested that cystatin C secreted from astrocytes protects neurite growth-associated molecules from degradation by cysteine proteases and thus facilitates regeneration (Ying et al., 2002). A recent report by Xu and colleagues (2005) demonstrated that administration of cystatin C prevents neuronal loss in a model of Parkinson's disease. They demonstrated that application of cystatin C to the substantia nigra prevented neuronal loss induced by the administration of 6-hydroxydopamine (Xu et al., 2005). Also, Nishiyama and colleagues (2005) demonstrated that expression of cystatin C in PC-12 cells confers protection against oxidative stress. These data suggest that cystatin C protects cells from death signals and that its expression is beneficial following brain injury.

There are, however, also several reports suggesting that instead of being a protective factor, cystatin C is detrimental to the brain and functions as a mediator of cell death. Cystatin C is upregulated in response to high oxygen levels, which typically induce apoptotic death of neurons in vitro (Nishio et al., 2000). This suggests that cystatin C is involved in the regulation of oxidative stress-induced apoptosis, which is related to variety of neurodegenerative diseases, such as AD, Parkinson's disease, and amyotrophic lateral sclerosis, as well as normal aging (Emerit et al., 2004). Further, Nagai and colleagues (2002) demonstrated that unilateral injection of cystatin C into the hippocampus leads to robust neuronal degeneration in the granule cell layer and hilus. Cystatin C knockout mice exhibit reduced neuronal cell death following focal cerebral ischemia (Olsson et al., 2004), which supports the hypothesis that cystatin C mediates neuronal death. In these same mice, however, global ischemia induces more damage in cystatin C deficient mice than in wild-type mice (Olsson et al., 2004). These conflicting data suggest that different pathological situations lead to different responses.

2.3.4 Cystatin C in neurological diseases

2.3.4.1 Hereditary cystatin C amyloid angiopathy (HCAA)

HCAA, formerly called hereditary cerebral haemorrhage with amyloidosis of Icelandic type, is a fatal disease in which a variant of cystatin C accumulates as amyloid fibrils in the walls of cerebral arteries (Jensson et al., 1987; Palsdottir et al., 1988). Amyloid deposits are also found outside the central nervous system, such as in lymph nodes, spleen, salivary glands, seminal vesicles, and skin, but they are not associated with any clinical symptoms (Jonsdottir and Palsdottir, 1993). HCAA occurs in the northwestern parts of Iceland and has an autosomal dominant inheritance; that is, a single copy of the mutated gene is sufficient to cause amyloidosis. Patients with HCAA

develop strokes and brain haemorrhages as young adults, which subsequently leads to paralysis and behavioural changes. Typically, the condition leads to death before 40 years of age as a result of massive brain haemorrhage (Ghiso et al., 1986). The underlying cause of the disease is a single base substitution in exon 2 (CAG instead of CTG), leading to an amino acid substitution from leucine to glutamine at position 68 (Palsdottir et al., 1988; Levy et al., 1989). This mutation abolishes the *Alu*I restriction site in the cystatin C gene, which is used for the diagnosis of HCAA. The variant of cystatin C protein also lacks the first 10 amino acids. Both forms of cystatin C are processed and secreted to a similar extent, but the variant form of cystatin C has a high tendency to form dimeric and tetrameric structures, which easily form amyloid fibrils (Abrahamson, 1996). The three-dimensional domain swapping is considered typical for amyloid-forming molecules and could thus be a prerequisite for the deposition of cystatin C in the cerebral arteries (Jaskolski, 2001). The formation of cystatin C fibrils can be prevented by inhibiting the domain swapping and the subsequent dimerization (Nilsson et al., 2004). The variant cystatin C is also less stable than normal cystatin C and is quickly degraded in the extracellular space (Wei et al., 1998). Enhanced proteolysis of the variant cystatin C might underlie the decreased levels of cystatin C in the CSF typically found in HCAA (Grubb et al., 1984). The variant cystatin C protein accumulates inside the cells, particularly in the ER (Bjarnadottir et al., 1998). This is most probably caused by the lack of N-terminal amino acids, which would normally comprise the leader sequence needed for proper trafficking of cystatin C.

2.3.4.2 Alzheimer's disease

Cystatin C is associated also with Alzheimer´s disease (AD), which is a highly debilitating disease in elderly people that affects their memory function. Cystatin C colocalizes with beta-amyloid protein (Aβ) in arteriolar lesions in the brains of patients with AD (Maruyama et al., 1990; Vinters et al., 1990). Levy and colleagues (Levy et al., 2001) further observed that cystatin C accumulates with Aβ in parenchymal and vascular amyloid deposits. Further, cystatin C immunoreactivity is observed in amyloid deposits in transgenic mice that overexpress amyloid precursor protein (Levy et al., 2001). Deng and coworkers (2001) reported that cystatin C immunoreactivity is increased in the neurons that are highly susceptible to AD-induced cell loss. These cells locate in pyramidal layers III and IV of the cerebral cortex, but also in the hippocampus and entorhinal cortex. They observed cystatin C in some cerebrovascular Aβ deposits, but not in cortical deposits, in contrast to previous findings. Steinhoff and colleagues (2001) observed high levels of cystatin C in transgenic mice with a double mutation in the amyloid precursor protein (SweAPP). Cystatin C is not present in the amyloid core but spherically surrounds it, and rarely colocalizes with Aβ as a

homogeneous mixture. They also observed that cystatin C-positive astrocytes assemble around the plaques after they had formed, and cystatin C is rarely present in neurons, as was also demonstrated by Deng (Steinhoff et al., 2001).

Besides the notion that cystatin C is part of the amyloid plaques in AD, polymorphisms of the cystatin C gene are thought to confer risk for late-onset AD (Crawford et al., 2000; Finckh et al., 2000). This is, however, a highly controversial issue. There are three polymorphic nucleotides in the cystatin C gene, two in the 5' untranslated sequence and one in exon 1 (Balbin and Abrahamson, 1991; Balbin et al., 1993). Two haplotypes arise from these polymorphisms, CST3 A with the sequence GAG, and CST3 B with CCA. Finckh and coworkers (2000) demonstrated that haplotype B is a risk factor for late-onset AD. Some studies demonstrated a similar association between cystatin C polymorphism and AD occurrence (Crawford et al., 2000; Beyer et al., 2001; Cathcart et al., 2005), whereas others have not (Maruyama et al., 2001; Roks et al., 2001; Dodel et al., 2002; Monastero et al., 2005). Therefore it remains a debate whether the proposed link really exists in AD. The associated polymorphism is located in the signal peptide, at -2 residues from the cleavage site, and results in a threonine for alanine substitution (Balbin et al., 1993). Recently, Benussi and colleagues (2003) demonstrated that haplotype B is associated with the reduced secretion of cystatin C and reduced cleavage of the signal peptide, which would result in reduced levels of cystatin C. This is, however, in contrast to the finding that the level of cystatin C in CSF is increased in AD patients (Carrette et al., 2003). Based on the existing data, it is difficult to draw a conclusion regarding the role of cystatin C in AD.

2.3.4.4 Epilepsy

Recently cystatin C was associated with epilepsy. Cystatin C gene expression is highly increased following SE induced by electrical stimulation of the hippocampus in rat (Hendriksen et al., 2001). Hendriksen and colleagues (2001) used a novel approach, serial analysis of gene expression, to analyze differentially expressed genes during epileptogenesis, at 8 days after SE induction. The increase in gene expression results in increased protein expression of cystatin C within the same time frame (Aronica et al., 2001). Using immunohistochemistry and Western blot analysis, Aronica and coworkers (2001) demonstrated that cystatin C protein expression is increased at 24 h after SE, and becomes maximal 1 to 3 weeks following SE. Cystatin C is expressed mainly in GFAP and vimentin-positive astrocytes and Ox-42-positive microglia in the hilus of the dentate gyrus and in the CA1 and CA3 subregions of hippocampus proper. Cystatin C is also observed in interneurons in the dentate gyrus and CA1, which simultaneously expressed parvalbumin and calbindin. In addition, pyramidal neurons in the CA1 and CA3 regions have punctuate cystatin C

immunoreactivity in the perinuclear cytoplasm. There is also increased cystatin C expression in the glial and neuronal cells of the entorhinal cortex, particularly in superficial layer II and deep layers of the entorhinal cortex (Aronica et al., 2001). Cystatin C upregulation persists in the hilus and CA1 region 3 months following SE, that is, at the time of spontaneous recurrent seizures. Based on these findings, cystatin C is suggested to have a role in epileptogenesis, perhaps functioning as an endogenous neuroprotectant to limit the neuronal degeneration induced by SE.

3. AIMS OF THE STUDY

Molecular alterations, such as changes in gene expression, are hypothesized to underlie the neuropathological alterations that occur during epileptogenesis. cDNA microarray analysis has revealed a vast number of differentially expressed genes during SE-induced epileptogenesis, many of which were not previously linked to seizures or epilepsy. Cystatin C, a cysteine proteinase inhibitor, whose mRNA expression is increased following SE, is one of such candidate epileptogenesis-related genes. The main purpose of this study was to investigate the expression of cystatin C protein in the normal brain as well as during epileptogenesis and epilepsy, and to elucidate its functional role in other neuropathological alterations that occur during epileptogenesis, such as neuronal death and neurogenesis.

This thesis was especially designed to answer the following questions:

1. What is the distribution and cellular localization of cystatin C in normal mouse, rat, and human brain? **(I, II, III, IV)**
2. What is the distribution and time course of cystatin C expression following an epileptogenic brain insult such as SE or photothrombotic stroke? **(I, II, IV)**
3. Is the cystatin C expression altered in chronic epilepsy? **(III)**
4. Is cystatin C expression associated with the neuropathological findings, such as neuronal cell death, granule cell dispersion, and neurogenesis that occur during epileptogenesis and epilepsy? **(II, III)**

4. MATERIALS AND METHODS

4.1 Subjects

Rats (I, III, IV). Adult male Sprague-Dawley rats (Harlan, Netherlands) weighing 290 to 360 g were individually housed in cages at standard temperature (22°C ± 1°C), humidity (50-60%), and a circadian light cycle. Standard food pellets and water were available *ad libitum*. All animal procedures were approved by the Animal Care and Use Committee of the University of Kuopio, Finland, and were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC.

Mice (II). Cystatin C wild-type ($cystC^{+/+}$) and knockout ($cystC^{-/-}$) mice (129SvJxC57BL/6 hybrid genotype) were kindly provided by Professor Anders Grubb (University of Lund, Sweden). The production and characterization of the cystatin C knockout mice were previously reported (Huh et al., 1999). Animals from the same generation and with similar ages (10 to 14 wk at the beginning of the experiment) were used from both strains. Mice were housed similarly as rats.

Human (III). Control specimens (n=11) were obtained from autopsies performed at the Kuopio University Hospital. Subjects had no clinical evidence of dementia or history of brain disease. The absence of neurologic disease was confirmed by a neuropathologist. Epileptic hippocampal specimens (n=61) were obtained from patients who were operated on for drug-refractory TLE at the Kuopio University Hospital. Permission for the study was obtained from the Ethics Committee of the Kuopio University Hospital and the National Authority for Medicolegal Affairs. Patient consent was obtained according to the Declaration of Helsinki.

4.2 Experimental animal models

4.2.1 Electrical stimulation of the amygdala (I, III)

Implantation of electrodes. Rats were anesthetized with a mixture of pentobarbital (60 mg/kg i.p.) and chloral hydrate (100 mg/kg i.p.) and inserted into a Kopf stereotaxic frame. A bipolar stimulation electrode (Franco Corradi, Milano, Italy) was implanted into the left lateral nucleus of the amygdala (3.6 mm posterior, 5.0 mm lateral to bregma, 6.5 mm ventral to the surface of the brain) according to a rat brain atlas of Paxinos and Watson (1986) and a recording screw electrode was positioned on the skull over the contralateral frontal cortex (3.0 mm anterior and 2.0 mm lateral to bregma). Ground and reference electrodes were placed over the cerebellum.

Induction of SE. Two weeks after the surgery, SE was induced as described by Nissinen *et al.* (2000). The lateral nucleus of the amygdala was stimulated for 20 to 40 min at 500-ms intervals with a train of pulses lasting 100 ms (1-ms 60-Hz bipolar pulses at 400 μA peak to peak) via

implanted electrodes. The development of SE was monitored using the Nervus EEG Recording system (Taugagreining, Reykjavik, Iceland), and the duration of SE was defined as the time between the first and last high-amplitude (>2x baseline) and –frequency (>8 Hz) discharge (HAFD) detected in EEG. Control animals had implanted electrodes but were not stimulated.

Detection of spontaneous seizures. To verify that the animals were in the epileptogenic phase, *i.e.*, did not yet have spontaneous seizures **(I)**, rats were continuously (24 h/d) monitored with a combined video-EEG system for two weeks. To detect the occurrence of spontaneous recurrent seizures, *i.e.*, to verify that the animals were epileptic **(III)**, rats were monitored daily (24 h/d) during weeks 9 and 13 after SE induction. Electrographic seizures were identified from the EEG recordings by browsing the EEG manually on the computer screen, and if an electrographic seizure (a high-amplitude (>2x baseline), high-frequency (>8 Hz) discharge either in the amygdala, cortex, or both, lasting for at least 5 s) was observed, behavioral severity was analyzed from the corresponding video-recording.

4.2.2 Systemic application of kainic acid (II)

Kainic acid (#K-0250; Sigma-Aldrich Chemical Co. , St. Louis, MO) was dissolved in 0.9% saline and injected intraperitoneally (30-35 mg/kg, i.p.) in mice. Control animals received 0.9% saline.

Behavioral seizures. After the kainic acid or saline injection, the animals were visually observed for 2 h for the occurrence of kainate-induced behavioural seizures. Seizures were scored according to a modified Racine's scale (Racine, 1972) as follows: score 1, staring, head nodding; score 2, unilateral forelimb clonus; score 3, bilateral forelimb clonus; score 4, bilateral forelimb clonus with rearing and falling (loss of balance). To estimate the severity of SE, the latency to the onset of behavioural seizures and generalized seizures, and the total duration of behavioural seizures during the 2 h follow-up were recorded.

Electrographic seizures. To monitor electrographic seizure activity, epidural electrodes were implanted 2 weeks prior to the kainate injection. Under pentobarbital-chloral hydrate anaesthesia, two stainless steel screw electrodes (made in-house) were implanted into the skull bilaterally above the frontal cortex (-1.0 mm to bregma and 1.0 mm left of midline or +1.0 mm to bregma and 1.0 mm right of midline), a reference electrode over the right hemisphere (-1.0 mm to bregma and 1 mm right of midline), and a ground electrode over the cerebellum. Baseline EEG was recorded for 15 min from each animal before SE induction. Video-EEG monitoring was continued for 48 h after kainate injection. Electrographic seizure activity was analysed manually by browsing the EEG on the computer screen. The duration of electrographic SE was defined as the time between the first and last electrographic seizure event. A spike analysis of EEG data using the Clampfit 9.0

program (Axon Instruments Inc., Union City, CA) was also performed. A graphoelement was defined as a spike if it had an amplitude of two times the baseline and a duration of less than 200 ms. Spikes were counted in 30-min epochs.

4.2.3 Photothrombotic stroke (IV)

A photosensitive dye Rose Bengal (#R-3877, Sigma), was used to produce cortical thrombosis and subsequent brain infarction in rats according to Watson *et al.* (1985) with minor modifications. Animals were anaesthetized with a mixture of sodium pentobarbital (60 mg/kg i.p.) and chloral hydrate (60 mg/kg i.p.). The skull was exposed and white cold light (diameter 4.0 mm) was positioned over the left frontoparietal cortex (-1.8 mm to bregma and 2.2 mm lateral from midline according to Paxinos and Watson, 1986). Freshly prepared Rose Bengal solution (20 mg/kg, dissolved in saline) was injected into the right saphenus vein at a rate of 150 µl/min for 2 min. Thereafter, the skull was illuminated for 10 min. Control animals (n=4) were injected with Rose Bengal and the skull was exposed, but not illuminated.

4.3 BrdU administration for study of neurogenesis (II)

5-Bromo-3'-deoxyuridine (BrdU; #280879; Roche Diagnostics Corp., Indianapolis, IN) was dissolved in 0.9% saline, filtered through a 0.2-µm filter, and injected intraperitoneally (50 mg/kg). BrdU is incorporated into the DNA of proliferating cells and can be used as a marker for neurogenesis (Miller and Nowakowski, 1988). BrdU injections (twice daily) were started on day 3 following saline or kainate treatment and continued for 4 consecutive days. Animals were sacrificed 24 h after the last BrdU injection.

4.4 Processing of tissue for histology (I-IV)

Rat (I, III, IV). Deeply anesthetized animals were transcardially perfused with ice-cold 0.9% saline (2 min, 30 ml/min) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PFA) for 30 min **(I, IV)** or by 0.37% sulphide for 10 min and 4% PFA for 10 min, according to the Sloviter *et al.* (1982) **(III)**. The brains were removed from the skull, postfixed in 4% PFA for 4 h, cryoprotected (20% glycerol in 0.02 M potassium buffered saline, pH 7.4) for 48 h, frozen, and stored at -70^{o}C. The brains were cut serially in the coronal plane into 25- or 30-µm-thick sections with a sliding microtome. The sections were stored in 10% formalin (for Nissl) or in tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer, for immunohistochemistry) until further processed.

Mouse (II). Deeply anesthetized animals were perfusion-fixed with saline (2 min; 5 ml/min) and 4% PFA (20 min; 5 ml/min). Postfixation, cryoprotection, and storage procedures were similar to

those described for rat. The brains were cut serially (1-in-7-series) in the coronal plane into 20-µm-thick sections. The sections were stored in formalin or tissue-collection solution as described above.

Human (III). Tissue available from autopsy and epilepsy surgery was immersed in 4% PFA in 0.1 M phosphate buffer (PB) for 72 h at 4°C. Thereafter, tissue was cryoprotected in 10% glycerol with 2% dimethylsulfoxide in PB for 24 h followed by 20% glycerol with 2% dimethylsulfoxide in PB for 3 d. The tissue was frozen in dry ice and coronally sectioned into 50-µm-thick sections (1-in-8 series) with a sliding microtome. The sections were stored in formalin or tissue-collection solution as described for rat.

4.5 Histology and immunohistochemistry

4.5.1 Nissl staining

The first series of sections was stained with 0.125% cresyl violet **(I, II, IV)** or 0.125% thionin **(III)** to identify the cytoarchitectonic boundaries and analyze neuronal death.

4.5.2 Fluoro-Jade B (II, IV)

To visualize degenerating neurons after kainate-induced SE **(II)** and photothrombotic stroke **(IV)**, one series of sections was stained with 0.001% Fluoro-Jade B (FJB, Histo-Chem Inc., Jefferson, AR) as described previously (Hopkins et al., 2000). Slides were examined with a fluorescence microscope using an I3 filter cube for fluorescein isothiocyanate (excitation band 450-490 nm).

4.5.3 Cystatin C immunohistochemistry (I-IV)

To study the expression of cystatin C protein in mouse, rat, and human brain, sections were stained with a polyclonal rabbit anti-human cystatin C antibody (#A0451; DAKO, Denmark). Free-floating sections were treated with 1% H_2O_2 (rat, mouse) or 3% H_2O_2 in 10% methanol (human) to remove endogenous peroxidase activity, after which human sections were treated with 1% sodium borohydride for 15 min. Sections were blocked with 10% normal goat serum (NGS) and 0.5 % Triton X-100 in 0.05 M Tris-buffered saline (TBS, pH 7.4) before incubation with a cystatin C antibody (1:40 000) for 2 d at +4°C. Sections were then washed (2% NGS in TBS), and incubated with biotinylated goat anti-rabbit IgG (1:200; #BA-1000; Vector Laboratories, Burlingame, CA) diluted in 1% NGS, 0.3% Triton X-100 in TBS. After washing (2% NGS in TBS), the sections were incubated with avidin-biotin-peroxidase complex according to the manufacturer's instructions (Vectastain ABC Standard, #PK-4000, Vector Laboratories). The peroxidase activity was visualized in 0.05% 3,3´-diaminobenzidine (DAB; Pierce Chemical Company, Rockford, IL) and

0.04% H_2O_2 in TBS or in nickel-3-3´-DAB solution [0.5% nickel ammonium sulfate, 0.5 mg/ml DAB, 10% ß-D(+)- glucose, 0.2% ammonium chloride, and 1 µl/ml glucose oxidase (#G-6891, Sigma)]. Rat and mouse sections were intensified with osmium tetroxide and thiocarbohydrazine according to Lewis *et al.* (1987) before coverslipping with Depex (BDH Chemical, Poole, UK).

The specificity of the antibody was tested either by omitting the primary antibody or by preincubating the primary antibody overnight with excess human cystatin C protein (0.5-10 µg/ml; Calbiochem, La Jolla, CA). This resulted in the disappearance of all specific immunostaining (glial-like) in a concentration-dependent manner. In rats, however, diffuse staining in the pyramidal layer was not abolished by preincubation and therefore it was considered non-specific for the antibody.

4.5.4 BrdU immunohistochemistry (II)

A horseradish peroxidase-coupled mouse monoclonal BrdU antibody (1:500; #MAB3566P, Chemicon International Inc., Temecula, CA) was used to visualize BrdU-positive newborn cells. The protocol was essentially the same as described for cystatin C, except the sections were first pretreated with 2 M HCl at 37°C for 30 min to denature DNA, and then washed twice in 0.1 M boric acid buffer (pH 8.5) to neutralize the residual HCl. The staining was visualized with nickel-3-3´-DAB solution. The sections were lightly counterstained with 0.0625% thionin.

4.5.5 Tuc-4 and PSA-NCAM immunohistochemistry (II, III)

A polyclonal rabbit anti-Tuc-4 antibody (1:5000; #AB5454; Chemicon) **(II)** and a mouse monoclonal anti-PSA-NCAM antibody (12E3 IgM; 1:600 [mouse], 1:800 [human]; a generous gift from Dr. Tatsunori Seki) **(II, III)** were used as additional markers of neurogenesis. The protocol was essentially the same as for cystatin C. The secondary antibodies were biotinylated goat anti-rabbit IgG (1:200; #BA-1000; Vector Laboratories) for Tuc-4 and a biotinylated goat anti-mouse IgM (1:300; #BA-2020; Vector Laboratories) for PSA-NCAM. The Tuc-4 immunoreactivity was visualized with 0.05% 3,3´-DAB with the 0.3% nickel-ammonium sulphate and 0.04% H_2O_2, and PSA-NCAM with the nickel-3-3´-DAB solution described above.

4.5.6 Double immunostaining for confocal microscopy (I-IV)

Double immunostaining of cystatin C and neuronal (NeuN), astrocytic (GFAP), and microglial (Ox-42) markers was used to examine the subcellular localization of cystatin C. Free-floating sections were blocked for 40 min in 10% NGS and 0.5% Triton X-100 in TBS, and incubated for 2 d at 4°C in a mixture of primary antibodies: a cystatin C antibody (1:40 000; DAKO) together with a mouse monoclonal anti-NeuN (1:15000; #MAB377; Chemicon), mouse anti-Ox-42 antibody

(1:4000; #MCA275G; Serotec, Oslo, Norway), or mouse anti-GFAP antibody (1:2000; #814369; Boehringer Mannheim, Germany), 0.5% Triton X-100, and 1% NGS in TBS. After washing (1% NGS, 0.5 % Triton X-100 in TBS), the sections were incubated overnight in a mixture of secondary antibodies: biotinylated goat anti-rabbit IgG (1:200; #BA-1000; Vector Laboratories), and Alexa 488-conjugated goat anti-mouse IgG (1:500; #A-11001; Molecular Probes, Leiden, The Netherlands) in 1% NGS, 0.5% Triton X-100 in TBS. Thereafter, the sections were incubated for 3 h in Cy5-conjugated streptavidin (1 µg/ml; Jackson Immunoresearch, West Grove, PA) diluted in TBS containing 1% NGS, washed with 0.05 M Tris, pH 7.4, and mounted on glass slides using Gel Mount (Biomeda, Foster City, CA). Images were captured either with a Leica confocal microscope (Leica GmbH, Germany) equipped with a krypton-argon laser **(I)** or a Nikon laser scanning confocal microscope (Nikon GmbH, Germany) equipped with Ultra View LCI confocal imaging system (Perkin Elmer, Fremont, CA) **(II-IV)**. The excitation/emission wavelengths of 488/525 nm (Alexa 488) and 647/700 nm (Cy5) were used.

To analyse the colocalization of cystatin C with a marker of a subpopulation of GABAergic interneurons **(II)**, double immunostaining of cystatin C and a mouse monoclonal anti-parvalbumin antibody (1:10000; #235; Swant, Bellinzona, Switzerland) was performed. The protocol was the same as for the cellular markers.

To examine the proportions of BrdU-positive cells that were also Tuc-4 or PSA-NCAM positive, double immunostaining with rat anti-BrdU (1:500; #MCA2060; Serotec) and anti-Tuc-4 or anti-PSA-NCAM antibody was performed **(II)**. The protocol was essentially the same as for the cellular markers. The sections were pretreated with 2 N HCl prior to the staining, as described for the BrdU staining. The mixture of secondary antibodies: Alexa 488 conjugated goat anti-rat IgG (1:500; #A-11006; Molecular Probes) together with a biotinylated goat anti-rabbit IgG (1:200; #BA-1000; Vector Laboratories) or biotinylated goat anti-mouse IgM (1:300; #BA-2020; Vector Laboratories) was applied overnight followed by Alexa 680-conjugated streptavidin (1:500; #S-21378; Molecular Probes).

4.6 Western Blot analysis (II)

Western blot analysis was performed to analyze the protein levels of FGF-2 and to test the specificity of the cystatin C antibody. Naïve animals were decapitated and hippocampi were dissected on ice and frozen in dry ice. The hippocampal tissue was homogenized in a lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 0.25% sodium dodecyl sulfate (SDS), 5 µg/ml leupeptin and aprotinin, and centrifuged. Supernatant was collected and the protein concentration was

determined using the Bradford method (Bio-Rad, Hercules, CA). Protein samples were separated on 12% SDS- polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were probed either with a cystatin C (1:40 000; DAKO) or a rabbit anti-FGF-2 antibody (1:500; #AB1459; Chemicon International) for 2 h at room temperature. Horseradish peroxidase-coupled secondary antibody (polyclonal goat anti-rabbit IgG; 1:20000; #81-6120, Zymed Laboratories, San Francisco, CA) and enhanced chemiluminescence (ECL+kit; #RPN2132, Amersham) were used to reveal the antigens. The blots were exposed to Fuji Super RX film (Tokyo, Japan), which were scanned and analysed with a GS-710 Calibrated Imaging Densitometer (Bio-Rad).

4.7 Data analysis

4.7.1 Degree and distribution of neuronal damage in the hippocampus

FJB (II, IV). The degree of neuronal damage in hippocampal subfields CA1, CA3a, CA3b, CA3c, dentate gyrus, and hilus were analysed semiquantitatively by scoring the presence of FJB-positive cells as follows: score 0, no FJB-positive cells present; score 1, <50 FJB cells; score 2, 50 to 100 FJB cells; score 3, >100 FJB cells within the analysed subfield. A mean score ± standard deviation (SD) was calculated.

Hilar cell death (III). The severity of neuronal loss in the hilus was estimated semiquantitatively and categorized into four classes as follows: normal (score 0, no neuronal loss detected), mild (score 1, less than 20% of neurons lost), moderate (score 2, 20% -50% of neurons lost), or severe (score 3, over 50% of neurons lost). The mean score ± SD was calculated from the scored sections.

Granule cell dispersion (III). In rats, granule cell dispersion was classified as present or not present, and the thickness of the granule cell layer was measured from the mid-portion of the temporal granule cell layer using Stereo Investigator software in the Neurolucida morphometry system (MicroBrightField, Colchester, VT). In humans, granule cell dispersion was analyzed based on the morphology and organization of the granule cell layer. The appearance of the granule cell layer was divided into five categories as follows: *control*, granule cell layer is formed from a dense band of granule cells that are close to each other and displays regular inner and outer borders; *epileptic with non-dispersed appearance*, granule cell layer in the epileptic specimens that resembles the organization in control specimens; *dispersion,* the granule cell bodies are dispersed into the molecular layer forming an irregular outer border; *double-layer*, granule cell bodies are organized in two layers; *death*, granule cell death prevails (modified from Houser, 1990).

4.7.2 Analysis of immunoreactivity

4.7.2.1 Cystatin C

(I-III) Cystatin C immunostaining was semiquantitatively scored in hippocampal areas CA1, CA3, and dentate gyrus (granule cell layer, molecular layer and hilus) as follows: score 0, no cystatin C-immunoreactive (ir) cells present; score 1, low; score 2, moderate number of cystatin C-ir cells; score 3, high number of cystatin C- ir cells present. Glial-like and neuronal-like staining was scored separately. A mean score ± SD was calculated from the analysed sections.

(IV) The quantitative analyses of cystatin C immunoreactivity in the hippocampal area CA1 and molecular layer of the dentate gyrus were performed using a fractionator with Stereo Investigator software in the Neurolucida morphometry system (MicroBrightField). The analysis was performed on three sections sampled at 300-µm intervals starting at the level where the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells (-3.30 from bregma according to Paxinos and Watson, 1986). The area was outlined and cystatin C-ir cells with glial morphology were counted in evenly-spaced x-y intervals (125x125µm) in unbiased counting frames (50x50µm). The entire thickness of the section was scanned, avoiding cells that were in focus at the surface of the section. The area of CA1 did not differ between groups and therefore the data were expressed as cells per mm^2. The area in the molecular layer of the dentate gyrus, however, did differ between groups and thus the data were expressed as mean number of cells per section rather than as density per unit area.

4.7.2.2 BrdU (II)

BrdU-immunopositive cells were manually counted from the SGZ starting at bregma level -1.60 mm according to Hof *et al.* (2000). The boundaries of the granule cell layer were drawn from the sections with a computer-aided digitizing system (Minnesota Datametrics, St. Paul, MN), and the length of the granule cell layer was measured from the line drawings using a digitizing tablet (Summasketch II Professional, Summagraphics Corp., Stanford, CT) and Sigma-Scan V3.92 MS-DOS software (Jandel Scientific, San Raphael, CA). No differences in the length of the granule cell layer were detected between the different genotypes or treatment groups and therefore the results were presented as the mean number of BrdU-labelled cells/mm ± SD.

4.7.2.3 Tuc-4 and PSA-NCAM

Mice (II). For analysis of Tuc-4 and PSA-NCAM immunoreactivity, the band of dentate granule cells was divided from the middle into two parts, the inner and outer granule cell layer, and the

immunoreactive cells were separately counted in these areas as described for BrdU. The results (ir-cells/mm) are presented as mean values of three sections ± SD.

Human (III). PSA-NCAM-immunopositive cells in the dentate gyrus were plotted and the boundaries of the dentate granule cell layer were drawn using a computer-aided digitizing system (Minnesota Datametrics). The length of the granule cell layer was measured from the line drawings using a digitizing tablet (Summasketch II Professional, Summagraphics Corp.) and Sigma-Scan V3.92 MS-DOS software (Jandel Scientific). The numbers of PSA-NCAM -positive cells in the SGZ, granule cell layer, and molecular layer of the dentate gyrus were calculated. The results are presented as the mean value (PSA-NCAM cells/mm) ± SD of three consecutive sections.

4.7.2.4 Confocal microscopy (I, II, IV)

(I) The number of cystatin C-ir cells with or without cellular markers was counted (50-100 cells per case) in the area of interest.

(II, IV) A stack of seven images (sampled 2-3 µm apart) was captured from 6 to 10 different frames from the analyzed area. The number of cystatin C-ir cells with or without cellular markers or BrdU-positive cells with or without Tuc-4 or PSA-NCAM was counted (minimum of 50 cells per case). Colocalization was considered when a double-labelled cell was visible in at least two successive images.

4.7.3 Statistical analysis

All data were analysed using SPSS for Windows (v11.5). Differences between two groups were assessed using a non-parametric Mann-Whitney U-test. The statistical significances of multiple groups were calculated using a non-parametric Kruskall-Wallis test followed by a *post-hoc* test for multiple comparisons (Siegel and Castellan, 1988) or by Mann-Whitney with Bonferroni's correction for multiple comparisons. Mortality was analysed using a two-tailed Fisher's exact test, hemispheric differences with a Wilcoxon signed rank test, differences in FGF-2 immunoblotting with a t- test, spike-analysis using repeated measures analysis of variance, and correlations using a two-tailed Spearman's correlation coefficient. A p value of less than 0.05 was considered significant.

5. RESULTS

5.1 Kainate-induced seizures in mice (II)

Systemic kainate administration was used to induce SE in mice. First, we determined the optimal dose of kainate to induce seizures in cystC$^{+/+}$ and cystC$^{-/-}$ mice using three different doses (30, 32, and 35 mg/kg). The latency to the first behavioral seizure and to the first generalized seizure, as well as the duration of behavioral seizures during the 2-h follow-up, were recorded to estimate SE severity. All doses that were tested induced behavioral seizures similarly in both mouse strains; that is, there were no differences in the seizure parameters between cystC$^{+/+}$ and cystC$^{-/-}$ mice (**II**, Table 1). Wild-type mice (cystC$^{+/+}$) had a higher mortality rate compared to cystC$^{-/-}$ mice with the highest dose ($p<0.05$), but the mortality was related to the toxicity of the kainate rather than to more severe SE because death occurred within minutes of kainate injection, well before the onset of seizures. The 32 mg/kg dose seemed to be optimal. For the next experiment, however, we used the lower dose (30 mg/kg) because electrode implantation reduces tolerance to kainate (Lähteinen et al., 2002).

Another set of animals was injected with kainate and electrographic seizure activity was recorded via cortical screw electrodes. During the experiment, we increased the dose of kainate from 30 to 32 mg/kg because, in contrast to the first set of animals, the lower dose did not consistently produce seizures. There was, however, no difference in the duration of electrographic seizures between the different doses, and therefore they were combined into one group in subsequent experiments. There were no differences between the mouse strains in kainate-induced electrographic seizure activity or duration of SE. Analysis of spiking activity performed in a set of animals indicated that the response to kainate did not differ between cystC$^{+/+}$ and cystC$^{-/-}$ mice (**II**, Fig.1C).

5.2 Cystatin C expression in normal rat, mouse, and human hippocampus (I-IV)

In normal rat hippocampus (**I, III, IV**), cystatin C-positive elements were observed in all layers of the CA1 and CA3 subregions as well as in the dentate gyrus (**I**, Fig.1A2; **III**, Fig.1B; **IV**, Fig.1B). The overall staining pattern in different hippocampal regions was low. The cystatin C-ir cells had a morphology resembling that of glial cells (**I**, Fig.2M, R; **III**, Fig.1B; **IV**, Fig.1I). This staining pattern was abolished by preincubation of cystatin C antibody with low concentrations of cystatin C protein (**I**, Fig.1C1). Diffuse staining within the pyramidal layers with this antibody was, however, considered nonspecific for the antibody, because it was not abolished by the preincubation. The glial cell type expressing cystatin C was verified by double-labelling of

cystatin C with different glial markers. In the *stratum radiatum* of CA1, cystatin C colocalized with the microglial marker Ox-42; thus, cystatin C was predominantly expressed in microglia (**I**, Fig.2I; **IV**, Fig.2C). The confocal system used in the first study **(I)** did not show clear colocalization of cystatin C and the astrocytic marker GFAP. Instead, the GFAP-stained processes were surrounded by cystatin C-positive elements (**I**, Fig.2A, C). With a newer confocal system, which enabled stack analysis **(IV)**, however, cystatin C colocalization was demonstrated also in GFAP-positive astrocytes in the *stratum radiatum* of the CA1 region (**IV**, Fig.2A).

Similar to rat, there were low levels of cystatin C-ir in all areas of the mouse hippocampus (**II**, Fig.2A). Cystatin C-ir cells localized especially to the *stratum oriens* and *stratum radiatum* of the CA1 and CA3 subregions and the molecular layer of the dentate gyrus (**II**, Fig.2D, F). The morphology of the cystatin C-ir cells resembled glia (**II**, Fig.2D). In double-labelling experiments, cystatin C colocalized mainly with GFAP (56%), but also with Ox-42 (37%). In mouse hippocampus, there were also a few intensely stained single neurons (also NeuN-positive) in the CA1 subregion (**II**, Fig.2D), which were presumably interneurons based on their location in non-pyramidal layers. Double-labelling experiments confirmed that they were parvalbumin-positive GABAergic interneurons (**II**, Fig.3D).

In the human hippocampus **(III)**, low levels of cystatin C immunoreactivity were also present in all layers of the dentate gyrus, as well as in the CA1, CA2, and CA3 subregions. The cystatin C-positive cells had a glial-like morphology (**III**, Fig.2E), but neuronal staining was also more prominent than in rat or mouse. Neuronal staining of cystatin C was observed in the hilus (**III**, Fig.2B, G) and pyramidal cells of the CA1, CA2, and CA3 subregions. Double-labelling also revealed that cystatin C colocalized with astrocytic GFAP and neuronal NeuN in human tissue (**III**, Fig.2F, H). Unfortunately, we could not perform double-staining with the microglial marker HLA-DR, because cystatin C staining could not be visualized after the microwave treatment required for microglial staining. Also, the presence of lipofuscin in the neurons and astrocytes of human brain prevented quantitation of cystatin C and cellular markers, due to the autofluorescence of lipofuscin, which was visible in all channels including those of the specific markers.

5.3 Cystatin C expression following an epileptogenic brain insult (I, II, IV)

5.3.1 Distribution and time course of cystatin C expression following SE (I, II)

The time course of cystatin C protein expression in the rat was investigated at 24 h, 4 d, 1 wk, and 2 wk following SE induced by electrical stimulation of the amygdala **(I)**. The number of cystatin C-ir cells appeared slightly decreased 24 h after SE compared with controls (**I**, Fig.1D). A robust

increase in cystatin C-positive glial cells, however, occurred 4 d after SE (**I**, Fig.1E). The increase was especially confined to the non-pyramidal layers of the CA1 and CA3 subregions, and was located in Ox-42-positive microglial cells with activated morphology (**II**, Fig.2K). At 4 d, cystatin C was also present in degenerating pyramidal cells, and at this time the staining was considered to be specific, unlike at other time points, because the staining could be abolished by preincubating cystatin C antibody with the recombinant cystatin C protein prior to staining. The number of cystatin C-ir cells was further increased at 1 wk and 2 wk (**I**, Table 2). At all time points, cystatin C predominantly colocalized with Ox-42, and microglia was thus considered the major cell type expressing cystatin C after SE (**I**, Fig.2J-L). As in the control brain, clear colocalization with astrocytic GFAP could not be demonstrated at any of the examined time points (**I**, Fig.2B, D).

In mice, cystatin C expression following kainate-induced SE was examined at two time points, 24 h and 1 wk after SE **(II)**. At 24 h, the number of cystatin C-ir cells in the CA1, CA3, and the molecular layer of the dentate gyrus was similar to that in the control brain; that is, low cystatin C staining was observed in all regions. The number of cystatin C- positive cells, however, increased robustly by 1 wk after SE as compared to controls (**II**, Fig.2B). The increase was present in the CA1 ($p<0.05$), CA3 ($p<0.01$), and the molecular layer of the dentate gyrus ($p<0.05$; **II**, Fig.2E, G). At this time point, cystatin C colocalized equally with astrocytes and microglia, and only slightly with neurons (**II**, Fig.3A-C). The apparent increase seemed to be due to the increased number of glial cells expressing cystatin C as well as denser staining of cystatin C in glial cells, which suggests increased protein levels in individual cells.

5.3.2 Distribution and time course of cystatin C expression after stroke (IV)

Cystatin C expression following photothrombotic stroke was investigated at 24 h, 4 d, 2 wk, and 2 mo after the stroke **(IV)**. The expression was examined in the hippocampal CA1 subregion and the molecular layer of the dentate gyrus. At 24 h following photothrombosis, there was no difference in the number of cystatin C-ir cells in the CA1 region as compared to controls (**IV**, Table 1, Fig.1E). The cells, however, had different morphological features from those in controls. The glial cells were more darkly stained with shorter and thicker branches, resembling activated glia (**IV**, Fig.1N,O). At this time point, there were also numerous, very darkly-stained cystatin C-positive cells in the *stratum oriens* of the CA1 (**IV**, Fig.1E,O). The morphology of the cystatin C-ir cells did not change in the molecular layer of the dentate gyrus. At 4 d, the number of cystatin C-ir cells was clearly increased in the CA1 area in comparison with controls ($p<0.05$; **IV**, Table 1, Fig.1H). The cystatin C-ir cells resembled those at the 24-h time point (**IV**, Fig.1P). There was also a tendency towards an increased number of cystatin C-positive cells in the molecular layer of the

dentate gyrus. At 2 wk, the number of cystatin C-ir cells in the CA1 as well as in the molecular layer of the dentate gyrus tended to be higher than that in controls, but returned to baseline levels by 2 mo. At that time, the morphology of the cystatin C-positive cells resembled that of controls (**IV**, Fig.1Q). At all time points examined, cystatin C colocalized with GFAP in astrocytes and with Ox-42 in microglia, demonstrating that astrocytes and microglia were the major cell types expressing cystatin C after photothrombosis.

5.4 Cystatin C expression in chronic epilepsy (III)

To investigate whether cystatin C expression is also altered during the chronic stage of epilepsy, we examined chronically epileptic rats at 3.5 mo after SE induced by electrical stimulation of the amygdala. Cystatin C expression was investigated both in the septal and temporal dentate gyrus. In the septal dentate gyrus of epileptic animals, there was glial-like cystatin C staining in all layers (**III**, Table 1). In comparison to non-epileptic animals, the cystatin C staining in the glial cells of the hilar region was increased in epileptic animals ($p<0.05$). Also, neuronal-like cystatin C-ir was present in all layers of the dentate gyrus except the molecular layer (**III**, Table 1). The density of cystatin C in the hilar neurons was decreased in epileptic animals ($p<0.01$). At the temporal end, cystatin C-ir was increased in the glial cells of the molecular layer as compared to the non-epileptic animals ($p<0.05$; **III**, Table 1, Fig. 1D). In contrast to the septal end, there was no change in the neuronal-like cystatin C staining.

The hippocampal samples obtained from the epilepsy surgery of patients with drug-refractory TLE offered a great opportunity to investigate cystatin C expression in human epilepsy. It also provided an interesting comparison between the rat data and human data. In epileptic patients, cystatin C-ir was observed in all layers of the hippocampus, but it was highly increased in glial-like cells in the molecular layer of the dentate gyrus compared to the non-epileptic control patients ($p<0.001$; **III**, Table 1, Fig.2D). Also, the CA1 ($p<0.05$), CA2 ($p<0.001$), and CA3 ($p<0.01$) subregions of the hippocampus had an increased number of cystatin C-ir cells compared with controls. Cystatin C-ir cells in these regions mainly resembled glia, but neuronal staining was also observed in the pyramidal cell layer. Neuronal-like staining was also seen in the hilus of the dentate gyrus, where the density of cystatin C-positive cells was decreased in comparison with controls ($p<0.05$, **III**, Table 1). The glial and neuronal localization of cystatin C in the human tissue was also verified by double-labelling. In the epileptic tissue, cystatin C localized in GFAP-positive astrocytes and NeuN-positive neurons. Because the patient population represented a heterogeneous group of epileptic patients, we also investigated whether increased cystatin C expression was related to a specific type of epilepsy. Increased cystatin C staining in the molecular layer of the dentate gyrus

was particularly associated with symptomatic TLE ($p<0.01$) and with hippocampal sclerosis ($p<0.01$).

5.5 Cystatin C expression and neuronal damage

5.5.1 Acute SE-induced damage in cystatin C-deficient mice (II)

Neuronal cell death is a major neuropathologic consequence of SE and occurs both in animal models and in human TLE. Previous reports suggest that cystatin C has a role in neuronal cell death, but the data remain controversial. To clarify the role of cystatin C in neuronal death, we investigated kainate-induced neuronal death in cystatin C-deficient mice compared with wild-type littermates **(II)**. For the detection of degenerated neurons in different hippocampal subregions, we used the fluorescent marker FJB (Schmued and Hopkins, 2000). No FJB-positive cells were visualized in control animals, suggesting that there was no neuronal damage (**II**, Fig.4B). At 24 h after kainate-induced SE, neuronal damage was present in both $cystC^{+/+}$ and $cystC^{-/-}$ mice (**II**, Fig.4C-F). The FJB-positive degenerated neurons were predominantly located in the CA3a subregion of the hippocampus in both strains, but the degree of damage differed. $CystC^{-/-}$ mice had clearly decreased damage compared with $cystC^{+/+}$ mice (**II**, Fig.4C-F). Also, if there was damage in the CA1 region, it was milder in $cystC^{-/-}$ mice compared to $cystC^{+/+}$ mice.

5.5.2 Association of cystatin C expression with hilar damage in chronic epilepsy (III)

Hilar cell death is a typical feature of neuronal damage in the amygdala stimulation model of TLE and in human patients with TLE and hippocampal sclerosis. Therefore, we investigated the relation between cystatin C expression in the molecular layer of the dentate gyrus and the degree of hilar cell death in chronic epilepsy. In the rat, we concentrated on the temporal dentate gyrus, because it corresponds to the human anterior dentate gyrus, which was removed in epilepsy surgery and was available for the current analysis. All animals had bilateral hilar cell death, and the degree of damage correlated with seizure number; that is, there was more pronounced hilar cell death with a higher frequency of spontaneous seizures ($r=0.853$, $p<0.05$). Although the increase in cystatin C expression in the molecular layer of the dentate gyrus correlated with the high seizure frequency ($r=0.737$, $p<0.05$), there was no direct association with the degree of hilar cell damage in the epileptic animals. In humans, however, there was a link between hilar cell death and cystatin C expression; that is, more pronounced cystatin C expression was observed in the molecular layer of the dentate gyrus when the hilar cell death was severe ($r=0.356$, $p<0.01$). There was, however, no correlation between seizure number and cystatin C expression in the human patients with TLE,

although a longer duration of epilepsy was associated with more severe hilar cell death ($r=0.329$, $p<0.05$).

5.5.3 Association of cystatin C expression with granule cell dispersion (III)

Granule cell dispersion, *i.e.*, disorganization of the granule cell layer, is another prominent feature of hippocampal sclerosis in human TLE. There are only a few reports of granule cell dispersion in the animal models of TLE. Thus, we examined whether granule cell dispersion occurs in the amygdala stimulation model of TLE, and whether dispersion is associated with cystatin C expression in the molecular layer of the dentate gyrus both in rat and in human patients with TLE. The granule cell layer in the temporal dentate gyrus was wider in 75% of the epileptic rats (6/8) (**III**, Fig.1C). The thickness of the granule cell layer ranged from 119 to 165 μm in epileptic rats compared to 51 to 77 μm in controls ($p<0.05$). The dispersion was located specifically at the temporal end and there was no dispersion observed at the septal end. Hilar cell death was associated with granule cell dispersion; animals with granule cell dispersion had more severe damage in the hilus than animals without dispersion ($p<0.01$). Also, the cystatin C expression in the molecular layer of the dentate gyrus was more intense in animals with granule cell dispersion ($p<0.05$).

In the human epileptic hippocampus, granule cell dispersion was classified into four categories based on the morphology and organization of the granule cell layer (**III**, Fig.4). Cystatin C expression in the molecular layer was most prominent when the granule cell layer exhibited dispersed organization ($p<0.01$; **III**, Fig.3, Fig.4F), meaning that the granule cells were scattered to the molecular layer, and the outer border of the granule cell layer was irregular (**III**, 4E). Also, when the granule cells formed a two-layered organization, the expression of cystatin C in the molecular layer tended to be higher than in non-epileptic control subjects (**III**, Fig.4G-H).

5.6 Cystatin C expression and neurogenesis

5.6.1 Proliferation and differentiation of neural stem cells in cystatin C-deficient mice (II)

Cystatin C is linked to neurogenesis, and is highly increased following SE. To clarify the functional role of cystatin C in neurogenesis, we studied the process of neurogenesis (proliferation of neuronal stem cells, commitment to the neuronal phenotype, and migration) in cystatin C-deficient mice compared to their wild-type littermates. In control mice, proliferation of neuronal stem cells, as assessed with BrdU, was reduced in $cystC^{-/-}$ mice compared with $cystC^{+/+}$ mice ($p<0.05$; **II**, Table 2, Fig.5A, Fig.6A, B). Kainate produced a large increase in proliferation in both mouse strains, although $cystC^{-/-}$ mice tended to have a lower number of BrdU-positive cells in the

SGZ in comparison with cystC$^{+/+}$ (**II**, Table 2, Fig.5A, Fig.6C, D). The proliferating BrdU-positive cells located within the SGZ. When we assessed neurogenesis with PSA-NCAM, control cystC$^{-/-}$ mice also had lower numbers of neuronal progenitor cells than cystC$^{+/+}$ mice (**II**, Table 2, Fig.5A). Kainate also increased the numbers of PSA-NCAM and Tuc-4-positive cells in both mouse strains (**II**, Table 2, Fig.5A).

In addition to proliferation, we investigated the commitment of neural stem cells to the neuronal phenotype by double-labelling BrdU-positive cells with markers of newborn neurons, Tuc-4 and PSA-NCAM. In the cystC$^{+/+}$ mice, 28% of BrdU cells that were localized to the SGZ expressed Tuc-4 and 50% expressed PSA-NCAM (**II**, Table 2). The percentages of BrdU/Tuc-4 or BrdU/PSA-NCAM double-positive cells were similar in cystC$^{-/-}$ mice; 38% and 42%, respectively. Kainate treatment did not affect the differentiation of neuronal progenitors, and the percentages of BrdU/Tuc-4 or BrdU/PSA-NCAM double-positive cells were similar to controls in both genotypes (**II**, Table 2).

5.6.2 Neurogenesis in human patients with drug-refractory TLE (III)

Although it is well documented that neurogenesis is induced by seizure activity in experimental animal models of TLE, the effects of prolonged recurrent seizures on neurogenesis in human remain controversial. We therefore examined the rate of neurogenesis in patients with drug-refractory TLE using an immature neuronal marker, PSA-NCAM, as an indirect marker of neurogenesis. In control patients, numerous PSA-NCAM positive cells were observed along the granule cell layer, locating to the SGZ (**III**, Table 2, Fig.5A, B). The number of PSA-NCAM positive cells was, however, substantially lower in epileptic patients compared with control patients ($p<0.01$; **III**, Table 2, Fig.5A, C). The patients with symptomatic TLE ($p<0.01$) and with hippocampal sclerosis ($p<0.01$) had especially low levels of neurogenesis.

5.6.3 Migration of neuronal progenitor cells (II, III)

To elucidate the effects of cystatin C on migration of newborn neurons in the dentate gyrus, we examined the distribution of Tuc-4 and PSA-NCAM-positive cells in cystatin C-deficient mice in comparison with wild-type mice. The presence of Tuc-4 and PSA-NCAM-positive cells in different parts of the granule cell layer (inner and outer granule cell layer) was recorded. In control animals, the majority of Tuc-4 and PSA-NCAM-labelled cells remained within the inner granule cell layer (95% and 93%, respectively), and only a few cells were located in the outer parts of the granule cell layer (**II**, Table 2, Fig.5B). Kainate induced the migration of newborn neurons towards the outer parts of the granule cell layer in both genotypes. CystC$^{-/-}$ mice, however,

demonstrated reduced migration of newborn neurons compared with cystC$^{+/+}$ mice ($p<0.05$; **III**, Fig.6E, F). The outer granule cell layer contained 18% of both Tuc-4 and PSA-NCAM- positive cells in cystC$^{-/-}$ mice in comparison with 33% and 26% in cystC$^{+/+}$ mice, respectively (**II**, Table 2, Fig.5B).

The effect of recurrent spontaneous seizures on the migration of newborn neurons was investigated in human epileptic patients using PSA-NCAM. The distribution of PSA-NCAM- positive cells in the SGZ, granule cell layer, and molecular layer of the dentate gyrus was recorded. In control patients, the newborn PSA-NCAM-positive cells mainly located within the SGZ (47% of all PSA-NCAM cells) and the granule cell layer (49%), whereas only a few PSA-NCAM cells were present in the molecular layer (3%) (**III**, Table 2). In patients with TLE, the distribution of PSA-NCAM-positive cells, however, differed from that in controls. The most striking difference was the increased presence of PSA-NCAM- positive cells in the molecular layer of the dentate gyrus. In epileptic patients, 12% of all PSA-NCAM- positive cells located in the molecular layer compared with 3% in the controls ($p<0.05$; **III**, Table 2). The abnormal migration of PSA-NCAM-positive cells to the molecular layer of the dentate gyrus was particularly evident in TLE patients with granule cell dispersion, in which 13% of all PSA-NCAM-positive cells located within the molecular layer ($p<0.05$; **III**, Table 2). Increased migration of PSA-NCAM cells to the molecular layer was also observed in cases with a depleted granule cell layer; *i.e.*, when granule cells died, the molecular layer comprised 28% of all PSA-NCAM-positive cells compared with 3% in controls ($p<0.01$). Also, the high number of PSA-NCAM-positive cells in the molecular layer was associated with high expression of cystatin C in the molecular layer ($r=0.371$, $p<0.05$).

5.6.4 Fibroblast growth factor-2 (FGF-2) expression in cystatin C-deficient mice (II)

The glycosylated form of cystatin C is required for the mitogenic activity of FGF-2 (Taupin et al., 2000). In an attempt to characterize the underlying mechanism of reduced neurogenesis in cystatin C-deficient mice, we investigated the protein levels of FGF-2 in cystatin C knockout mice compared with wild-type mice. The polyclonal FGF-2 antibody recognized a 24-kDa form of FGF-2 in Western blot analysis. The protein levels of this isomer were reduced in cystC$^{-/-}$ mice compared with cystC$^{+/+}$ mice ($p<0.01$; **II**, Fig.7).

6. DISCUSSION

The aim of this thesis work was to investigate the temporal and spatial expression of cystatin C protein during epileptogenesis and epilepsy, and to elucidate its role in the neuropathological alterations that underlie network reorganization during epileptogenesis such as neuronal cell death and neurogenesis. The present series of experiments demonstrated the following: First, cystatin C is expressed at low levels in the normal hippocampus in all species investigated. The expression is confined mainly to glial cells. Second, cystatin C protein levels are upregulated in response to epileptogenic brain insult, being increased in activated astrocytes and microglia 4 to 7 d after the insult. Third, cystatin C upregulation also occurs during the chronic stages of epilepsy, both in the rat model of TLE and in human patients with drug-refractory TLE. Finally, cystatin C-deficient mice are less susceptible to SE-induced neuronal degeneration and have impaired proliferation and migration of neuronal progenitor cells in the dentate gyrus, suggesting that cystatin C functions both as a mediator of neuronal cell death and as a migratory cue for newborn neurons. Also, in human patients with TLE, the migration of newborn neurons to the molecular layer is enhanced in the presence of high cystatin C expression, suggesting that cystatin C upregulation underlies the development of the dispersed granule cell layer.

6.1 Methodological considerations

6.1.1 Kainate model of TLE in mice

The kainate model of TLE was the model of choice for mice in the present study because our group has extensive experience with systemic application of kainate and performing EEG recordings in mice (Lähteinen et al., 2002). The problem with the application of the kainate model in mice is that mouse strains with different genetic backgrounds vary in their response to kainate-induced neuronal cell death (Ferraro et al., 1995; Schauwecker and Steward, 1997; McKhann et al., 2003). Some strains are vulnerable to kainate (DBA/2, FVB/N and 129SvJ) whereas other strains are highly resistant (C57BL/6 and Balb/c). Also, the rate of neurogenesis might differ between the commonly used strains (Kempermann et al., 1997a). The problem with transgenic animals is that they typically have a genetic background that is hybrid from the parent strains and might respond differently to kainate than their parent strains. Therefore, it is essential to have appropriate control animals with a similar genetic background. In the present study, cystatin C-deficient mice were hybrids of the vulnerable 129SvJ strain and the resistant C57BL/6 strain, and therefore we were able to demonstrate kainate-induced neuronal death in these mice. As controls, we used wild-type littermates with a similar genetic background. To control the possible effects of SE severity on neuronal death, we characterized the response of cystatin C-deficient mice and their

wild-type littermates to kainate. These mouse strains did not differ in the kainate-induced seizure severity, as determined both behaviourally or using EEG. In addition, to control for variables that might hamper the interpretation of the results, only animals that experienced behavioural or electrographic seizures for at least 80 min were included in the study.

6.1.2 Human tissue samples

The human material used in the present experiments was derived from epilepsy surgery of patients with drug-refractory TLE. The selection of patients represented a highly selected population of patients in which the disease was well advanced and the seizure frequency was high. Also, the underlying causes of epilepsy are different in these patients. Furthermore, AED treatment had been ongoing, the effects of which cannot be excluded. The control tissue was obtained from autopsies and there is a possibility that the post-mortem interval affected the results. Previous reports described problems with cystatin C staining performed with human autopsy samples (Bernstein et al., 1988). In the present experiments, however, we demonstrated uniform cystatin C staining in autopsied human specimens, regardless of the post-mortem interval.

6.1.3 Specificity of the immunohistochemical stainings

In immunohistochemistry, the specificity of the cystatin C antibody was controlled by pre-incubation of the antibody with cystatin C protein. This resulted in the disappearance of glial-like immunoreactivity in a concentration-dependent manner. In rat, the antibody diffusely stained the pyramidal layer, but since this type of staining was not abolished by preincubation we considered it to be non-specific for the antibody. In contrast, however, the neuronal staining in human specimens was specific. We also tested the specificity of cystatin C antibody using cystatin C-deficient mice as controls, and there was no glial-like immunoreactivity in the hippocampus of cystatin C-deficient mice. The confocal microscope used in the first study for the analysis of double-stained sections was an older model with which we were not able to perform the stack analysis, and this might be the reason why we were not able to demonstrate the colocalization of cystatin C and GFAP in astrocytes in the first study (I). This problem was overcome by using a newer model capable of stack analysis, and thus the colocalization with GFAP and cystatin C was demonstrated in other studies (II-IV). Double-labelling with fluorescent markers was problematic when using human material because of the presence of lipofuscin in neurons and astrocytes. Lipofuscin is a non-degradable intralysosomal substance that accumulates in aging neurons and astrocytes (Terman and Brunk, 2004). The particular problem with lipofuscin is that it gives a non-specific autofluorescence in all channels and therefore can mimic any fluorescent label and complicate the distinction between specific and nonspecific labelling (Schnell et al., 1999).

Therefore we were not able to quantify human double-labelled sections. Regardless of this problem with human tissue, we were able to demonstrate the colocalization of cystatin C with GFAP and NeuN after carefully examining the cells and the subcellular location of the staining to discriminate lipofuscin from the specific signal.

6.2 Normal pattern of cystatin C expression in the hippocampus

Brain contains relatively high amounts of cystatin C, but reports of the cellular localization of cystatin C expression are sparse or controversial. To clarify the spatial and subcellular localization of cystatin C, we investigated cystatin C immunoreactivity in normal rat, mouse, and human brain. Light cystatin C-ir cells with glial-like morphology were present throughout the hippocampus in all species investigated, particularly in the *stratum oriens* and *stratum radiatum* of the CA1 and CA3 subregions and in the hilus and molecular layer of the dentate gyrus. In mouse, there were a number of non-pyramidal neurons in the CA1, which were also cystatin C-positive. These were parvalbumin-expressing interneurons, consistent with the observations by Aronica and colleagues (2001). Cystatin C staining located in astrocytes and microglia, which was confirmed by double-labelling with cellular markers. In study I, we were unable to demonstrate clear colocalization of cystatin C and GFAP in the rat, but this was later achieved in study IV. Further, in humans, the microglial origin of cystatin C was not demonstrated due to difficulties with the staining protocol, but cannot be excluded based on the findings in rat and mouse.

Previously, Yasuhara and colleagues (1993) described cystatin C expression in astrocytes, but not in microglia in the rat, monkey, or human brain. Microglial cystatin C was, however, demonstrated by Miyake and colleagues (1996) in the rat facial nucleus and by Aronica and coworkers (2001) in the rat hippocampus. Furthermore, Palm and colleagues (1995) demonstrated only subtle neuronal staining without a trace in astrocytes or microglia. Our results further extend these observations by clearly demonstrating that cystatin C is expressed in both astrocytes and microglia. Neurons that were positive for cystatin C were rarely observed in rat or mouse, but were more numerous in human, which is consistent with Yasuhara's observations (1993). In the human brain, cystatin C expression was previously described in choroid plexus epithelial cells, cerebral and cerebellar neurons, and astrocytes (Lignelid et al., 1997).

The different patterns of cystatin C immunoreactivity in different studies might be related to differences in staining protocols and antibodies used. In our study, we used a similar staining protocol and same antibody dilution in all species and therefore the staining pattern was highly similar in rat, mouse, and human brain. Cystatin C was diffusely expressed mainly in astrocytes

and microglia and to a lesser extent in neurons in normal rat, mouse, and human brain, with a high degree of similarity between species.

6.3 Delayed increase in cystatin C expression after an epileptogenic brain injury

Cystatin C gene expression was upregulated in response to SE. To verify the upregulation of cystatin C protein after SE, we investigated the temporal and spatial expression of cystatin C in the hippocampus using two different models of SE and two different animal species **(I, II)**. Also, to elucidate whether cystatin C upregulation is a common epileptogenesis-related response, we applied another model with a different precipitating injury, the photothrombotic stroke model of post-stroke epileptogenesis **(IV)**. We hypothesized that similarities in cystatin C expression between these models indicates a role for cystatin C in epileptogenesis-related cellular alterations.

Our data demonstrated a robust upregulation of cystatin C protein following SE, which was independent of the method used to induce SE and of the species investigated; meaning that the increased cystatin C immunoreactivity occurred both after electrically-induced SE in the rat and after kainate-induced SE in the mice. The upregulation of cystatin C protein expression occurred in a delayed manner, and was highly increased at 4 to 7 d after SE both in rats and mice. The upregulation occurred in reactive astrocytes and microglia, and was spatially located in the non-pyramidal layers of CA1 and CA3 regions. Also in mice, there was a robust upregulation in the molecular layer of the dentate gyrus.

In addition to SE, cystatin C upregulation was also observed following photothrombotic stroke in rats. The temporal and spatial time course of cystatin C expression was similar to that seen after SE: it occurred in the activated glial cells of the CA1 subregion and peaked 4 d following photothrombosis. The number of cystatin C-ir cells still tended to be higher than in controls at 2 wk. In contrast to SE, there was, however, no change in the molecular layer of the dentate gyrus, thus indicating a transient and area-dependent upregulation of cystatin C following photothrombotic stroke.

Our data are consistent with previous reports describing brain injury-induced cystatin C upregulation in microglia following facial nucleus axotomy in rat (Miyake et al., 1996), in astrocytes after perforant path transection in mice (Ying et al., 2002), and in both astrocytes and microglia after SE in rat (Aronica et al., 2001). Cystatin C locates in the degenerating neurons in CA1 after global ischemia in rat and gerbil (Palm et al., 1995; Ishimaru et al., 1996), in hypothalamus after hypophysectomy in the rat (Katakai et al., 1997), and in the striatum and

substantia nigra after lesioning of dopaminergic neurons with 6-hydroxydopamine (Xu et al., 2005). In support of this, we also observed neuronal cystatin C staining in the pyramidal layer of CA1 4 d following electrically-induced SE in rat. Additionally, we observed cystatin C upregulation in the neuropil in the rat after SE, which indicates that cystatin C is released from glial cells and the main function of cystatin C is extracellular. Microglial and astrocytic secretion of cystatin C occurs in vitro (Zucker-Franklin et al., 1987).

In conclusion, our data together with previous observations clearly demonstrate that damage-induced gliosis might serve as a substantial source of cystatin C in the hippocampus. Because cystatin C is present in the hippocampus, which undergoes several cellular and molecular alterations during epileptogenesis, it is likely to have a role in some of these alterations, such as neuronal death, gliosis, axonal and dendritic plasticity, and neurogenesis.

6.4 Persistent cystatin C upregulation in epilepsy

Cystatin C upregulation occurs at the time of network reorganization during epileptogenesis. The plasticity of neuronal networks is suggested to continue in the chronic phase of epilepsy (Pitkänen and Sutula, 2002). Therefore, we were interested in whether alterations in cystatin C expression also occur in chronic epilepsy. For this, we investigated chronically epileptic rats and human patients with drug-refractory TLE **(III)**. In addition, the availability of human material alongside the animal material provided us a means to compare the results from both models, which is a relevant question when modelling human disease.

Our data demonstrated that cystatin C expression was increased 3.5 mo after SE induced by electrical stimulation of the amygdala in rat. The expression had a differential pattern along the septo-temporal axis of the hippocampus, being increased in the hilus of the septal dentate gyrus and in the molecular layer of the temporal dentate gyrus. The cystatin C-ir cells had glial-like morphology, similar to that observed acutely after SE. These results are in accordance with previous observations by Aronica and coworkers (2001) who demonstrated that cystatin C expression is persistently upregulated in the hilus of the dentate gyrus 3 mo following SE induced by electrical stimulation of the hippocampus.

We also demonstrated in study **III** that cystatin C upregulation occurred in human patients with drug-refractory TLE. A striking similarity with the rat model was observed in the molecular layer of the dentate gyrus, where cystatin C expression was highly increased in TLE patients with hippocampal sclerosis. Cystatin C-ir cells were mainly glial cells but some neurons were also

stained, especially in the hilus and in CA1, CA2, and CA3 regions. The similarity between rat and human was quite surprising given the fact that human patients with TLE had various underlying etiologies, were undergoing antiepileptic medication, and were of variable age. In contrast, rats in our experiments were of the same age and had SE with a common etiology.

Our results are the first to report cystatin C upregulation in human epilepsy. Previously, increased cystatin C immunoreactivity was described only in the vulnerable neurons in AD (Deng et al., 2001). In conclusion, cystatin C expression is increased in chronic epilepsy with similarities between the rat model of TLE and human patients with TLE, indicating that network reorganization is an ongoing process in epileptic brain and consists of pathological alterations in which cystatin C might participate.

6.5 Functional implications of cystatin C expression in epileptogenesis and epilepsy

6.5.1 Association of cystatin C with neuronal death

In study **I**, the increase in cystatin C expression following SE was more prominent in the areas of severe damage, indicating that upregulation of cystatin C occurred in association with neuronal damage and subsequent gliosis. A review of the literature revealed that previous results concerning the role of cystatin C in cell death are highly controversial. It has been suggested that cystatin C might be protective factor because its major function is to regulate the activities of lysosomal cysteine proteinases, which can be extremely harmful if released from degenerating neurons. Furthermore, cathepsin B secreted from microglia can activate caspase 3 and induce neuronal apoptosis (Kingham and Pocock, 2001). The upregulation of cystatin C and its release from glial cells might thus be an endogenous attempt to limit degeneration following injury. This is further supported by the notion that selective cathepsin inhibitors reduce neuronal death following ischemia (Tsuchiya et al., 1999; Seyfried et al., 2001). Furthermore, cystatin C might be protective by upregulating the expression of IL-6 after seizures. IL-6 expression is increased in the hippocampus following kainate-induced seizures in rat and contributes to the protection from kainate-induced neuronal death (Penkowa et al., 2001; Lehtimäki et al., 2003). Cystatin C also upregulates IL-6 production in human gingival fibroblasts and murine splenocytes (Kato *et al.*, 2000).

On the other hand, cystatin C might also promote neuronal death. If cystatin C is injected into the hippocampus, it induces neuronal degeneration in the granule cell layer and hilus of the dentate gyrus (Nagai et al., 2002). Furthermore, cystatin C expression is induced concomitantly with neuronal death following oxidative stress in vitro (Nishio et al., 2000). These observations led us

to examine the functional role of cystatin C in neuronal death by investigating the pattern of cell death in the hippocampus of cystatin C-deficient mice after kainate-induced SE **(II)**.

In cystatin C knockout mice there was less neuronal death following kainate-induced SE than in wild-type littermates, suggesting that instead of being neuroprotective, cystatin C might promote neuronal damage following SE. This is consistent with findings by Olsson and coworkers (2004), who demonstrated that neuronal death is reduced following global ischemia in the same cystatin C-deficient mouse strain. Their observations, however, also revealed that the degree of neuronal death increases following focal ischemia (Olsson et al., 2004). Recently, it was demonstrated that cystatin C prevents 6-hydroxydopamine-induced degeneration of dopaminergic neurons in the substantia nigra (Xu et al., 2005). Also, the expression of cystatin C in PC-12 cells in vitro prevents neuronal apoptosis after oxidative stress (Nishiyama et al., 2005). Together these results suggest that cystatin C has differential effects on neuronal death following different brain injuries. The signals and mechanisms that would determine which pathway cystatin C takes remain to be elucidated.

6.5.2 Association of cystatin C with neurogenesis

The participation of cystatin C is also implicated in the regulation of neurogenesis that occurs throughout life in the mammalian hippocampus (Taupin and Gage, 2002). Animal experiments further demonstrate that neurogenesis is highly increased in response to seizures (Parent et al., 1997; Gray and Sundstrom, 1998; Parent et al., 1998). Because cystatin C expression is also increased in response to seizures, we hypothesized that cystatin C is involved in the regulation of neurogenesis. Therefore, we investigated the proliferation and migration of neuronal progenitor cells in cystatin C-deficient mice in comparison with their wild-type littermates **(II)**. The proliferation was assessed with BrdU, which incorporates into the DNA and can be visualized with immunohistochemistry (Miller and Nowakowski, 1988).

Our results demonstrated that cystatin C-deficient mice had reduced proliferation of neural progenitor cells in the SGZ of the dentate gyrus in comparison with wild-type mice. This observation was consistent with previous observations by Taupin and colleagues (2000) demonstrating a 60% reduction in the proliferation rate in cystatin C knockout mice. The reduction that we observed was slightly less (~30 %), which is most likely related to the different protocols used for BrdU administration, because BrdU is available for incorporation in dividing cells only for a certain period of time after which it is cleared from the brain.

Our results extended earlier observations by demonstrating that there was no change in the proliferation rate between cystatin C knockout mice and wild-type mice after SE. Cystatin C knockout mice, however, tended to have a lower number of BrdU-positive proliferating cells in the SGZ after kainate than wild-type mice. This indicates, as expected, that cystatin C is not the only factor contributing to seizure-induced neurogenesis. Several factors contribute to the regulation of neurogenesis, such as growth factors, neurotransmitters, stress, running, and learning (Fuchs and Gould, 2000). Our results also demonstrated that the commitment of newborn cells to the neuronal phenotype was not affected by a lack of cystatin C, suggesting that cystatin C is not needed for the differentiation of newborn neurons.

Newborn neurons born in the SGZ migrate to the granule cell layer and develop the characteristics of mature granule cells (Kuhn et al., 1996). Thus, we investigated the effects of cystatin C on migration of newborn neurons using Tuc-4 and PSA-NCAM as markers for neurogenesis **(II, III)**. Our results demonstrated that following kainate administration, the newborn neurons migrated further to the outer parts of the granule cell layer, which is consistent with the observation by Parent and colleagues (Parent et al., 1997) that newborn cells are present in the hilus and molecular layer of the dentate gyrus following pilocarpine-induced SE. This is further supported by our findings in human patients with TLE in which there were increased numbers of PSA-NCAM-positive cells in the molecular layer of the dentate gyrus compared with control patients. These results indicate that seizures induce aberrant migration of newborn neurons. Cystatin C-deficient mice, however, had decreased migration compared to wild-type mice, meaning that a lower number of Tuc-4 or PSA-NCAM-positive cells was detected in the outer part of the granule cell layer in the cystatin C knockout mice. In other words, migration of newborn neurons to the outer parts of the granule cell layer is impaired when cystatin C is not expressed, suggesting that cystatin C expressed by glial cells in the molecular layer of the dentate gyrus functions as an exogenous cue for migrating newborn neurons. Both microglia and astrocytes might secrete factors that induce proliferation, migration, and differentiation of neural progenitor cells (Song et al., 2002; Aarum et al., 2003). Cystatin C is required for the neurogenic actions of FGF-2, a growth factor involved in the regulation of neurogenesis (Taupin et al., 2000; Yoshimura et al., 2001). Our data further demonstrated that FGF-2 protein levels were decreased concomitantly with cystatin C deficiency, indicating that the neurogenic functions of cystatin C might be partly mediated via FGF-2. The exact mechanisms underlying cystatin C-induced guidance of newborn neurons remain to be elucidated.

Cystatin C expression was increased in the CA1 region of the hippocampus following photothrombotic stroke **(IV)**. The functional significance of increased cystatin C expression after stroke remains unknown. In addition to seizures, ischemia increases neurogenesis in the SVZ of the lateral ventricles from where the newborn cells migrate to the CA1 region and differentiate into mature neurons (Nakatomi et al., 2002). Photothrombosis increases neurogenesis in the cortex surrounding the lesion, but its effects on hippocampal neurogenesis are unknown (Gu et al., 2000). The previous and present observations suggest that cystatin C expressed in the glial cells of the CA1 region provide migratory cues for SVZ progenitor cells on their way to the CA1 region after stroke. Whether this occurs in photothrombosis requires further study.

6.5.3 Association of cystatin C with granule cell dispersion

The dispersion of granule cells to the molecular layer of the dentate gyrus is a typical morphological alteration that occurs in TLE in association with hippocampal sclerosis (Houser, 1990). Neurogenesis and impaired migration of newborn neurons is suggested to underlie granule cell dispersion (Lurton et al., 1997). The results of previous studies of neurogenesis in human patients with TLE are, however, controversial. This is due mainly to the use of different cellular markers for detecting proliferating cells in the human hippocampus (see chapter 2.2.4.2 and Table 2). Therefore, we investigated the degree of neurogenesis in human patients with TLE using PSA-NCAM as a marker for immature newborn neurons **(III)**.

Our results demonstrated that the rate of neurogenesis was greatly reduced in patients with TLE compared with control patients. This was especially prominent in cases of severe hippocampal damage. These observations are clearly in contrast to the findings from experimental studies that demonstrated greatly increased neurogenesis in response to seizures (Parent et al., 1997; Gray and Sundstrom, 1998). These studies, however, assessed neurogenesis only acutely after seizures and hence cannot be directly compared to the human situation. Our findings are consistent with the recent study by Hattiangady and coworkers (2004) who demonstrated that neurogenesis is reduced in epileptic rats 5 mo after kainate-induced SE as assessed by doublecortin. Decreased neurogenesis was also demonstrated in pediatric epilepsy patients using PSA-NCAM (Mathern et al., 2002). Recent studies performed on tissue obtained from drug-refractory TLE patients, however, demonstrated increased numbers of proliferating cells in the neurogenic area SGZ (Crespel et al., 2005; Thom et al., 2005). In contrast to our study, these studies used markers of proliferating cells that are expressed at an earlier stage in neurogenesis than PSA-NCAM or doublecortin. Our results, together with previous data, indicate that the survival and differentiation of newborn cells to neurons is diminished in human patients with TLE, although proliferation

might be enhanced. Based on animal experiments, the majority of newborn neurons die within 4 wk (Ekdahl et al., 2002).

A recent study by Thom and colleagues (2005) demonstrated increased proliferation of neural progenitors in the SGZ in patients with TLE and granule cell dispersion. Our results further extend this by demonstrating that the surviving newborn neurons aberrantly migrate to the molecular layer of the dentate gyrus in epileptic patients compared with controls. We hypothesized that upregulation of cystatin C in the molecular layer of the dentate gyrus underlies the development of the dispersed granule cell layer. This was supported by the observations that cystatin C expression is highly increased in the region where the newborn cells migrate. Further, the increase in cystatin C expression occurred concomitantly with granule cell dispersion both in the rat model of TLE and in human patients with epilepsy. Also in rat, cystatin C expression was not upregulated in the septal dentate gyrus where there was no granule cell dispersion. These results, together with the notion that migration is impaired in cystatin C-deficient mice, suggest that cystatin C functions as a migratory cue for newborn neurons and that upregulation of cystatin C expression is an underlying factor in granule cell dispersion.

6.6 Signals leading to upregulation of cystatin C

In the first three studies, upregulation of cystatin C expression occurred together with neuronal cell death in the hippocampus, which is accompanied by reactive gliosis, *i.e.*, proliferation of glial cells, morphologic activation, and secretion of a wide variety of molecules. The glial activation in response to neuronal death might be a triggering factor for cystatin C upregulation. Our results demonstrated that cystatin C upregulation was due to an increased number of cystatin C-expressing cells as well as to increased cystatin C content in individual cells, which was demonstrated as denser immunoreactivity. In epileptic animals, although there was dense gliosis in the hilus of the dentate gyrus, there was no increase in cystatin C expression, indicating that gliosis is not the only factor regulating cystatin C. Cystatin C regulation might also be activated directly by seizure activity. Seizure activity, however, is not enough to trigger upregulation, and the development of SE following electrical stimulation of the hippocampus is required (Aronica et al., 2001). This also suggests the involvement of neuronal death and gliosis.

In study **IV**, we investigated whether photothrombotic stroke, similar to SE, leads to upregulated cystatin C expression. Our results demonstrated a robust upregulation 4 d following photothrombosis, which was similar to the time course following SE. The striking difference was that it occurred without neuronal degeneration in the hippocampus, indicating that neuronal death

is not required for the upregulation of cystatin C after cortical stroke. Because the upregulation was most prominent when the cortical lesion extended to the subcortical white matter, we hypothesized that cystatin C was upregulated by signals leaking from the lesion site to the hippocampus. Ischemic stroke is typically associated with inflammatory reactions and microglial and monocytic cell infiltration to the ischemic site (Lehrmann et al., 1998; del Zoppo et al., 2001). Microglia are capable of secreting various inflammatory cytokines, such as TGF-β (Lehrmann et al., 1998). TGF- β upregulates cystatin C gene expression in serum- free mouse embryo cells (Solem et al., 1990), in decidual cultures (Afonso et al., 2002), and in 3T3-fibroblasts (Sokol and Schiemann, 2004). The promoter of the cystatin C gene contains regulatory sites for hormones (androgens) as well as for inflammatory mediators such as interferons (Huh et al., 1995; Tamura et al., 2005). Also, blood-brain barrier breakdown and subsequent vasogenic edema contribute to the formation of cortical lesion after the photothrombotic stroke (Dietrich et al., 1986; Dietrich et al., 1987a; Laursen et al., 1993). The time course of edema formation is similar to cystatin C upregulation in the CA1; that is, it becomes maximal by 24 h and ceases by 4 d after photothrombotic stroke (Laursen et al., 1993). The edema is typically confined to the lesion site. Whether cystatin C is upregulated in response to TGF- β leaking from the lesion site or in the aftermath of edema due to the extravasation of blood-borne cystatin C remains unknown.

7. SUMMARY AND CONCLUSIONS

The principal aim of this study was to investigate the expression pattern of cystatin C protein during epileptogenesis and epilepsy, and to elucidate the functional role of cystatin C in neuronal cell death and neurogenesis, which occur during epileptogenesis. The main findings can be summarized as follows:

1. Cystatin C was present at low levels in normal rat, mouse, and human hippocampus; and expression was confined mainly to the glial cells, both microglia and astrocytes.

2. A robust increase in cystatin C expression occurred within 4 to 7 d after SE induced by electrical stimulation of the amygdala in rat and kainate administration in mice. The upregulation was most prominent in the reactive astrocytes and microglia of the CA1 and CA3 regions and in the molecular layer of the dentate gyrus. Cystatin C expression was also increased in the CA1 region of the hippocampus 4 d after photothrombotic stroke, another epileptogenic brain insult.

3. Cystatin C upregulation persisted during the chronic phase of epilepsy, and was highly increased in the molecular layer of the dentate gyrus both in epileptic rats and human patients with TLE.

4. Cystatin C-deficient mice demonstrated reduced neuronal death, and impaired proliferation and migration of neuronal progenitor cells after kainate administration compared with wild-type littermates, suggesting that cystatin C functions to promote neuronal death and as a cofactor and/or a migratory cue for newborn neurons. Furthermore, in human patients with TLE the robust cystatin C expression in the molecular layer of the dentate gyrus was coincident with abnormal migration of newborn neurons to the molecular layer and granule cell dispersion, indicating that cystatin C participates in the development of the dispersed granule cell layer.

In conclusion, cystatin C upregulation is a common brain injury-related response that occurs mainly in the glial cells of the hippocampus. The present results demonstrated that cystatin C has a bifunctional role in the neuropathological alterations that occur during epileptogenesis, regulating both neuronal cell death and birth. Whether cystatin C underlies the development of a lowered seizure threshold and subsequent epilepsy is, however, unknown. The finding that cystatin C upregulation also occurs at the chronic phase of epilepsy indicates that reorganization is an ongoing process that continues beyond the appearance of spontaneous seizures.

8. REFERENCES

Aarum, J., Sandberg, K., Haeberlein, S. L. and Persson, M. A. (2003). Migration and differentiation of neural precursor cells can be directed by microglia. Proc Natl Acad Sci U S A. *100*, 15983-15988.

Abrahamson, M. (1994). Cystatins. Meth Enzymol. *244*, 685-700.

Abrahamson, M. (1996). Molecular basis for amyloidosis related to hereditary brain hemorrhage. Scand J Clin Lab Invest. *226*, 47-56.

Abrahamson, M., Alvarez-Fernandez, M. and Nathanson, C. M. (2003). Cystatins. Biochem Soc Symp, 179-199.

Abrahamson, M., Buttle, D. J., Mason, R. W., Hansson, H., Grubb, A., Lilja, H. and Ohlsson, K. (1991a). Regulation of cystatin C activity by serine proteinases. Biomed Biochim Acta. *50*, 587-593.

Abrahamson, M., Grubb, A., Olafsson, I. and Lundwall, A. (1987). Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine proteinase inhibitor cystatin C. FEBS Lett. *216*, 229-233.

Abrahamson, M., Islam, M. Q., Szpirer, J., Szpirer, C. and Levan, G. (1989). The human cystatin C gene (CST3), mutated in hereditary cystatin C amyloid angiopathy, is located on chromosome 20. Hum Genet. *82*, 223-226.

Abrahamson, M., Mason, R. W., Hansson, H., Buttle, D. J., Grubb, A. and Ohlsson, K. (1991b). Human cystatin C. role of the N-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leucocyte elastase. Biochem J. *273*, 621-626.

Abrahamson, M., Olafsson, I., Palsdottir, A., Ulvsback, M., Lundwall, A., Jensson, O. and Grubb, A. (1990). Structure and expression of the human cystatin C gene. Biochem J. *268*, 287-294.

Abrous, D. N., Koehl, M. and Le Moal, M. (2005). Adult neurogenesis: from precursors to network and physiology. Physiol Rev. *85*, 523-569.

Afonso, S., Tovar, C., Romagnano, L. and Babiarz, B. (2002). Control and expression of cystatin C by mouse decidual cultures. Mol Reprod Dev. *61*, 155-163.

Altman, J. and Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. *124*, 319-335.

Alvarez-Fernandez, M., Barrett, A. J., Gerhartz, B., Dando, P. M., Ni, J. and Abrahamson, M. (1999). Inhibition of mammalian legumain by some cystatins is due to a novel second reactive site. J Biol Chem. *274*, 19195-19203.

Amaral, D. G. (1993). Emerging principles of intrinsic hippocampal organization. Curr Opin Neurobiol. *3*, 225-229.

Amaral, D. G. and Insausti, R., 1990. Hippocampal formation. In: Paxinos, G. (Ed.), The Human Nervous System. Academic Press, Inc., San Diego, pp. 711-755.

Amaral, D. G. and Witter, M. P., 1995. Hippocampal formation. In: Paxinos, G. (Ed.), The Rat Nervous System. Academic Press, Inc., San Diego, pp. 443-493.

Amato, E. D., Kokala, Z., Nanobashvili, A., Reeben, M., Lehesjoki, A.-E., Saarma, M. and Lindvall, O. (2000). Seizures induce widespread upregulation of cystatin B, the gene mutated in progressive myoclonus epilepsy, in rat forebrain neurons. Eur J Neurosci. *12*, 1687-1695.

Araki, T., Simon, R. P., Taki, W., Lan, J. Q. and Henshall, D. C. (2002). Characterization of neuronal death induced by focally evoked limbic seizures in the C57BL/6 mouse. J Neurosci Res. *69*, 614-621.

Armstrong, D. D. (1993). The neuropathology of temporal lobe epilepsy. J Neuropathol Exp Neurol. *52*, 433-443.

Aronica, E., van Vliet, E. A., Hendriksen, E., Troost, D., Lopes Da Silva, F. H. and Gorter, J. A. (2001). Cystatin C, a cysteine protease inhibitor, is persistently up-regulated in neurons and glia in a rat model for mesial temporal lobe epilepsy. Eur J Neurosci. *14*, 1485-1491.

Babb, T. L. and Pretorius, J. K., 1996. Pathologic Substrates of Epilepsy. In: Wyllie, E. (Ed.), The Treatment of Epilepsy: principles and practice. Williams & Wilkins, Baltimore, pp. 106-121.

Balbin, M. and Abrahamson, M. (1991). SstII polymorphic sites in the promoter region of the human cystatin C gene. Hum Genet. *87*, 751-752.

Balbin, M., Grubb, A. and Abrahamson, M. (1993). An Ala/Thr variation in the coding region of the human cystatin C gene (CST3) detected as a SstII polymorphism. Hum Genet. *92*, 206-207.

Barrett, A. J., Davies, M. E. and Grubb, A. (1984). The place of human gamma-trace (cystatin C) amongst the cysteine proteinase inhibitors. Biochem Biophys Res Commun. *120*, 631-636.

Becker, A. J., Chen, J., Zien, A., Sochivko, D., Normann, S., Schramm, J., Elger, C. E., Wiestler, O. D. and Blumcke, I. (2003). Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy. Eur J Neurosci. *18*, 2792-2802.

Beghi, E., 2004. Aetiology of Epilepsy. In: Shorvon, S. et al. (Eds.), The Treatment of Epilepsy. Blackwell Science Ltd., Massachusetts, USA, pp. 50-63.

Ben-Ari, Y. (1985). Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience. *14*, 375-403.

Ben-Ari, Y., Tremblay, E., Ottersen, O. P. and Meldrum, B. S. (1980). The role of epileptic activity in hippocampal and "remote" cerebral lesions induced by kainic acid. Brain Res. *191*, 79-97.

Bengzon, J., Kokaia, Z., Elmer, E., Nanobashvili, A., Kokaia, M. and Lindvall, O. (1997). Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. Proc Natl Acad Sci U S A. *94*, 10432-10437.

Benkovic, S. A., O'Callaghan, J. P. and Miller, D. B. (2004). Sensitive indicators of injury reveal hippocampal damage in C57BL/6J mice treated with kainic acid in the absence of tonic-clonic seizures. Brain Res. *1024*, 59-76.

Bennett, M. J., Choe, S. and Eisenberg, D. (1994). Domain swapping: entangling alliances between proteins. Proc Natl Acad Sci U S A. *91*, 3127-3131.

Benussi, L., Ghidoni, R., Steinhoff, T., Alberici, A., Villa, A., Mazzoli, F., Nicosia, F., Barbiero, L., Broglio, L., Feudatari, E., Signorini, S., Finckh, U., Nitsch, R. M. and Binetti, G. (2003). Alzheimer disease-associated cystatin C variant undergoes impaired secretion. Neurobiol Dis. *13*, 15-21.

Berdowska, I. (2004). Cysteine proteases as disease markers. Clin Chim Acta. *342*, 41-69.

Bernstein, H. G., Jarvinen, M., Pollanen, R., Schirpe, H., Knofel, B. and Rinne, R. (1988). Cystatin C containing neurons in human postmortem hypothalamus. Neurosci Lett. *88*, 131-134.

Beyer, K., Lao, J. I., Gomez, M., Riutort, N., Latorre, P., Mate, J. L. and Ariza, A. (2001). Alzheimer's disease and the cystatin C gene polymorphism: an association study. Neurosci Lett. *315*, 17-20.

Bezvenyuk, Z., Nissinen, J. and Pitkänen, A. (2003). Photothrombotic brain infarction induces epileptogenesis in rats. 2003 Abstract Viewer Washington, DC: Society of Neurosciece. *Program No. 533.15*.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. Nature. *321*, 209-213.

Bjarnadottir, M., Wulff, B. S., Sameni, M., Sloane, B. F., Keppler, D., Grubb, A. and Abrahamson, M. (1998). Intracellular accumulation of the amyloidogenic L68Q variant of human cystatin C in NIH/3T3 cells. Mol Pathol. *51*, 317-326.

Bjorck, L., Grubb, A. and Kjellen, L. (1990). Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus. J Virol. *64*, 941-943.

Blümcke, I., Beck, H., Lie, A. A. and Wiestler, O. D. (1999). Molecular neuropathology of human mesial temporal lobe epilepsy. Epilepsy Res. *36*, 205-223.

Blümcke, I., Schewe, J. C., Normann, S., Brustle, O., Schramm, J., Elger, C. E. and Wiestler, O. D. (2001). Increase of nestin-immunoreactive neural precursor cells in the dentate gyrus of pediatric patients with early-onset temporal lobe epilepsy. Hippocampus. *11*, 311-321.

Blümcke, I., Thom, M. and Wiestler, O. D. (2002). Ammon's horn sclerosis: a maldevelopmental disorder associated with temporal lobe epilepsy. Brain Pathol. *12*, 199-211.

Bobek, L. A. and Levine, M. J. (1992). Cystatins-inhibitors of cysteine proteinases. Crit Rev Oral Biol Med. *3*, 307-332.

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. and Turk, V. (1988). The 2.0 A X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. EMBO J. *7*, 2593-2599.

Bollengier, F. (1987). Cystatin C, alias post-gamma-globulin: a marker for multiple sclerosis? J Clin Chem Clin Biochem. *25*, 589-593.

Bouilleret, V., Ridoux, V., Depaulis, A., Marescaux, C., Nehlig, A. and Le Gal La Salle, G. (1999). Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience. *89*, 717-729.

Brown, J. P., Couillard-Despres, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L. and Kuhn, H. G. (2003). Transient expression of doublecortin during adult neurogenesis. J Comp Neurol. *467*, 1-10.

Browne, T. R. and Holmes, G. L., 2000. Handbook of epilepsy. Lippincott Williams& Wilkins, Philadelphia, PA.

Bruton, C. J., 1988. The neuropathology of temporal lobe epilepsy. Oxford University Press, New York.

Brzin, J., Popovic, T., Turk, V., Borchart, U. and Machleidt, W. (1984). Human cystatin, a new protein inhibitor of cysteine proteinases. Biochem Biophys Res Commun. *118*, 103-109.

Buck, M. R., Karustis, D. G., Day, N. A., Honn, K. V. and Sloane, B. F. (1992). Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. Biochem J. *282*, 273-278.

Cai, J., Xue, H., Zhan, M. and Rao, M. S. (2004). Characterization of progenitor-cell-specific genes identified by subtractive suppression hybridization. Dev Neurosci. *26*, 131-147.

Cameron, H. A., McEwen, B. S. and Gould, E. (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. J Neurosci. *15*, 4687-4692.

Cameron, H. A. and McKay, R. D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol. *435*, 406-417.

Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. (1993). Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience. *56*, 337-344.

Carrette, O., Demalte, I., Scherl, A., Yalkinoglu, O., Corthals, G., Burkhard, P., Hochstrasser, D. F. and Sanchez, J. C. (2003). A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. Proteomics. *3*, 1486-1494.

Cathcart, H. M., Huang, R., Lanham, I. S., Corder, E. H. and Poduslo, S. E. (2005). Cystatin C as a risk factor for Alzheimer disease. Neurology. *64*, 755-757.

Cha, B. H., Akman, C., Silveira, D. C., Liu, X. and Holmes, G. L. (2004). Spontaneous recurrent seizure following status epilepticus enhances dentate gyrus neurogenesis. Brain Dev. *26*, 394-397.

Chapman, H. A., Riese, R. J. and Shi, G. P. (1997). Emerging roles for cysteine proteases in human biology. Annu Rev Physiol. *59*, 63-88.

Cole, T., Dickson, P. W., Esnard, F., Averill, S., Risbridger, G. P., Gauthier, F. and Schreiber, G. (1989). The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. Eur J Biochem. *186*, 35-42.

Commission on Classification and Terminology of the International League Against Epilepsy (1989): Proposal for revised classification of epilepsies and epileptic syndromes. Epilepsia. *30*, 389-399.

Commission on Classification and Terminology of the International League Against Epilepsy (1981): Proposal for revised clinical and electroencephalographic classification of epileptic seizures. Epilepsia. *22*, 489-501.

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H. G. and Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci. *21*, 1-14.

Coulter, D. A., McIntyre, D. C. and Loscher, W. (2002). Animal models of limbic epilepsies: what can they tell us? Brain Pathol. *12*, 240-256.

Covolan, L., Ribeiro, L. T., Longo, B. M. and Mello, L. E. (2000). Cell damage and neurogenesis in the dentate granule cell layer of adult rats after pilocarpine- or kainate-induced status epilepticus. Hippocampus. *10*, 169-180.

Cox, J. L., Sexton, P. S., Green, T. J. and Darmani, N. A. (1999). Inhibition of B16 melanoma metastasis by overexpression of the cysteine proteinase inhibitor cystatin C. Melanoma Res. *9*, 369-374.

Crawford, F. C., Freeman, M. J., Schinka, J. A., Abdullah, L. I., Gold, M., Hartman, R., Krivian, K., Morris, M. D., Richards, D., Duara, R., Anand, R. and Mullan, M. J. (2000). A polymorphism in the cystatin C gene is a novel risk factor for late-onset Alzheimer's disease. Neurology. *55*, 763-768.

Crespel, A., Coubes, P., Rousset, M. C., Brana, C., Rougier, A., Rondouin, G., Bockaert, J., Baldy-Moulinier, M. and Lerner-Natoli, M. (2002). Inflammatory reactions in human medial temporal lobe epilepsy with hippocampal sclerosis. Brain Res. *952*, 159-169.

Crespel, A., Rigau, V., Coubes, P., Claude Rousset, M., de Bock, F., Okano, H., Baldy-Moulinier, M., Bockaert, J. and Lerner-Natoli, M. (2005). Increased number of neural progenitors in human temporal lobe epilepsy. Neurobiol Dis.

Dahl, A., Eriksson, P. S., Davidsson, P., Persson, A. I., Ekman, R. and Westman-Brinkmalm, A. (2004). Demonstration of multiple novel glycoforms of the stem cell survival factor CCg. J Neurosci Res. *77*, 9-14.

Dash, P. K., Mach, S. A. and Moore, A. N. (2001). Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. J Neurosci Res. *63*, 313-319.

del Zoppo, G. J., Becker, K. J. and Hallenbeck, J. M. (2001). Inflammation after stroke: is it harmful? Arch Neurol. *58*, 669-672.

Deng, A., Irizarry, M. C., Nitsch, R. M., Growdon, J. H. and Rebeck, G. W. (2001). Elevation of cystatin c in susceptible neurons in alzheimer's disease. Am J Pathol. *159*, 1061-1068.

Dickinson, D. P., Thiesse, M. and Hicks, M. J. (2002). Expression of type 2 cystatin genes CST1-CST5 in adult human tissues and the developing submandibular gland. DNA Cell Biol. *21*, 47-65.

Dietrich, W. D., Busto, R., Watson, B. D., Scheinberg, P. and Ginsberg, M. D. (1987a). Photochemically induced cerebral infarction. II. Edema and blood-brain barrier disruption. Acta Neuropathol (Berl). *72*, 326-334.

Dietrich, W. D., Ginsberg, M. D., Busto, R. and Watson, B. D. (1986). Photochemically induced cortical infarction in the rat. 1. Time course of hemodynamic consequences. J Cereb Blood Flow Metab. *6*, 184-194.

Dietrich, W. D., Watson, B. D., Busto, R., Ginsberg, M. D. and Bethea, J. R. (1987b). Photochemically induced cerebral infarction. I. Early microvascular alterations. Acta Neuropathol (Berl). *72*, 315-325.

Dodel, R. C., Du, Y., Depboylu, C., Kurz, A., Eastwood, B., Farlow, M., Oertel, W. H., Muller, U. and Riemenschneider, M. (2002). A polymorphism in the cystatin C promoter region is not associated with an increased risk of AD. Neurology. *58*, 664.

Eisch, A. J., Barrot, M., Schad, C. A., Self, D. W. and Nestler, E. J. (2000). Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci U S A. *97*, 7579-7584.

Ekdahl, C. T., Claasen, J. H., Bonde, S., Kokaia, Z. and Lindvall, O. (2003a). Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A. *100*, 13632-13637.

Ekdahl, C. T., Mohapel, P., Elmer, E. and Lindvall, O. (2001). Caspase inhibitors increase short-term survival of progenitor-cell progeny in the adult rat dentate gyrus following status epilepticus. Eur J Neurosci. *14*, 937-945.

Ekdahl, C. T., Mohapel, P., Weber, E., Bahr, B., Blomgren, K. and Lindvall, O. (2002). Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. Eur J Neurosci. *16*, 1463-1471.

Ekdahl, C. T., Zhu, C., Bonde, S., Bahr, B. A., Blomgren, K. and Lindvall, O. (2003b). Death mechanisms in status epilepticus-generated neurons and effects of additional seizures on their survival. Neurobiol Dis. *14*, 513-523.

Ekiel, I. and Abrahamson, M. (1996). Folding-related dimerization of human cystatin C. J Biol Chem. *271*, 1314-1321.

Ekiel, I., Abrahamson, M., Fulton, D. B., Lindahl, P., Storer, A. C., Levadoux, W., Lafrance, M., Labelle, S., Pomerleau, Y., Groleau, D., LeSauteur, L. and Gehring, K. (1997). NMR structural studies of human cystatin C dimers and monomers. J Mol Biol. *271*, 266-277.

El Bahh, B., Lespinet, V., Lurton, D., Coussemacq, M., Le Gal La Salle, G. and Rougier, A. (1999). Correlations between granule cell dispersion, mossy fiber sprouting, and hippocampal cell loss in temporal lobe epilepsy. Epilepsia. *40*, 1393-1401.

Elliott, R. C., Miles, M. F. and Lowenstein, D. H. (2003). Overlapping microarray profiles of dentate gyrus gene expression during development- and epilepsy-associated neurogenesis and axon outgrowth. J Neurosci. *23*, 2218-2227.

El-Sukkari, D., Wilson, N. S., Håkansson, K., Steptoe, R. J., Grubb, A., Shortman, K. and Villadangos, J. A. (2003). The protease inhibitor cystatin C is differentially expressed among dendritic cell populations, but does not control antigen presentation. J Immunol. *171*, 5003-5011.

Emerit, J., Edeas, M. and Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. Biomed Pharmacother. *58*, 39-46.

Engel, J., Jr. (1996). Introduction to temporal lobe epilepsy. Epilepsy Res. *26*, 141-150.

Engel, J., Jr. (2001). A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. Epilepsia. *42*, 796-803.

Engel, J. J., 1989. Seizures and Epilepsy. F.A. Davis Company, Philadelphia.

Esnard, F., Esnard, A., Faucher, D., Capony, J. P., Derancourt, J., Brillard, M. and Gauthier, F. (1990). Rat cystatin C: the complete amino acid sequence reveals a site for N-glycosylation. Biol Chem Hoppe Seyler. *371 Suppl*, 161-166.

EUCARE (2003). European White Paper on Epilepsy. Epilepsia. *44 Suppl 6*, 1-88.

Falconer, M. A., Serafetinides, E. A. and Corsellis, J. A. (1964). Etiology and Pathogenesis of Temporal Lobe Epilepsy. Arch Neurol. *10*, 233-248.

Ferland, R. J., Gross, R. A. and Applegate, C. D. (2002). Increased mitotic activity in the dentate gyrus of the hippocampus of adult C57BL/6J mice exposed to the flurothyl kindling model of epileptogenesis. Neuroscience. *115*, 669-683.

Ferraro, T. N., Golden, G. T., Smith, G. G. and Berrettini, W. H. (1995). Differential susceptibility to seizures induced by systemic kainic acid treatment in mature DBA/2J and C57BL/6J mice. Epilepsia. *36*, 301-307.

Finckh, U., von Der Kammer, H., Velden, J., Michel, T., Andresen, B., Deng, A., Zhang, J., Muller-Thomsen, T., Zuchowski, K., Menzer, G., Mann, U., Papassotiropoulos, A., Heun, R., Zurdel, J., Holst, F., Benussi, L., Stoppe, G., Reiss, J., Miserez, A. R., Staehelin, H. B., Rebeck, G. W., Hyman, B. T., Binetti, G., Hock, C., Growdon, J. H. and Nitsch, R. M. (2000). Genetic association of a cystatin C gene polymorphism with late-onset alzheimer disease. Arch Neurol. *57*, 1579-1583.

Fisher, R. S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P. and Engel, J., Jr. (2005). Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia. *46*, 470-472.

Frotscher, M. (1998). Cajal-Retzius cells, Reelin, and the formation of layers. Curr Opin Neurobiol. *8*, 570-575.

Frotscher, M., Haas, C. A. and Forster, E. (2003). Reelin controls granule cell migration in the dentate gyrus by acting on the radial glial scaffold. Cereb Cortex. *13*, 634-640.

Fuchs, E. and Gould, E. (2000). Mini-review: in vivo neurogenesis in the adult brain: regulation and functional implications. Eur J Neurosci. *12*, 2211-2214.

Gage, F. H., Kempermann, G., Palmer, T. D., Peterson, D. A. and Ray, J. (1998). Multipotent progenitor cells in the adult dentate gyrus. J Neurobiol. *36*, 249-266.

Gauguier, D., Kaisaki, P. J., Rouard, M., Wallis, R. H., Browne, J., Rapp, J. P. and Bihoreau, M. T. (1999). A gene map of the rat derived from linkage analysis and related regions in the mouse and human genomes. Mamm Genome. *10*, 675-686.

Ghiso, J., Jensson, O. and Frangione, B. (1986). Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C). Proc Natl Acad Sci U S A. *83*, 2974-2978.

Glass, M. and Dragunow, M. (1995). Neurochemical and morphological changes associated with human epilepsy. Brain Res Brain Res Rev. *21*, 29-41.

Gloor, P., 1991. Mesial Temporal Sclerosis: Historical Background and an Overview from a modern perspective. In: Lüders, H. (Ed.), Epilepsy Surgery. Raven Press, Ltd., New York, pp. 689-703.

Gould, E., Cameron, H. A., Daniels, D. C., Woolley, C. S. and McEwen, B. S. (1992). Adrenal hormones suppress cell division in the adult rat dentate gyrus. J Neurosci. *12*, 3642-3650.

Gould, E. and Tanapat, P. (1997). Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult rat. Neuroscience. *80*, 427-436.

Gould, E. and Tanapat, P. (1999). Stress and hippocampal neurogenesis. Biol Psychiatry. *46*, 1472-1479.

Gould, E., Tanapat, P., Hastings, N. B. and Shors, T. J. (1999). Neurogenesis in adulthood: a possible role in learning. Trends Cogn Sci. *3*, 186-192.

Graffagnino, C., Herbstreith, M. H., Schmechel, D. E., Levy, E., Roses, A. D. and Alberts, M. J. (1995). Cystatin C mutation in an elderly man with sporadic amyloid angiopathy and intracerebral hemorrhage. Stroke. *26*, 2190-2193.

Gray, W. P. and Sundstrom, L. E. (1998). Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. Brain Res. *790*, 52-59.

Gross, C. G. (2000). Neurogenesis in the adult brain: death of a dogma. Nat Rev Neurosci. *1*, 67-73.

Grubb, A., Jensson, O., Gudmundsson, G., Arnason, A., Lofberg, H. and Malm, J. (1984). Abnormal metabolism of gamma-trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. N Engl J Med. *311*, 1547-1549.

Grubb, A. and Lofberg, H. (1982). Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. Proc Natl Acad Sci U S A. *79*, 3024-3027.

Gu, J., Lynch, B. A., Anderson, D., Klitgaard, H., Lu, S., Elashoff, M., Ebert, U., Potschka, H. and Loscher, W. (2004). The antiepileptic drug levetiracetam selectively modifies kindling-induced alterations in gene expression in the temporal lobe of rats. Eur J Neurosci. *19*, 334-345.

Gu, W., Brannstrom, T. and Wester, P. (2000). Cortical neurogenesis in adult rats after reversible photothrombotic stroke. J Cereb Blood Flow Metab. *20*, 1166-1173.

Haas, C. A., Dudeck, O., Kirsch, M., Huszka, C., Kann, G., Pollak, S., Zentner, J. and Frotscher, M. (2002). Role for reelin in the development of granule cell dispersion in temporal lobe epilepsy. J Neurosci. *22*, 5797-5802.

Hagihara, H., Hara, M., Tsunekawa, K., Nakagawa, Y., Sawada, M. and Nakano, K. (2005). Tonic-clonic seizures induce division of neuronal progenitor cells with concomitant changes in expression of neurotrophic factors in the brain of pilocarpine-treated mice. Brain Res Mol Brain Res.

Håkansson, K., Huh, C., Grubb, A., Karlsson, S. and Abrahamson, M. (1996). Mouse and rat cystatin C: Escherichia coli production, characterization and tissue distribution. Comp Biochem Physiol B Biochem Mol Biol. *114*, 303-311.

Harrington, C. A., Rosenow, C. and Retief, J. (2000). Monitoring gene expression using DNA microarrays. Curr Opin Microbiol. *3*, 285-291.

Hastings, N. B. and Gould, E. (1999). Rapid extension of axons into the CA3 region by adult-generated granule cells. J Comp Neurol. *413*, 146-154.

Hattiangady, B., Rao, M. S. and Shetty, A. K. (2004). Chronic temporal lobe epilepsy is associated with severely declined dentate neurogenesis in the adult hippocampus. Neurobiol Dis. *17*, 473-490.

Hauser, W. A. and Hesdorffer, D. C., 2001. Epidemiology of intractable epilepsy. In: Lüders, H. O. and Comair, Y. G. (Eds.), Epilepsy Surgery. Lippincott, Williams and Wilkins, Philadelphia, PA, pp. 55-61.

Hendriksen, H., Datson, N. A., Ghijsen, W. E., van Vliet, E. A., da Silva, F. H., Gorter, J. A. and Vreugdenhil, E. (2001). Altered hippocampal gene expression prior to the onset of spontaneous seizures in the rat post-status epilepticus model. Eur J Neurosci. *14*, 1475-1484.

Henshall, D. C., Chen, J. and Simon, R. P. (2000). Involvement of Caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. J Neurochem. *74*, 1215-1223.

Hof, P. R., Young, W. G., Bloom, F. E., Belichenko, P. V. and Celio, M. R., 2000. Comparative cytoarchitectonic atlas of the C57BL/6 and 129/SV mouse brains. Elsevier, The Netherlands.

Hopkins, K. J., Wang, G. and Schmued, L. C. (2000). Temporal progression of kainic acid induced neuronal and myelin degeneration in the rat forebrain. Brain Res. *864*, 69-80.

Houser, C. R. (1990). Granule cell dispersion in the dentate gyrus of humans with temporal lobe epilepsy. Brain Res. *535*, 195-204.

Houser, C. R., Miyashiro, J. E., Swartz, B. E., Walsh, G. O., Rich, J. R. and Delgado-Escueta, A. V. (1990). Altered patterns of dynorphin immunoreactivity suggest mossy fiber reorganization in human hippocampal epilepsy. J Neurosci. *10*, 267-282.

Houser, C. R., Swartz, B. E., Walsh, G. O. and Delgado-Escueta, A. V. (1992). Granule cell disorganization in the dentate gyrus: possible alterations of neuronal migration in human temporal lobe epilepsy. Epilepsy Res Suppl. *9*, 41-48.

Hu, R. Q., Koh, S., Torgerson, T. and Cole, A. J. (1998). Neuronal stress and injury in C57/BL mice after systemic kainic acid administration. Brain Res. *810*, 229-240.

Huh, C., Nagle, J. W., Kozak, C. A., Abrahamson, M. and Karlsson, S. (1995). Structural organization, expression and chromosomal mapping of the mouse cystatin-C-encoding gene (Cst3). Gene. *152*, 221-226.

Huh, C. G., Hakansson, K., Nathanson, C. M., Thorgeirsson, U. P., Jonsson, N., Grubb, A., Abrahamson, M. and Karlsson, S. (1999). Decreased metastatic spread in mice homozygous for a null allele of the cystatin C protease inhibitor gene. Mol Pathol. *52*, 332-340.

Ishimaru, H., Ishikawa, K., Ohe, Y., Takahashi, A. and Maruyama, Y. (1996). Cystatin C and apolipoprotein E immunoreactivities in CA1 neurons in ischemic gerbil hippocampus. Brain Res. *709*, 155-162.

Jacobsson, B., Lignelid, H. and Bergerheim, U. S. (1995). Transthyretin and cystatin C are catabolized in proximal tubular epithelial cells and the proteins are not useful as markers for renal cell carcinomas. Histopathology. *26*, 559-564.

Janowski, R., Kozak, M., Jankowska, E., Grzonka, Z., Grubb, A., Abrahamson, M. and Jaskolski, M. (2001). Human cystatin C, an amyloidogenic protein, dimerizes through three- dimensional domain swapping. Nat Struct Biol. *8*, 316-320.

Jaskolski, M. (2001). 3D domain swapping, protein oligomerization, and amyloid formation. Acta Biochim Pol. *48*, 807-827.

Jensson, O., Gudmundsson, G., Arnason, A., Blondal, H., Petursdottir, I., Thorsteinsson, L., Grubb, A., Lofberg, H., Cohen, D. and Frangione, B. (1987). Hereditary cystatin C (gamma-trace) amyloid angiopathy of the CNS causing cerebral hemorrhage. Acta Neurol Scand. *76*, 102-114.

Jiang, W., Wan, Q., Zhang, Z. J., Wang, W. D., Huang, Y. G., Rao, Z. R. and Zhang, X. (2003). Dentate granule cell neurogenesis after seizures induced by pentylenetrazol in rats. Brain Res. *977*, 141-148.

Jonsdottir, S. and Palsdottir, A. (1993). Molecular diagnosis of hereditary cystatin C amyloid angiopathy. Biochem Med Metab Biol. *49*, 117-123.

Jutila, L., Immonen, A., Partanen, K., Partanen, J., Mervaala, E., Ylinen, A., Alafuzoff, I., Paljärvi, L., Karkola, K., Vapalahti, M. and Pitkänen, A. (2002). Neurobiology of epileptogenesis in the temporal lobe. Adv Tech Stand Neurosurg. *27*, 5-22.

Karhunen, H., Pitkänen, A., Virtanen, T., Gureviciene, I., Pussinen, R., Ylinen, A., Sivenius, J., Nissinen, J. and Jolkkonen, J. (2003). Long-term functional consequences of transient occlusion of the middle

cerebral artery in rats: a 1-year follow-up of the development of epileptogenesis and memory impairment in relation to sensorimotor deficits. Epilepsy Res. *54*, 1-10.

Katakai, K., Shinoda, M., Kabeya, K., Watanabe, M., Ohe, Y., Mori, M. and Ishikawa, K. (1997). Changes in distribution of cystatin C, apolipoprotein E and ferritin in rat hypothalamus after hypophysectomy. J Neuroendocrinol. *9*, 247-253.

Kato, T., Imatani, T., Miura, T., Minaguchi, K., Saitoh, E. and Okuda, K. (2000). Cytokine-inducing activity of family 2 cystatins. Biol Chem. *381*, 1143-1147.

Kee, N., Sivalingam, S., Boonstra, R. and Wojtowicz, J. M. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods. *115*, 97-105.

Kee, N. J., Preston, E. and Wojtowicz, J. M. (2001). Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. Exp Brain Res. *136*, 313-320.

Kelly, K. M., Kharlamov, A., Hentosz, T. M., Kharlamova, E. A., Williamson, J. M., Bertram, E. H., 3rd, Kapur, J. and Armstrong, D. M. (2001). Photothrombotic brain infarction results in seizure activity in aging Fischer 344 and Sprague Dawley rats. Epilepsy Res. *47*, 189-203.

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1997a). Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc Natl Acad Sci U S A. *94*, 10409-10414.

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1997b). More hippocampal neurons in adult mice living in an enriched environment. Nature. *386*, 493-495.

Kemppainen, S. and Pitkänen, A. (2004). Damage to the amygdalo-hippocampal projection in temporal lobe epilepsy: a tract-tracing study in chronic epileptic rats. Neuroscience. *126*, 485-501.

Kharlamov, E. A., Jukkola, P. I., Schmitt, K. L. and Kelly, K. M. (2003). Electrobehavioral characteristics of epileptic rats following photothrombotic brain infarction. Epilepsy Res. *56*, 185-203.

Kingham, P. J. and Pocock, J. M. (2001). Microglial secreted cathepsin B induces neuronal apoptosis. J Neurochem. *76*, 1475-1484.

Konduri, S. D., Yanamandra, N., Siddique, K., Joseph, A., Dinh, D. H., Olivero, W. C., Gujrati, M., Kouraklis, G., Swaroop, A., Kyritsis, A. P. and Rao, J. S. (2002). Modulation of cystatin C expression impairs the invasive and tumorigenic potential of human glioblastoma cells. Oncogene. *21*, 8705-8712.

Kotagal, P., 1991. Seizure symptomatology of temporal lobe epilepsy. In: Lüders, H. (Ed.), Epilepsy Surgery. Raven Press, Ltd., New York, pp. 143-156.

Kuhn, H. G., Dickinson-Anson, H. and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. *16*, 2027-2033.

Kumada, T., Hasegawa, A., Iwasaki, Y., Baba, H. and Ikenaka, K. (2004). Isolation of cystatin C via functional cloning of astrocyte differentiation factors. Dev Neurosci. *26*, 68-76.

Lähteinen, S., Pitkänen, A., Koponen, E., Saarelainen, T. and Castren, E. (2003). Exacerbated status epilepticus and acute cell loss, but no changes in epileptogenesis, in mice with increased brain-derived neurotrophic factor signaling. Neuroscience. *122*, 1081-1092.

Lähteinen, S., Pitkänen, A., Saarelainen, T., Nissinen, J., Koponen, E. and Castren, E. (2002). Decreased BDNF signalling in transgenic mice reduces epileptogenesis. Eur J Neurosci. *15*, 721-734.

Laursen, H., Hansen, A. J. and Sheardown, M. (1993). Cerebrovascular permeability and brain edema after cortical photochemical infarcts in the rat. Acta Neuropathol (Berl). *86*, 378-385.

Lehesjoki, A. (2003). Molecular background of progressive myoclonus epilepsy. EMBO J. *22*, 3473-3478.

Lehrmann, E., Kiefer, R., Christensen, T., Toyka, K. V., Zimmer, J., Diemer, N. H., Hartung, H. P. and Finsen, B. (1998). Microglia and macrophages are major sources of locally produced transforming growth factor-beta1 after transient middle cerebral artery occlusion in rats. Glia. *24*, 437-448.

Lehtimäki, K. A., Peltola, J., Koskikallio, E., Keränen, T. and Honkaniemi, J. (2003). Expression of cytokines and cytokine receptors in the rat brain after kainic acid-induced seizures. Brain Res Mol Brain Res. *110*, 253-260.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell. *60*, 585-595.

Leung-Tack, J., Tavera, C., Er-Raki, A., Gensac, M. C. and Colle, A. (1990a). Rat cystatin C: inhibitor of granulocyte phagocytic functions. Biol Chem Hoppe Seyler. *371 Suppl*, 255-258.

Leung-Tack, J., Tavera, C., Martinez, J. and Colle, A. (1990b). Neutrophil chemotactic activity is modulated by human cystatin C, an inhibitor of cysteine proteases. Inflammation. *14*, 247-258.

Levy, E., Lopez-Otin, C., Ghiso, J., Geltner, D. and Frangione, B. (1989). Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, an inhibitor of cysteine proteases. J Exp Med. *169*, 1771-1778.

Levy, E., Sastre, M., Kumar, A., Gallo, G., Piccardo, P., Ghetti, B. and Tagliavini, F. (2001). Codeposition of cystatin C with amyloid-beta protein in the brain of Alzheimer disease patients. J Neuropathol Exp Neurol. *60*, 94-104.

Lignelid, H., Collins, V. P. and Jacobsson, B. (1997). Cystatin C and transthyretin expression in normal and neoplastic tissues of the human brain and pituitary. Acta Neuropathol (Berl). *93*, 494-500.

Lindholt, J. S., Erlandsen, E. J. and Henneberg, E. W. (2001). Cystatin C deficiency is associated with the progression of small abdominal aortic aneurysms. Br J Surg. *88*, 1472-1475.

Liu, S., Wang, J., Zhu, D., Fu, Y., Lukowiak, K. and Lu, Y. (2003). Generation of functional inhibitory neurons in the adult rat hippocampus. J Neurosci. *23*, 732-736.

Lukasiuk, K., Kontula, L. and Pitkänen, A. (2003). cDNA profiling of epileptogenesis in the rat brain. Eur J Neurosci. *17*, 271-279.

Lukasiuk, K., Pirttilä, T. J. and Pitkänen, A. (2002). Upregulation of cystatin C expression in the rat hippocampus during epileptogenesis in the amygdala stimulation model of temporal lobe epilepsy. Epilepsia. *43 Suppl 5*, 137-145.

Lukasiuk, K. and Pitkänen, A. (2004). Large-scale analysis of gene expression in epilepsy research: Is synthesis already possible? Neurochem Res. *29*, 1169-1178.

Luo, Z. and Geschwind, D. H. (2001). Microarray applications in neuroscience. Neurobiol Dis. *8*, 183-193.

Lurton, D., El Bahh, B., Sundstrom, L. and Rougier, A. (1998). Granule cell dispersion is correlated with early epileptic events in human temporal lobe epilepsy. J Neurol Sci. *154*, 133-136.

Lurton, D., Sundstrom, L., Brana, C., Bloch, B. and Rougier, A. (1997). Possible mechanisms inducing granule cell dispersion in humans with temporal lobe epilepsy. Epilepsy Res. *26*, 351-361.

Madsen, T. M., Treschow, A., Bengzon, J., Bolwig, T. G., Lindvall, O. and Tingstrom, A. (2000). Increased neurogenesis in a model of electroconvulsive therapy. Biol Psychiatry. *47*, 1043-1049.

Margerison, J. H. and Corsellis, J. A. (1966). Epilepsy and the temporal lobes. A clinical, electroencephalographic and neuropathological study of the brain in epilepsy, with particular reference to the temporal lobes. Brain. *89*, 499-530.

Maruyama, H., Izumi, Y., Oda, M., Torii, T., Morino, H., Toji, H., Sasaki, K., Terasawa, H., Nakamura, S. and Kawakami, H. (2001). Lack of an association between cystatin C gene polymorphisms in Japanese patients with Alzheimer's disease. Neurology. *57*, 337-339.

Maruyama, K., Ikeda, S., Ishihara, T., Allsop, D. and Yanagisawa, N. (1990). Immunohistochemical characterization of cerebrovascular amyloid in 46 autopsied cases using antibodies to beta protein and cystatin C. Stroke. *21*, 397-403.

Maslov, A. Y., Barone, T. A., Plunkett, R. J. and Pruitt, S. C. (2004). Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. J Neurosci. *24*, 1726-1733.

Mason, R. W., Sol-Church, K. and Abrahamson, M. (1998). Amino acid substitutions in the N-terminal segment of cystatin C create selective protein inhibitors of lysosomal cysteine proteinases. Biochem J. *330*, 833-838.

Mathern, G. W., Babb, T. L., Leite, J. P., Pretorius, K., Yeoman, K. M. and Kuhlman, P. A. (1996). The pathogenic and progressive features of chronic human hippocampal epilepsy. Epilepsy Res. *26*, 151-161.

Mathern, G. W., Kuhlman, P. A., Mendoza, D. and Pretorius, J. K. (1997). Human fascia dentata anatomy and hippocampal neuron densities differ depending on the epileptic syndrome and age at first seizure. J Neuropathol Exp Neurol. *56*, 199-212.

Mathern, G. W., Leiphart, J. L., De Vera, A., Adelson, P. D., Seki, T., Neder, L. and Leite, J. P. (2002). Seizures decrease postnatal neurogenesis and granule cell development in the human fascia dentata. Epilepsia. *43*, 68-73.

McKhann, G. M., 2nd, Wenzel, H. J., Robbins, C. A., Sosunov, A. A. and Schwartzkroin, P. A. (2003). Mouse strain differences in kainic acid sensitivity, seizure behavior, mortality, and hippocampal pathology. Neuroscience. *122*, 551-561.

Meencke, H. J. and Veith, G., 1991. Hippocampal sclerosis in epilepsy. In: Lüders, H. (Ed.), Epilepsy surgery. Raven Press, Ltd., New York, pp. 705-715.

Mello, L. E., Cavalheiro, E. A., Tan, A. M., Pretorius, J. K., Babb, T. L. and Finch, D. M. (1992). Granule cell dispersion in relation to mossy fiber sprouting, hippocampal cell loss, silent period and seizure frequency in the pilocarpine model of epilepsy. Epilepsy Res Suppl. *9*, 51-59.

Merz, G. S., Benedikz, E., Schwenk, V., Johansen, T. E., Vogel, L. K., Rushbrook, J. I. and Wisniewski, H. M. (1997). Human cystatin C forms an inactive dimer during intracellular trafficking in transfected CHO cells. J Cell Physiol. *173*, 423-432.

Mikkonen, M., Soininen, H., Kälviäinen, R., Tapiola, T., Ylinen, A., Vapalahti, M., Paljarvi, L. and Pitkänen, A. (1998). Remodeling of neuronal circuitries in human temporal lobe epilepsy: increased expression of highly polysialylated neural cell adhesion molecule in the hippocampus and the entorhinal cortex. Ann Neurol. *44*, 923-934.

Miller, M. W. and Nowakowski, R. S. (1988). Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. *457*, 44-52.

Minturn, J. E., Geschwind, D. H., Fryer, H. J. and Hockfield, S. (1995). Early postmitotic neurons transiently express TOAD-64, a neural specific protein. J Comp Neurol. *355*, 369-379.

Miyake, T., Gahara, Y., Nakayma, M., Yamada, H., Uwabe, K.-I. and Kitamura, T. (1996). Up-regulation of cystatin C by microglia in the rat facial nucleus following axotomy. Mol Brain Res. *37*, 273-282.

Monastero, R., Camarda, C., Cefalu, A. B., Caldarella, R., Camarda, L. K., Noto, D., Averna, M. R. and Camarda, R. (2005). No association between the cystatin C gene polymorphism and Alzheimer's disease: A case-control study in an Italian population. J Alzheimers Dis. *7*, 291-295.

Monje, M. L., Toda, H. and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. Science. *302*, 1760-1765.

Morimoto, K., Fahnestock, M. and Racine, R. J. (2004). Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol. *73*, 1-60.

Mussap, M. and Plebani, M. (2004). Biochemistry and clinical role of human cystatin C. Crit Rev Clin Lab Sci. *41*, 467-550.

Nacher, J., Rosell, D. R. and McEwen, B. S. (2000). Widespread expression of rat collapsin response-mediated protein 4 in the telencephalon and other areas of the adult rat central nervous system. J Comp Neurol. *424*, 628-639.

Nadler, J. V. (1979). Kainic acid: neurophysiological and neurotoxic actions. Life Sci. *24*, 289-299.

Nadler, J. V., Perry, B. W., Gentry, C. and Cotman, C. W. (1980). Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J Comp Neurol. *192*, 333-359.

Nagai, A., Murakawa, Y., Terashima, M., Shimode, K., Umegae, N., Takeuchi and Kobayashi, S. (2000). Cystatin C and cathepsin B in CSF from patients with inflammatory neurologic diseases. Neurology. *55*, 1828-1832.

Nagai, A., Ryu, J. K., Kobayash, S. and Kim, S. U. (2002). Cystatin C induces neuronal cell death in vivo. Ann N Y Acad Sci. *977*, 315-321.

Nakagawa, E., Aimi, Y., Yasuhara, O., Tooyama, I., Shimada, M., McGeer, P. L. and Kimura, H. (2000). Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. Epilepsia. *41*, 10-18.

Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S., Hatano, O., Kawahara, N., Tamura, A., Kirino, T. and Nakafuku, M. (2002). Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell. *110*, 429-441.

Narkilahti, S. and Pitkänen, A. (2005). Caspase 6 expression in the rat hippocampus during epileptogenesis and epilepsy. Neuroscience. *131*, 887-897.

Nilsson, M., Wang, X., Rodziewicz-Motowidlo, S., Janowski, R., Lindstrom, V., Onnerfjord, P., Westermark, G., Grzonka, Z., Jaskolski, M. and Grubb, A. (2004). Prevention of domain swapping inhibits dimerization and amyloid fibril formation of cystatin C: use of engineered disulfide bridges, antibodies, and carboxymethylpapain to stabilize the monomeric form of cystatin C. J Biol Chem. *279*, 24236-24245.

Nishikawa, H., Ozaki, Y., Nakanishi, T., Blomgren, K., Tada, T., Arakawa, A. and Suzumori, K. (2004). The role of cathepsin B and cystatin C in the mechanisms of invasion by ovarian cancer. Gynecol Oncol. *92*, 881-886.

Nishio, C., Yoshida, K., Nishiyama, K., Hatanaka, H. and Yamada, M. (2000). Involvement of cystatin C in oxidative stress-induced apoptosis of cultured rat CNS neurons. Brain Res. *873*, 252-262.

Nishiyama, K., Konishi, A., Nishio, C., Araki-Yoshida, K., Hatanaka, H., Kojima, M., Ohmiya, Y., Yamada, M. and Koshimizu, H. (2005). Expression of cystatin C prevents oxidative stress-induced death in PC12 cells. Brain Res Bull. *67*, 94-99.

Nissinen, J., Halonen, T., Koivisto, E. and Pitkänen, A. (2000). A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. Epilepsy Res. *38*, 1-29.

O'Connor, W. M., Masukawa, L., Freese, A., Sperling, M. R., French, J. A. and O'Connor, M. J. (1996). Hippocampal cell distributions in temporal lobe epilepsy: a comparison between patients with and without an early risk factor. Epilepsia. *37*, 440-449.

Okano, H., Imai, T. and Okabe, M. (2002). Musashi: a translational regulator of cell fate. J Cell Sci. *115*, 1355-1359.

Olney, J. W., Rhee, V. and Ho, O. L. (1974). Kainic acid: a powerful neurotoxic analogue of glutamate. Brain Res. *77*, 507-512.

Olsson, S. L., Pihlgren, U., Ploen, L. and Bjork, I. (2000). Tissue distribution of bovine cystatin C analysed by in situ hybridisation. Eur J Histochem. *44*, 171-178.

Olsson, T., Nygren, J., Hakansson, K., Lundblad, C., Grubb, A., Smith, M. L. and Wieloch, T. (2004). Gene deletion of cystatin C aggravates brain damage following focal ischemia but mitigates the neuronal injury after global ischemia in the mouse. Neuroscience. *128*, 65-71.

Omar, A. I., Senatorov, V. V. and Hu, B. (2000). Ethidium bromide staining reveals rapid cell dispersion in the rat dentate gyrus following ouabain-induced injury. Neuroscience. *95*, 73-80.

Ono, S., Shimizu, N., Imai, T., Mihori, A. and Nagao, K. (2000). Increased cystatin C immunoreactivity in the skin in amyotrophic lateral sclerosis. Acta Neurol Scand. *102*, 47-52.

Palm, D. E., Knuckey, N. W., Primiano, M. J., Spangenberger, A. G. and Johanson, C. E. (1995). Cystatin C, a protease inhibitor, in degenerating rat hippocampal neurons following transient forebrain ischemia. Brain Res. *691*, 1-8.

Palsdottir, A., Abrahamson, M., Thorsteinsson, L., Arnason, A., Olafsson, I., Grubb, A. and Jensson, O. (1988). Mutation in cystatin C gene causes hereditary brain haemorrhage. Lancet. *2*, 603-604.

Paraoan, L., Grierson, I. and Maden, B. E. (2003). Fate of cystatin C lacking the leader sequence in RPE cells. Exp Eye Res. *76*, 753-756.

Paraoan, L., Ratnayaka, A., Spiller, D. G., Hiscott, P., White, M. R. and Grierson, I. (2004). Unexpected intracellular localization of the AMD-associated cystatin C variant. Traffic. *5*, 884-895.

Parent, J. M. (2002). The role of seizure-induced neurogenesis in epileptogenesis and brain repair. Epilepsy Res. *50*, 179-189.

Parent, J. M., Janumpalli, S., McNamara, J. O. and Lowenstein, D. H. (1998). Increased dentate granule cell neurogenesis following amygdala kindling in the adult rat. Neurosci Lett. *247*, 9-12.

Parent, J. M., Tada, E., Fike, J. R. and Lowenstein, D. H. (1999). Inhibition of dentate granule cell neurogenesis with brain irradiation does not prevent seizure-induced mossy fiber synaptic reorganization in the rat. J Neurosci. *19*, 4508-4519.

Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S. and Lowenstein, D. H. (1997). Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci. *17*, 3727-3738.

Paxinos, G. and Watson, C., 1986. The Rat brain in stereotaxic coordinates. Academic Press, San Diego.

Penkowa, M., Molinero, A., Carrasco, J. and Hidalgo, J. (2001). Interleukin-6 deficiency reduces the brain inflammatory response and increases oxidative stress and neurodegeneration after kainic acid-induced seizures. Neuroscience. *102*, 805-818.

Pierre, P. and Mellman, I. (1998). Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell. *93*, 1135-1145.

Pitkänen, A., Kharatishvili, I., Nissinen, J. and McIntosh, T. K., 2005. Post-traumatic epilepsy induced by lateral fluid-percussion injury in rats. In: Pitkänen, A. et al. (Eds.), Animal models of epilepsy.

Pitkänen, A., Nissinen, J., Nairismägi, J., Lukasiuk, K., Gröhn, O. H., Miettinen, R. and Kauppinen, R. (2002). Progression of neuronal damage after status epilepticus and during spontaneous seizures in a rat model of temporal lobe epilepsy. Prog Brain Res. *135*, 67-83.

Pitkänen, A. and Sutula, T. P. (2002). Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet Neurol. *1*, 173-181.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M. and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev. *8*, 9-22.

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. *32*, 281-294.

Rankin, S. L., Partlow, G. D., McCurdy, R. D., Giles, E. D. and Fisher, K. R. (2004). The use of proliferating cell nuclear antigen immunohistochemistry with a unique functional marker to detect postnatal neurogenesis in paraffin-embedded sections of the mature pig brain. Brain Res Brain Res Protoc. *13*, 69-75.

Rao, M. S. and Shetty, A. K. (2004). Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. Eur J Neurosci. *19*, 234-246.

Rawlings, N. D. and Barrett, A. J. (1990). Evolution of proteins of the cystatin superfamily. J Mol Evol. *30*, 60-71.

Ribak, C. E. and Dashtipour, K. (2002). Neuroplasticity in the damaged dentate gyrus of the epileptic brain. Prog Brain Res. *136*, 319-328.

Riban, V., Bouilleret, V., Pham-Le, B. T., Fritschy, J. M., Marescaux, C. and Depaulis, A. (2002). Evolution of hippocampal epileptic activity during the development of hippocampal sclerosis in a mouse model of temporal lobe epilepsy. Neuroscience. *112*, 101-111.

Roks, G., Cruts, M., Slooter, A. J., Dermaut, B., Hofman, A., Van Broeckhoven, C. and Van Duijn, C. M. (2001). The cystatin C polymorphism is not associated with early onset Alzheimer's disease. Neurology. *57*, 366-367.

Sandberg, R., Yasuda, R., Pankratz, D. G., Carter, T. A., Del Rio, J. A., Wodicka, L., Mayford, M., Lockhart, D. J. and Barlow, C. (2000). From the cover: regional and strain-specific gene expression mapping in the adult mouse brain. Proc Natl Acad Sci U S A. *97*, 11038-11043.

Sanders, A., Jeremy Craven, C., Higgins, L. D., Giannini, S., Conroy, M. J., Hounslow, A. M., Waltho, J. P. and Staniforth, R. A. (2004). Cystatin forms a tetramer through structural rearrangement of domain-swapped dimers prior to amyloidogenesis. J Mol Biol. *336*, 165-178.

Sarkisian, M. R. (2001). Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. Epilepsy Behav. *2*, 201-216.

Scharfman, H. E. (1999). The role of nonprincipal cells in dentate gyrus excitability and its relevance to animal models of epilepsy and temporal lobe epilepsy. Adv Neurol. *79*, 805-820.

Scharfman, H. E. (2004). Functional implications of seizure-induced neurogenesis. Adv Exp Med Biol. *548*, 192-212.

Scharfman, H. E., Goodman, J. H. and Sollas, A. L. (2000). Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. J Neurosci. *20*, 6144-6158.

Scharfman, H. E., Sollas, A. L. and Goodman, J. H. (2002). Spontaneous recurrent seizures after pilocarpine-induced status epilepticus activate calbindin-immunoreactive hilar cells of the rat dentate gyrus. Neuroscience. *111*, 71-81.

Schauwecker, P. E. (2002). Modulation of cell death by mouse genotype: differential vulnerability to excitatory amino acid-induced lesions. Exp Neurol. *178*, 219-235.

Schauwecker, P. E. and Steward, O. (1997). Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc Natl Acad Sci U S A. *94*, 4103-4108.

Schinder, A. F. and Gage, F. H. (2004). A hypothesis about the role of adult neurogenesis in hippocampal function. Physiology. *19*, 253-261.

Schmidt-Hieber, C., Jonas, P. and Bischofberger, J. (2004). Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature. *429*, 184-187.

Schmued, L. C. and Hopkins, K. J. (2000). Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. *874*, 123-130.

Schnell, S. A., Staines, W. A. and Wessendorf, M. W. (1999). Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. J Histochem Cytochem. *47*, 719-730.

Schnittger, S., Rao, V. V., Abrahamson, M. and Hansmann, I. (1993). Cystatin C (CST3), the candidate gene for hereditary cystatin C amyloid angiopathy (HCCAA), and other members of the cystatin gene family are clustered on chromosome 20p11.2. Genomics. *16*, 50-55.

Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J Cell Physiol. *182*, 311-322.

Schwob, J. E., Fuller, T., Price, J. L. and Olney, J. W. (1980). Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience. *5*, 991-1014.

Scott, B. W., Wang, S., Burnham, W. M., De Boni, U. and Wojtowicz, J. M. (1998). Kindling-induced neurogenesis in the dentate gyrus of the rat. Neurosci Lett. *248*, 73-76.

Scott, B. W., Wojtowicz, J. M. and Burnham, W. M. (2000). Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures. Exp Neurol. *165*, 231-236.

Seki, T. (2002). Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. J Neurosci Res. *70*, 327-334.

Seki, T. and Arai, Y. (1993). Highly polysialylated neural cell adhesion molecule (NCAM-H) is expressed by newly generated granule cells in the dentate gyrus of the adult rat. J Neurosci. *13*, 2351-2358.

Seki, T. and Arai, Y. (1995). Age-related production of new granule cells in the adult dentate gyrus. Neuroreport. *6*, 2479-2482.

Sexton, P. S. and Cox, J. L. (1997). Inhibition of motility and invasion of B16 melanoma by the overexpression of cystatin C. Melanoma Res. *7*, 97-101.

Seyfried, D. M., Veyna, R., Han, Y., Li, K., Tang, N., Betts, R. L., Weinsheimer, S., Chopp, M. and Anagli, J. (2001). A selective cysteine protease inhibitor is non-toxic and cerebroprotective in rats undergoing transient middle cerebral artery ischemia. Brain Res. *901*, 94-101.

Shapiro, L. A. and Ribak, C. E. (2005). Integration of newly born dentate granule cells into adult brains: hypotheses based on normal and epileptic rodents. Brain Res Brain Res Rev. *48*, 43-56.

Shi, G. P., Sukhova, G. K., Grubb, A., Ducharme, A., Rhode, L. H., Lee, R. T., Ridker, P. M., Libby, P. and Chapman, H. A. (1999). Cystatin C deficiency in human atherosclerosis and aortic aneurysms. J Clin Invest. *104*, 1191-1197.

Siegel, S. and Castellan, N. J. J., 1988. Nonparametric Statistics for the Behavioural Sciences. McGraw-Hill Book Company, New York, pp. 215-216.

Sloviter, R. S. (1982). A simplified Timm stain procedure compatible with formaldehyde fixation and routine paraffin embedding of rat brain. Brain Res Bull. *8*, 771-774.

Sokol, J. P. and Schiemann, W. P. (2004). Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. Mol Cancer Res. *2*, 183-195.

Solem, M., Rawson, C., Lindburg, K. and Barnes, D. (1990). Transforming growth factor beta regulates cystatin C in serum-free mouse embryo (SFME) cells. Biochem Biophys Res Commun. *172*, 945-951.

Song, H., Stevens, C. F. and Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. Nature. *417*, 39-44.

Sperk, G. (1994). Kainic acid seizures in the rat. Prog Neurobiol. *42*, 1-32.

Sperk, G., Lassmann, H., Baran, H., Kish, S. J., Seitelberger, F. and Hornykiewicz, O. (1983). Kainic acid induced seizures: neurochemical and histopathological changes. Neuroscience. *10*, 1301-1315.

Steinhoff, T., Moritz, E., Wollmer, M. A., Mohajeri, M. H., Kins, S. and Nitsch, R. M. (2001). Increased cystatin c in astrocytes of transgenic mice expressing the k670n-m671l mutation of the amyloid precursor protein and deposition in brain amyloid plaques. Neurobiol Dis. *8*, 647-654.

Sun, Q. (1989). Growth stimulation of 3T3 fibroblasts by cystatin. Exp Cell Res. *180*, 150-160.

Sutula, T., Cascino, G., Cavazos, J., Parada, I. and Ramirez, L. (1989). Mossy fiber synaptic reorganization in the epileptic human temporal lobe. Ann Neurol. *26*, 321-330.

Sutula, T., Zhang, P., Lynch, M., Sayin, U., Golarai, G. and Rod, R. (1998). Synaptic and axonal remodeling of mossy fibers in the hilus and supragranular region of the dentate gyrus in kainate-treated rats. J Comp Neurol. *390*, 578-594.

Suzuki, F., Heinrich, C., Boehrer, A., Mitsuya, K., Kurokawa, K., Matsuda, M. and Depaulis, A. (2005). Glutamate receptor antagonists and benzodiazepine inhibit the progression of granule cell dispersion in a mouse model of mesial temporal lobe epilepsy. Epilepsia. *46*, 193-202.

Tamura, T., Thotakura, P., Tanaka, T. S., Ko, M. S. and Ozato, K. (2005). Identification of target genes and a unique cis-element regulated by IRF-8 in developing macrophages. Blood. *106*, 1938-1947.

Tanaka, T., Tanaka, S., Fujita, T., Takano, K., Fukuda, H., Sako, K. and Yonemasu, Y. (1992). Experimental complex partial seizures induced by a microinjection of kainic acid into limbic structures. Prog Neurobiol. *38*, 317-334.

Tanapat, P., Hastings, N. B., Reeves, A. J. and Gould, E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci. *19*, 5792-5801.

Tang, Y., Lu, A., Aronow, B. J., Wagner, K. R. and Sharp, F. R. (2002). Genomic responses of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. Eur J Neurosci. *15*, 1937-1952.

Taupin, P. and Gage, F. H. (2002). Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res. *69*, 745-749.

Taupin, P., Ray, J., Fischer, W. H., Suhr, S. T., Hakansson, K., Grubb, A. and Gage, F. H. (2000). FGF-2-Responsive neural stem cell proliferation requires CCg, a novel Autocrine/Paracrine cofactor. Neuron. *28*, 385-397.

Tavera, C., Leung-Tack, J., Prevot, D., Gensac, M. C., Martinez, J., Fulcrand, P. and Colle, A. (1992). Cystatin C secretion by rat glomerular mesangial cells: autocrine loop for in vitro growth-promoting activity. Biochem Biophys Res Commun. *182*, 1082-1088.

Tavera, C., Prevot, D., Girolami, J. P., Leung-Tack, J. and Colle, A. (1990). Tissue and biological fluid distribution of cysteine proteinases inhibitor: rat cystatin C. Biol Chem Hoppe Seyler. *371 Suppl*, 187-192.

Terman, A. and Brunk, U. T. (2004). Lipofuscin. Int J Biochem Cell Biol. *36*, 1400-1404.

Thom, M., Martinian, L., Williams, G., Stoeber, K. and Sisodiya, S. M. (2005). Cell proliferation and granule cell dispersion in human hippocampal sclerosis. J Neuropathol Exp Neurol. *64*, 194-201.

Tinuper, P., Provini, F., Marini, C., Cerullo, A., Plazzi, G., Avoni, P. and Baruzzi, A. (1996). Partial epilepsy of long duration: changing semiology with age. Epilepsia. *37*, 162-164.

Tsuchiya, K., Kohda, Y., Yoshida, M., Zhao, L., Ueno, T., Yamashita, J., Yoshioka, T., Kominami, E. and Yamashima, T. (1999). Postictal blockade of ischemic hippocampal neuronal death in primates using selective cathepsin inhibitors. Exp Neurol. *155*, 187-194.

Tu, G. F., Aldred, A. R., Southwell, B. R. and Schreiber, G. (1992). Strong conservation of the expression of cystatin C gene in choroid plexus. Am J Physiol. *263*, R195-200.

Turk, B., Turk, D. and Turk, V. (2000). Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta. *1477*, 98-111.

Turk, V. and Bode, W. (1991). The cystatins: protein inhibitors of cysteine proteinases. FEBS. *285*, 213-219.

Turski, W. A., Cavalheiro, E. A., Schwarz, M., Czuczwar, S. J., Kleinrok, Z. and Turski, L. (1983). Limbic seizures produced by pilocarpine in rats: behavioural, electroencephalographic and neuropathological study. Behav Brain Res. *9*, 315-335.

Tuunanen, J., Lukasiuk, K., Halonen, T. and Pitkänen, A. (1999). Status epilepticus-induced neuronal damage in the rat amygdaloid complex: distribution, time-course and mechanisms. Neuroscience. *94*, 473-495.

van Praag, H., Christie, B. R., Sejnowski, T. J. and Gage, F. H. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A. *96*, 13427-13431.

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D. and Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. Nature. *415*, 1030-1034.

Verdot, L., Lalmanach, G., Vercruysse, V., Hartmann, S., Lucius, R., Hoebeke, J., Gauthier, F. and Vray, B. (1996). Cystatins up-regulate nitric oxide release from interferon-gamma- activated mouse peritoneal macrophages. J Biol Chem. *271*, 28077-28081.

Verdot, L., Lalmanach, G., Vercruysse, V., Hoebeke, J., Gauthier, F. and Vray, B. (1999). Chicken cystatin stimulates nitric oxide release from interferon-gamma- activated mouse peritoneal macrophages via cytokine synthesis. Eur J Biochem. *266*, 1111-1117.

Vinters, H. V., Nishimura, G. S., Secor, D. L. and Pardridge, W. M. (1990). Immunoreactive A4 and gamma-trace peptide colocalization in amyloidotic arteriolar lesions in brains of patients with Alzheimer's disease. Am J Pathol. *137*, 233-240.

Voutsinos-Porche, B., Koning, E., Kaplan, H., Ferrandon, A., Guenounou, M., Nehlig, A. and Motte, J. (2004). Temporal patterns of the cerebral inflammatory response in the rat lithium-pilocarpine model of temporal lobe epilepsy. Neurobiol Dis. *17*, 385-402.

Warfel, A. H., Zucker-Franklin, D., Frangione, B. and Ghiso, J. (1987). Constitutive secretion of cystatin C (gamma-trace) by monocytes and macrophages and its downregulation after stimulation. J Exp Med. *166*, 1912-1917.

Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S. and Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol. *17*, 497-504.

Wei, L., Berman, Y., Castano, E. M., Cadene, M., Beavis, R. C., Devi, L. and Levy, E. (1998). Instability of the amyloidogenic cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type. J Biol Chem. *273*, 11806-11814.

Wei, L. H., Walker, L. C. and Levy, E. (1996). Cystatin C. Icelandic-like mutation in an animal model of cerebrovascular beta-amyloidosis. Stroke. *27*, 2080-2085.

White, H. S. (2002). Animal models of epileptogenesis. Neurology. *59*, S7-S14.

Xu, L., Sheng, J., Tang, Z., Wu, X., Yu, Y., Guo, H., Shen, Y., Zhou, C., Paraoan, L. and Zhou, J. (2005). Cystatin C prevents degeneration of rat nigral dopaminergic neurons: in vitro and in vivo studies. Neurobiol Dis. *18*, 152-165.

Yang, H. Y., Wilkening, S. and Iadarola, M. J. (2001). Spinal cord genes enriched in rat dorsal horn and induced by noxious stimulation identified by subtraction cloning and differential hybridization. Neuroscience. *103*, 493-502.

Yano, M., Hirai, K., Naito, Z., Yokoyama, M., Ishiwata, T., Shiraki, Y., Inokuchi, M. and Asano, G. (2001). Expression of cathepsin B and cystatin C in human breast cancer. Surg Today. *31*, 385-389.

Yasuhara, O., Hanai, K., Ohkubo, I., Sasaki, M., McGeer, P. L. and Kimura, H. (1993). Expression of cystatin C in rat, monkey and human brains. Brain Res. *628*, 85-92.

Ying, G. X., Huang, C., Jiang, Z. H., Liu, X., Jing, N. H. and Zhou, C. F. (2002). Up-regulation of cystatin C expression in the murine hippocampus following perforant path transections. Neuroscience. *112*, 289-298.

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C., Bakowska, J. C., Breakefield, X. O. and Moskowitz, M. A. (2001). FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. Proc Natl Acad Sci U S A. *98*, 5874-5879.

Zavasnik-Bergant, T., Repnik, U., Schweiger, A., Romih, R., Jeras, M., Turk, V. and Kos, J. (2005). Differentiation- and maturation-dependent content, localization, and secretion of cystatin C in human dendritic cells. J Leukoc Biol. *78*, 122-134.

Zhu, H., Dahlstrom, A. and Hansson, H. A. (2005). Characterization of cell proliferation in the adult dentate under normal conditions and after kainate induced seizures using ribonucleotide reductase and BrdU. Brain Res. *1036*, 7-17.

Zhu, H., Wang, Z. Y. and Hansson, H. A. (2003). Visualization of proliferating cells in the adult mammalian brain with the aid of ribonucleotide reductase. Brain Res. *977*, 180-189.

Zucker-Franklin, D., Warfel, A., Grusky, G., Frangione, B. and Teitel, D. (1987). Novel monocyte-like properties of microglial/astroglial cells. Constitutive secretion of lysozyme and cystatin-C. Lab Invest. *57*, 176-185.

Zurdel, J., Finckh, U., Menzer, G., Nitsch, R. M. and Richard, G. (2002). CST3 genotype associated with exudative age related macular degeneration. Br J Ophthalmol. *86*, 214-219.

APPENDIX:

ORIGINAL PUBLICATIONS

I – IV