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Magnetic Resonance Imaging Study of Induced Epileptogenesis in Animal Models of Epilepsy

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium Sky 1, Microtower building, University of Kuopio, on Friday 19th May 2006, at 12 noon

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KUOPIO 2006

ABSTRACT

Epilepsy, manifested by spontaneous recurrent epileptic seizures, is a very common neurological disorder affecting some 1 % of the population. The aim of the present study was to characterize spatiotemporal changes in several epilepsy-sensitive brain structures in animal models both during epileptogenesis and later on in fully expressed epilepsy. The primary hypothesis claimed whether amygdalohippocampal damage precedes fully expressed epilepsy. Status epilepticus and the subsequent epilepsy were induced either by kainic acid (KA) or pilocarpine injections or by electric stimulation of the amygdala in rats. Both conventional (T_2, diffusion (D_{av}), and anatomical T_1 imaging) and novel ($T_{1\rho}$, manganese enhanced MRI (MEMRI)) magnetic resonance imaging (MRI) techniques were used acutely and in long-term follow-up of these epilepsy models. The MRI data were compared with video-EEG monitoring of seizure frequency and severity as well as post mortem histological assessments of cell death (Nissl, Fluoro-Jade B) and mossy fiber sprouting (Timm staining).

The present study demonstrated that neurodegeneration and plasticity changes took place side by side in both the hippocampus and the amygdala in animal models of epilepsy. Mossy fiber sprouting, determined by histology, was evident during the appearance of seizure activity. Sprouting was monitored non-invasively by MEMRI in vivo. This MR imaging method probes both increases in neuronal activity as well as anatomical changes such as the reorganization of neuronal pathways. Increases in the D_{av} values within the hippocampus were delayed until spontaneous seizures appeared some 1.5 months after the induction of status epilepticus, while amygdala and adjacent brain cortex showed changes in T_2, D_{av} and $T_{1\rho}$ measurements, probably due to edema and plausible tissue damage a few days later. MRI parameters, determined in the early phase of epileptogenesis, did not have a clear association with seizure severity and frequency in the amygdala stimulation model. Present results also increase our understanding of juvenile epilepsy, as P12 animals went through neurodegeneration and the subsequent development of brain atrophy and epileptogenesis by diverse factors after lithium pilocarpine administration. The involvement of the thalamus in the epileptogenesis process was unveiled by abnormal T_2, D_{av} and $T_{1\rho}$ MRI signals occurring after amygdala stimulation, as well as by MEMRI in a KA model.

The MRI methods used here were able to show progressive changes in brain tissue undergoing epileptogenesis and during epilepsy. The exact underpinning cellular and physio-chemical mechanisms for these alterations at tissue level remain to be studied. As mossy fiber sprouting took place before occurrence of spontaneous seizures in the KA model of epilepsy, MEMRI can be proposed as a possible tool for predicting epileptic brain damage during epileptogenesis in experimental animal models. Future studies exploiting advanced MRI techniques, such as $T_{1\rho}$ and MEMRI, and combined with epilepsy models compatible with NMR are likely to provide insights into the fundamental pathological processes of epileptogenesis. The data provided by non-invasive imaging surrogate markers from ongoing epileptogenesis is envisaged to play a role in the assessment of the efficacy of antiepileptogenic treatment strategies.

In beloving memory of,
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ACKNOWLEDGEMENTS

ABBREVIATIONS

ADC	= apparent diffusion coefficient
AED	= antiepileptic drug
AHP	= adiabatic half passage (radio frequency pulse)
BBB	= blood-brain barrier
BOLD	= blood oxygen level dependent
CA	= Cornu Ammonis (Ammon's horn)
Ca^{2+}	= calcium-ion
CBF	= cerebral blood flow
CCSI	= constant current stimulus isolator
CT	= computer tomography
D_{av}	= 1/3 of the trace of the diffusion tensor
DTI	= diffusion tensor imaging
DWI	= diffusion weighed imaging
EEG	= electroencephalography
FID	= free induction decay
fMRI	= functional magnetic resonance imaging
FOV	= field of view
GABA	= gamma-aminobutyric acid
GE	= gradient echo
GLN	= glutamine
GLU	= glutamic acid, i.e glutamate
HAFD	= high-amplitude and frequency discharges
^{1H}MRS	= proton magnetic resonance spectroscopy
ILAE	= International League against Epilepsy
i.p.	= intraperitoneally
KA	= kainic acid
MCAo	= medial cerebral artery occlusion
MEMRI	= manganese enhanced magnetic resonance imaging
Mn^{2+}	= manganese ion
MR	= magnetic resonance
MRI	= magnetic resonance imaging
MRS	= magnetic resonance spectroscopy
MRSI	= magnetic resonance spectroscopic imaging
N_2O	= nitric dioxide
NAA	= N-acetyl aspartate

NMDA	= N-methyl-D-aspartate
NMR	= nuclear magnetic resonance
PET	= positron-emission tomography
ppm	= parts per million
ROI	= region of interest
SE	= spin echo
SNR	= signal-to-noise ratio
SPECT	= single-photon-emission computed tomography
STE	= status epilepticus
T_1	= longitudinal relaxation time
$T_{1\rho}$	= longitudinal relaxation time in the rotating frame
T_2	= transverse relaxation time
T_2^*	= transverse relaxation time, measured with gradient refocusing
TE	= time to echo
TLE	= temporal lobe epilepsy
TR	= time to repetition

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred by their corresponding Roman numerals:

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

II   **Nairismägi J.**, Gröhn O.H.J., Kettunen M.I., Nissinen J., Kauppinen R.A., Pitkänen A. "Progression of brain damage after status epilepticus and its association with epileptogenesis: a quantitative MRI study in a rat model of temporal lobe epilepsy." Epilepsia. 2004;45:1024-34.

III   **Nairismägi J.**, Pitkänen A., Kettunen M.I., Kauppinen R.A., Kubova H. "Status epilepticus in 12-day-old rats leads to temporal lobe neurodegeneration and volume reduction: a histologic and MRI study." Epilepsia. 2006;47:479-88.

IV   **Nairismägi J.**, Pitkänen A., Narkilahti S., Huttunen J., Kauppinen R.A., Gröhn O.H.J. "Manganese-enhanced magnetic resonance imaging of mossy fiber plasticity in vivo." NeuroImage. 2006;30:130-135.

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1. INTRODUCTION

Epilepsy is one of the most common neurological disorders worldwide, affecting 1-2 % of people in the population at some point in their lifetime. Human temporal lobe epilepsy (TLE) is associated with many pathological features such as gliosis, neuronal cell death, neurogenesis, mossy fibre sprouting and pathological reorganization of the neuronal networks and the pathways interconnecting them. Following the initial insult, completely developed epilepsy is often prefaced by a preclinical stage called epileptogenesis, described as the latency period adjusting the bases for occurrence of spontaneous epileptic seizures. Epilepsies are commonly initiated by head traumas, tumors and other external factors. In contrast, the exact mechanisms of genetic predisposition, as well as other initiating causes of epilepsy remain still unclear.

Status epilepticus entails continuous epileptic seizures without full recovery of consciousness between seizures. Several antiepileptic drugs (AED) are effective in stopping status epilepticus, which is as one of the chief risk factors of epilepsy. To date, no routinely used surrogate markers, that could be detected non-invasively *in vivo*, are available for the identification of ongoing epileptogenesis. The beginning of the latency period is usually clinically unrecognizable, there are no specific symptoms expressed until the epilepsy appears. In future, identification of disease progression surrogate markers for development of the brain damage during the latency period will be required to set up antiepileptogenic drug treatments during early stages of the disease.

Magnetic resonance imaging (MRI) has been widely used as a noninvasive imaging method for both the observation of different stages of epilepsy and the planning of surgery aimed at alleviating the symptoms of the disease. The present study focuses on epileptogenesis and its association with clinical long-term outcome with a special reference to epilepsy development exploiting both young and adult animal models of epilepsy.

2. REVIEW OF THE LITERATURE

2.1. Epilepsy

Epilepsy is a chronic brain disorder characterized by recurrent seizures as episodes of abnormal neuronal activity in the central nervous system (Engel, 1989). Status epilepticus (STE) is a neurological emergency, expressing prolonged seizure or repeated seizures of 30 minutes or more duration without full recovery of consciousness.

Epilepsies are a diverse collection of neurological disorders, affecting almost 1% of the human population worldwide. The clinical manifestation of epilepsy depends on the anatomical regions of the brain involved. Therefore epileptic events can be experienced, for example, as spontaneous movements, impaired sensory hallucinations or psychic actions with all of its modalities. Epileptic seizures can also be divided into partial or generalized seizures with different subgroups, based on the spreading of abnormal electrical activity in EEG.

Temporal lobe epilepsy is the most common form of human symptomatic epilepsy (Engel, 1996). It is characterized by seizures originating from the mesial temporal lobe structures. The clinical symptomatology of TLE involves epigastric rising, emotional changes as well as olfactory and gustatory hallucinations during the simple seizures. Complex temporal lobe seizures can be manifested as altered responsiveness, staring, oroalimentary and gestural automatisms (Engel, 1996). The neuropathological changes associated with TLE occur in several temporal lobe structures as the hippocampal CA1, CA3 and dentate gyrus subfields (Engel, 1989; Siesjo and Wieloch, 1986; Sloviter, 1991; Cavazos et al., 1991; Sankar et al., 1998), the entorhinal cortex (I; II; Du et al., 1995; Roch et al.; 2002a) and the amygdaloid complex (I; II; Sankar et al., 1998; Tuunanen et al., 1999).

2.2. Epileptogenesis

STE has a general incidence of 0.1% in the human population that results in approximately 180 000 and 365 000 new cases in the USA and in Europe each year, respectively (Shorvon, 1994). STE has a high risk of mortality (20-60%) as well as morbidity, including epileptogenesis (Hesdorffer et al., 1998) and permanent cognitive decline (Shorvon, 1994). Data from humans and experimental models of STE indicate that both risks of epilepsy as well as the severity of cognitive impairment are associated with brain damage caused by prolonged seizure activity (Shorvon, 1994).

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Several studies using different experimental models indicate that STE lasting for 30-40 min in rats (Lukasiuk and Pitkanen, 1998; Lukasiuk and Pitkanen, 2000) or 80 min in non-human primates (Meldrum and Horton, 1973) can initiate neurodegeneration. It is not known for how long the neurodegenerative process advances after STE in different brain regions, and what are accurate temporal profiles of the different features of epileptogenesis until the complete disease has developed.

Although our knowledge of epileptogenic mechanisms is still incomplete, previous research has given us some valuable insights into the pathophysiology of the disease. For instance, recent histological data from animal models suggest that STE-induced damage continues for several weeks (I). Epileptogenesis involves many different brain structures including different hippocampal areas (Smith and Dudek, 2001; Meldrum and Horton, 1973; Nadler et al., 1978; Nadler, 1981; Masukawa et al., 1995; Covolan et al., 2000; Roch et al., 2002a), the amygdala (Pitkanen et al., 1997; Nissinen et al., 2000; Parent et al., 1998; Lukasiuk and Pitkanen, 1998; Tuunanen et al., 1999; Fujikawa, 1996), the thalamus (Roch et al., 2002a; Dreifuss et al., 2001; Fabene et al., 2003) and several cortical areas such as the neocortex and the cingulate cortex with the most affected layers being II, III, V and VI. The participation of cortex has been demonstrated indirectly by a notable decrease in cortical blood volume after STE (Fabene et al., 2003). The widespread damage in cortical and subcortical regions develops within a few days after either chemically or electrically induced STE (Tuunanen et al., 1999; Fujikawa, 1996).

Possible involvement in epileptogenesis has been more intensively studied in the following brain areas, the amygdala, the hippocampus and the thalamus. The thalamus has been found to undergo very early blood-brain barrier disintegration, which is one of the most sensitive areas to damage after STE (Roch et al., 2002a). In addition, reduced thalamic and striatal volumes have been reported in patients with chronic epilepsy, which further indicates the involvement of these structures in epilepsy (Dreifuss et al., 2001). Several nuclei of amygdala (Tuunanen et al., 1999; Lukasiuk and Pitkanen, 1998; Nissinen et al., 2000) as well as the hippocampus often undergo serious damage during epileptogenesis (Smith and Dudek, 2001; Meldrum and Horton, 1973; Nadler et al., 1978; Nadler, 1981; Masukawa et al., 1995; Covolan et al., 2000; Roch et al., 2002a). Subregions of the hippocampus such as the hilus and the CA1 can lose a majority of their neurons under a very short time after STE. This cell loss is followed by mossy fiber sprouting and the development of new recurrent excitatory circuits inside the hippocampus (Smith and Dudek, 2001). A growing number of follow up

studies in humans using MRI indicate that hippocampal atrophy progresses over weeks, months, or even years after the first STE (Pitkanen et al., 1999b).

Markers for epileptogenesis after STE are critically important in determining the window for optimal neuroprotective treatment. For example, leviracetam, phenobarbital, valproic acid and pregabalin administered at strategic times have been shown to have a modifying effect on epileptogenesis in KA (kainic acid) and pilocarpine models of epilepsy (Yan et al., 2005; Bolanos et al., 1998; Mikati et al., 1994; Andre et al., 2003). Pharmacological inhibition of NMDA receptors has been proposed as a potential therapeutic antiepileptogenic strategy. However, the usefulness of this approach has not yet been established because of the adverse effects of NMDA receptor antagonists at therapeutic doses (Kornhuber and Weller, 1997; Chapman, 1998; Dannhardt and Kohl, 1998; Sanchez et al., 2000). Furthermore, growing interest in antiepileptogenic treatment is evident both in pharmacotherapy (Silver et al., 1991; Temkin et al., 2001; Loscher et al., 1998; Yan et al., 2005) as well as in alternative ways of epilepsy management, for example by studying the effects of a ketogenic diet (Sankar, 2004).

2.2.1. Mossy fiber sprouting and cell damage

Mossy fiber sprouting has been known as one of the principal features of mossy fiber pathway restructuring that could play a critical role in the development of recurrent spontaneous seizures after status epilepticus and the appearance of epileptogenesis (Nissinen et al., 2001; Pitkanen et al., 2000; Pitkanen et al., 1999a; Wenzel et al., 2000a). One possible cause of this abnormal axonal growth has been explained by a correlation between cell loss in the hippocampal hilar region after seizures (Masukawa et al., 1995), as hilar cells are known as one of the main anatomical output targets for granule cells. Sprouting has also been found to be present throughout the whole extent of the septotemporal axis of the hippocampus in epileptic rats (Jolkkonen et al., 1997).

However, the presence of sprouting is not necessarily associated with the occurrence of spontaneous seizures. In addition, the brains of rats electrically stimulated in the amygdala region, without observed seizures, have been shown to express sprouting (Nissinen et al., 2001). Nor is mossy fiber sprouting directly dependent on granule cell loss (Covolan et al., 2000) or seizure-induced neurogenesis (Radley and Jacobs, 2003). Therefore, it is not clear what functions sprouted fibers actually have. It has been shown in electron microscopy studies that sprouted fibers make synapses and appear to target mainly granule cells (Buckmaster et al., 2002) and GABAergic neurons (Okazaki et al., 1995; Kotti et al., 1997; Zhang and Houser, 1999; Wenzel et al., 2000a; Scharfman, 2002). Interestingly, surviving

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mossy cells become hyperexcitable (Scharfman et al., 2001), which could have important implications for network activity in later stages of epileptogenesis. Another common theory of mossy fiber pathway pathology development refers to the fact, that seizures *per se* can produce seizures (Berg and Shinnar, 1997), while mossy fiber activity can trigger mossy cell loss causing mossy fiber sprouting (Masukawa et al., 1999). This process is influenced by abnormal excitability of both interneurons and principal cells (Cobb et al., 1995; Aradi and Soltesz, 2002; Buhl et al., 1996; Brooks-Kayal et al., 1998; Nusser et al., 1998; Chen et al., 1999; Coulter, 2001; Cossart et al., 2000; Cossart et al., 2001; Wittner et al., 2001; Santhakumar et al., 2001; Chen et al., 2001). Additionally, since mossy fiber sprouting can be blocked by cycloheximide, reflecting the need for *de novo* protein synthesis to happen, this compound does not prevent epileptogenesis (Longo and Mello, 1997). Furthermore, recent data challenge the role and whole involvement of granule cell and mossy fibers in seizure genesis because of their low electrical activity during seizures (Harvey and Sloviter, 2005).

Sprouting has been demonstrated also in CA1 hippocampal area (Perez et al., 1996) and in some extra-hippocampal structures such as neocortex (Salin et al., 1995; Scharfman, 2002), but the histological detection and grading of sprouting are more complicated in these regions.

2.2.2. Other pathologies

GLIOSIS

Gliosis is related to many different pathological processes in brain. The role of gliosis in epileptogenesis and epilepsy is unclear, but it has been shown that microglia play both neuroprotective and damage promoting roles in the central nervous system (Siao et al., 2003). It has also been observed that many neuromodulators are synthesized in glial cells, including growth factors, cytokines, kynurenines and that seizure onset can increase their synthesis (Du et al., 1993; Vezzani et al., 1999). These findings indicate that glial cells may have a substantial influence on both neuronal function and fate after seizures (Scharfman, 2002).

NEUROGENESIS

In 1962 Altman and co-workers showed that new neurons can also be born in the adult brain (Altman, 1962). By using a high dose of BrdU along with a second S-phase marker, [^{3}H] thymidine, it has been proven now that young adult rats have approximately 9000 dividing cells proliferating with a cell cycle time of 25 hours, which would generate 9000 new cells each day, or more than 250000 per month (Cameron and McKay, 2001). These cells are not

randomly distributed through the brain and reside in certain special areas. One of the locations, where adult neurogenesis is prominent, is the granule cell layer of the dentate gyrus in hippocampus (Gage et al., 1998). Subsets of newborn dentate granule cells survive and mature into dentate granule neurons that modulate the physiology and hence the psychic functions of hippocampus (Porter et al., 2004).

The dentate gyrus is one of the few areas of brain that undergoes neurogenesis throughout life. New neurons appear to be born in the subgranular zone, which locates directly below the granular cell layer. The stem cells of this layer may also be affected in epilepsy since granule cell neurogenesis can be promoted by different types of induced seizures (Bengzon et al., 1997; Parent et al., 1998; Gray and Sundstrom, 1998; Scott et al., 1998; Covolan et al., 2000; Madsen et al., 2000; Nakagawa et al., 2000; Sankar et al., 2000). Interestingly, electroconvulsive seizures used as a clinical treatment for depression induce the proliferation of brain endothelial cells (Hellsten et al., 2004). This would lead to an increase in the availability of growth factors and result ultimately in neuroprotection instead of the neurogenesis (Kondratyev et al., 2001).

Neurogenesis following the STE is persistent over a long time. For example, it has been shown that after the injection of convulsants, such as pilocarpine and KA, the rate of granule cell neurogenesis is increased from days to weeks afterwards (Parent et al., 1998; Gray and Sundstrom, 1998; Nakagawa et al., 2000; Sankar et al., 2000). Although short-term proliferation of neural progenitors is independent of the severity of the STE, the long-term outcome of neurogenesis is also influenced by the degree of insult-induced degeneration in the dentate gyrus (Mohapel et al., 2004). Several active metabolites (Cameron et al., 1998) modify rearrangement of hippocampal physiology, because newborn granule cells, developing into mature neurons, integrate both anatomically and functionally into the circuitry within their vicinity (Scharfman et al., 2000; Scharfman et al., 2002). Interestingly, although most of the new cells develop normally (Scharfman et al., 2000; Markakis and Gage, 1999; Scharfman, 2002), many of them appear to migrate incorrectly, and they can be found deep in the hilar region. This notion of aberrantly traveled neurons may also turn out to be untrue, since according to another working hypothesis these neurons might actually be born in hilus, rather than migrate there (Parent et al., 1998; Scharfman et al., 2000; Scharfman et al., 2002).

Morphologically, these new granule cells develop normally, but there are also some remarkable exceptions. Although the size and shape of the soma and the axon of newly born granule cells are difficult to distinguish from a normal granule cell, there are often extensive

dendrites on both sides of the soma and these neurons develop both basal and apical dendrites (Scharfman et al., 2000; Scharfman, 2002). In spite of that, they are well integrated into a circuitry, which can both precipitate and participate in seizure activity (Scharfman et al., 2002). However, despite all of this evidence, the blocking of neurogenesis does not prevent spontaneously recurrent seizures and therefore it is likely that the birth of new neurons or the structures, which they go on to form, are not directly connected to epilepsy (Radley and Jacobs, 2003).

CELLULAR DAMAGE AND CELL DEATH

Cell death takes place under the epileptogenic process both through apoptotic and necrotic pathways. Autophagocytosis, which is usually an intracellular lysosome-mediated catabolic mechanism of neuronal cell death during brain development and in many different types of brain injury (Zhu et al., 2006; Kanzawa et al., 2005, Jellinger and Stadelmann 2001), has neither been studied in epilepsy nor in experimental seizure models. Programmed cell death, or as it is often called, apoptosis, is an active energy requiring process where the cell commits suicide in a strictly controlled manner. Necrosis is a form of uncontrolled cell death, usually initiated by the cell or tissue exposed to acute insult such as toxins, hyper-excitation, ischemia or physical injury.

Cell death leading to atrophy in different brain structures is one of the most prominent changes found in epileptic brain. Some of the brain structures are damaged immediately after the STE, others can be affected at later stage of epileptogenesis. In a rat model of TLE, the breakdown of the blood-brain barrier is observed 2 hours after STE in the thalamus (Roch et al., 2002a). Atrophy of the whole thalamus *in vivo* is difficult to explore due to its complex structure including a number of different nuclei. According to MRI studies there is a long-lasting reorganization starting after STE (I; II). Both the piriform and entorhinal cortices are affected first, with the hippocampus undergoing a delayed damage process (Roch et al., 2002a), which continues for months after the STE (I; II; Wieshmann et al., 1997b). Cell death in those brain structures plays a critical role for the installation of epileptic networks and development of epilepsy (Roch et al., 2002b). Histologic studies report that approximately 60% of the hilar somastostatin-immunoreactive neurons die in the septal end and 50% in the temporal end of the hippocampus after KA induced STE (Jolkkonen et al., 1997). In foci of maximal neuronal activity, the extracellular space undergoes a reduction by more than 30% (Lux et al., 1986), but cortical thickness can increase in epileptic rats (Fabene et al., 2003), probably because of edema as revealed by MRI. Separation of the necrosis and apoptosis *in vivo* is also problematic, caused by unknown variables that affect the damaging processes. It

has been proposed that due to the multiple mechanisms of cell death the anticonvulsant drugs can only slow down development rate of epilepsy without preventing it. This may enhance apoptosis later on (Kondratyev and Gale, 2004). Also NMDA antagonists should be avoided because of their propensity to increase severity of apoptotic damage (Pohl et al., 1999), but NMDA receptor blockade has been shown to be effective in preventing the stress-induced dendritic remodeling (McEwen and Sapolsky, 1995; McEwen, 1999). Sensitivity of the cells to the apoptosis and necrosis after STE can be partially explained by their genetic background or hormonal influence. For example, hippocampal neurons express receptors for circulating adrenal steroids (McEwen et al., 1968), which mediate a variety of effects on neuronal excitability, chemistry and structural plasticity (De Kloet et al., 1998). Another example of hormone dependence is possible involvement of the sex steroid hormones as can be inferred from various sexual bias patterns observed in TLE: men with TLE have more severe brain atrophy than women with the same condition. In addition, seizure frequency contributes to reduced brain volumes in men but not in women (Briellmann et al., 2000).

2.2.3. Epileptogenic alterations in the developing brain

Both STE and isolated seizures (Lanska et al., 1995) occur with the highest incidence in infancy and childhood rather than later in life (Hauser, 1990): over one fifth of the cases occur during the first year of life, and 64% within the first 5 years (Maytal et al., 1989). However, it is still under debate whether STE causes developmental deterioration, injury, or epileptogenesis in a maturing brain.

Previously published clinical studies investigating the morbidity associated with STE have revealed the complex nature of the disease. For example, neurological defects, such as motor disability or epilepsy, and mental abnormalities, for instance IQ scores under 80, are observed in between 20-33% of previously normal infants and children following STE (Aicardi and Chevrie, 1983; Fujiwara et al., 1979). These studies were performed before the widespread availability of modern intensive care and aggressive treatment of STE. Recent studies have, in fact, suggested lower mortality and morbidity from STE in children and infants, which can be exemplified by motor or cognitive disability occurring with an incidence of 9.1% among survivors (Maytal et al., 1989). Incidence of these deficits was particularly low in children with idiopathic or febrile STE. Children with STE with no further acute neurological insult or progressive neurological disorder, had also favorable outcomes (Yager et al., 1988), and only 5.8% of children with idiopathic STE had neuro-developmental sequealae. These data support a hypothesis that structural and functional outcomes from STE may depend on the type of STE and its association with treatment, other illnesses or predisposition for epilepsy.

Neuroimaging studies have suggested that either febrile or non-febrile STE can cause hippocampal injury in infants and children. It has been observed that brain T_2 relaxation values increase and edema develops 3-5 days after STE, however, the changes may reverse (Scott et al., 2002; Dube et al., 2004). Atrophy of the amygdala and hippocampus has been demonstrated by MRI volumetry in all adult patients with uncontrolled TLE and preceding history of prolonged febrile seizures in childhood (Cendes et al., 1993). In addition, underlying structural brain abnormalities may have an important role in long-term outcome in patients with early life STE. Taken together, these findings support the idea that certain subpopulations of infants that have had various long-term detrimental consequences such as prolonged seizure activity or inadequate treatment and this is likely to lead to an unfavorable outcome.

Experimental models of epilepsy have been developed to examine different characteristics and interrelationship between the severity of epileptic event with both clinical and histological long-term outcomes. Interestingly, STE at P12 in the rat seemed to affect the brain with milder long-term clinical consequences. At the same time the STE occurring at P16 or P20 induces neurodegeneration and mossy fiber sprouting in hippocampus as well as cell loss in several brain structures, including the amygdala, the mediodorsal nucleus of the thalamus and the IIb and III sublayers of the piriform cortex. This is important because damage to these areas has been implicated in the cognitive impairment in adulthood observed in different experimental models of STE (Cilio et al., 2003; Sankar et al., 1997; Druga et al., 2003; Kubova et al., 2000; Kubova et al., 2004). Furthermore, at postnatal day 20 (P20) rats exposed to seizures show an increased susceptibility to the phenomenon of kindling in adulthood (Cilio et al., 2003). However, acute hyperthermic damage does not necessarily lead to chronic atrophy as assessed, either histologically or by T_2-weighted MRI (Dube et al., 2000; Dube et al., 2004). Finally, it has been observed that about 25% of rats with STE at P12 or 40% of animals with prolonged hyperthermic seizures at P10 will develop spontaneous seizures within 3-6 months (Kubova et al., 2004). Despite this evidence, explanations about the underlying mechanisms covering both the vulnerability and the repairing mechanisms of the infant brain are still controversial.

At the cellular level, STE during postnatal development can increase both the genesis and death of dentate granule cells (Porter et al., 2004). McCabe and coworkers have proposed that STE occurrence in the neonatal age reduces the neurogenesis rate of granule cells, which is opposite to the situation in the adult rat, where STE promotes neurogenesis (McCabe et al., 2001). Since the blood-brain barrier becomes functional around 2 weeks after birth in rats and

in 4 months in humans (Dobbing, 1968), the developmental stage of this organ could influence the outcome of STE due to the leakage of various hormones, neuromodulators, proteins and other cellular metabolites. These observations have lead to a hypothesis that, where neurodegeneration is associated with epileptogenesis, a subpopulation of animals with STE at P12 has a measurable atrophy of the temporal lobe in adulthood that can be detected by MRI and associate with functional impairments. However, comparative MRI data describing brain tissue responses to epileptic events from infant and adult experimental animals is not available.

2.3. MRI techniques applied to the imaging of epilepsy and epileptogenesis

Nuclear magnetic resonance (NMR) is based on the physical properties of the nucleus and it occurs, when certain nuclei in a static magnetic field are exposed to a second oscillating magnetic field. The composition of the nucleus includes positively charged protons and neutrons with no charge. The spin is a natural property of nucleus, which arises out of the specific composition of the neucleus, in terms of protons and neutrons. It is associated with an intrinsic angular momentum, which is a measure for the extent to which, and the direction in which, the proton rotates about a reference point. If the nucleus has either an even atomic weight (the sum of the protons and neutrons in the nucleus) or an even atomic number (number of protons), the spin angular momentum or as it is usually called, the spin, is 0, the nucleus is incapable of interacting with external magnetic fields and is undetectable by NMR. In contrast, nuclei with an odd number of protons or an even atomic weight with an odd atomic number have a spin and are thus detectable by NMR. The most common atoms used in NMR studies are ^{1}H, ^{13}C and ^{31}P. In living tissues, hydrogen, due to its high natural abundance (99.9%), high water content and high intrinsic sensitivity, is commonly exploited for *in vivo* NMR applications.

For the generation of an MR image, the protons of water molecules in the tissue, located in a static, uniform and strong magnetic field, have to be excited by a radiofrequency for generating the NMR detectable signal. During the return to the equilibrium energy state, the so-called relaxation event, the nuclei emit energy. MR scanners have usually a superconducting magnet for generating magnetic fields and detecting the NMR signals. The same coil used for excitation, can also be used to collect the NMR signal from the tissue. Spatial encoding of nuclei is accomplished by the measurement of disturbances to the magnetic field gradients using the gradient coils. A typical MRI pulse sequence is a combination of radio frequency and gradient pulses that are used to affect the tissue magnetization for the generation of the signal and coding its location to produce a MR image

or spectrum. Afterwards, the collected signal from the tissue has to be processed to generate an actual image. Since, direct contact between transmission coil and target is not necessary and radiofrequency waves have low energy and occur at the non-ionizing form of radiation, NMR technology is truly noninvasive (Kuzniecky and Jackson, 2005; Gadian, 1995; Jin, 1999).

2.3.1. T_1 and T_2 weighted magnetic resonance imaging

T_1 is the time characterizing return of magnetization to 63% of its original value after the excitation pulse of magnetic energy. The T_1 relaxation can also be called a spin-lattice or longitudinal relaxation time because the process involves exchange of energy between the spins and their molecular framework (the lattice). The primary use of T_1-weighted image sequences in clinical brain imaging is to produce contrast aimed at discriminating anatomical details. For instance, high resolution T_1-weighted images give a good contrast between gray and white matter of the brain. T_1-weighted images are also acquired in conjunction with a tissue compartment specific exogenous contrast agents that locally shorten T_1 values. One type of compounds used to achieve this are the water soluble Gd^{3+}-based contrast agents. Contrast agents of this type are used, for instance, in the assessment of blood-brain barrier integrity, tissue perfusion and the visualization of tumors.

T_2 relaxation is a process describing the loss of the transverse magnetization. It is also called a spin-spin relaxation because it involves energy transfer between protons nearby to each other. T_2 is the time when the transverse magnetization has decayed to 37% of its maximum value. To measure the T_2 relaxation, a 180° refocusing pulse has to be applied to compensate for signal loss due to static magnetic field inhomogeneities. The simplest imaging sequence for T_2 contrast is a spin echo imaging sequence (SE) where an initial pulse, applied at 90° is followed by another at 180°, that is applied half way through the time when the echo is expected. The other main imaging sequence is called the gradient echo imaging sequence (GE), when the excitation pulse tilts the magnetization by a flip angle and the readout period occurs during free induction decay (FID). To accomplish this, the echo is generated by dephasing the spins of the nuclei with a gradient before they are rephased by another gradient with an equal area but opposite polarity. T_2-weighted imaging is valuable both for anatomical imaging and to detect pathological changes. An important application of anatomical T_2-weighted MRI in epilepsy is hippocampal volumetry for detecting loss of volume in this organ as a marker for sclerosis. A T_2* signal is obtained with gradient refocusing, without refocusing radio frequency pulse and it includes also the static dephasing effects. It is therefore particularly sensitive to changes in blood oxygenation levels and it is often used for

blood oxygenation level dependent (BOLD) imaging (Ogawa et al., 1993; Ogawa et al., 1990; van Bruggen and Roberts, 2003; Kuzniecky and Jackson, 2005; Gadian, 1995; Jin, 1999).

The imaging of acute and chronic epileptic processes provide non-invasive tools for determining changes in the functional integrity, water homeostasis, and the progressing structural damage over a period of time in experimental animals (Hossmann and Hoehn-Berlage, 1995). The usefulness of relaxation time-based contrast of tissue in epileptic animals is dependent mostly on the pathophysiology occurring within the tissue such as cytotoxic edema and micro-environmental changes. These features of the disease processes leading to the cell death share common factors with those occurring in the brain after ischemia, a disease where NMR imaging has enjoyed greater use and has led to valuable insights into that disease's nature and progression.

2.3.2. Diffusion imaging

Molecular diffusion is a result of thermal, Brownian type motion leading to small displacement of molecules. In living tissue, these movements are partially limited by cellular structures, making water molecule movements dependent on structures such as membranes and organelles. This effect of cell microenvironment-dependent proton movements is measurable by diffusion NMR. The diffusion weighted (DW) contrast is commonly generated by a SE sequence, incorporating a dephasing and rephasing gradient pulse symmetrically around the $180°$ rf-pulse. The first field gradient pulse is applied between a $90°$ and $180°$ and the second one after the $180°$ rf-pulse before the echo (Gadian, 1995). The first gradient pulse causes protons in different locations to precess at different frequencies leading to coherence dephasing. The second identical gradient rephases the spins, but only if the molecule undergoes no displacement during the time between the gradient pulses. However, when a molecule moves there is partial loss in phase coherence and this leads to a loss in the NMR detectable signal. By changing the amplitude of the diffusion gradients, the apparent diffusivity of water can be quantified (van Bruggen and Roberts, 2003; Kuzniecky and Jackson, 2005; Gadian, 1995; Jin, 1999) and this can be used to build up a picture of changes going on in an imaged tissue.

Diffusion NMR probes translational mobility and behavior of different water pools in tissue. Interestingly, it has been found that water ADC (apparent diffusion coefficient) is indirectly dependent on energy metabolism. Several mechanisms are involved in the decline of ADC in ischemia. The shift in water molecules into the intracellular space alone cannot satisfactory explain the reduced cerebral diffusion upon energy failure. During the epileptic activity, the

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extracellular space shrinks due to a water influx into cells at the area of maximum neuronal activity (Lux et al., 1986), which could explain the decrease in diffusion (Warach et al., 1995; Wieshmann et al., 1997a). Much of our understanding of changes covering tissue damage such as changes in diffusion constant and relaxation times comes from experimental models of stroke (Baird and Warach, 1999). These studies show a decrease in the ADC of water in the acute phase of cerebral ischemia, starting within minutes of vessel occlusion. This decline in ADC is associated with depolarization of the cells in the tissue due to energy failure which is followed by changes in the intra- and extracellular water homeostasis, as well as a decreased diffusivity in the cell interior and an increased extracellular tortuosity (Nicolay et al., 2001; Fabene et al., 2003). The decrease in diffusion is followed by a period of pseudo-normalization which in turn is superceded by increased diffusion in the chronic phase, which reflects increased water content in the tissue, disruption and lysis of cellular structures with developing necrosis and gliosis later on. Acute diffusion changes, however, do not unambiguously reflect the actual outcome of tissue from ischemic event (Wieshmann et al., 1997a).Imaging of seizure activity in epilepsy and STE indicate that similar events in cellular and tissue environments occur in this disease as well. Decreased ictal diffusivity leads often to increased diffusion in affected brain regions in the chronic phase (Righini et al., 1994). However, the decrease in diffusion after STE is short lasting and the temporal profile of ADC in the tissue differs from that observed in ischemia. Finally, it should be noted that in epilepsy models, low ADC is not inevitably followed by cell death (Wieshmann et al., 1997a).

The partial orientation dependent limitation of water molecule movements in a living tissue leads to an anisotropy of diffusion, depending on the planar directions of different membranes. This phenomenon can be captured in diffusion tensor imaging (DTI), which is one of the most promising novel NMR approaches for neurosciences, providing images from bundles of parallel axons and fibers for high-resolution anatomical studies on brain tissue both *ex vivo* and *in vivo*. DTI can also identify some white and gray matter substructures, as well as their shape and volumes (Zhang et al., 2002; Beaulieu, 2002; Li et al., 2003) along with the direction of fiber tracts, which could be crucial for understanding both the network damages as well as the subsequent pathophysiological reorganizations that occur in epilepsy.

2.3.3 Functional MRI

Functional MRI (fMRI) has a growing importance in both experimental and clinical MR-imaging. The most commonly used fMRI method is the blood oxygenation level dependent (BOLD) fMRI. It is based on the detection of the disproportionately high increase in blood flow to local vasculature that accompanies neural activity in the brain. The increase in blood

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flow results in a corresponding local reduction in deoxyhemoglobin level because brain oxygen consumption is not proportional to the increase in blood flow. This results in a decline in the oxygen extraction ratio. Since deoxyhemoglobin is paramagnetic in intact erythrocytes, locally decreased amount of deoxyhemoglobin increases the T_2^* weighted signal and therefore can be detected by NMR imaging techniques.

fMRI can be utilized to image brain activity related to the specific task of sensory stimulation. In the context of epilepsy, BOLD fMRI reflects changes in the oxygenation level of the blood, which is influenced by neural activity and can be used to retrieve information on brain areas involved in seizures because of the indirectly increased local CBF occurring at these sites (Ogawa et al., 1990; Opdam et al., 2002).

2.3.4. Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) is a modality of *in vivo* NMR used to reveal tissue metabolites. It is non-invasive and used increasingly in clinical practice. This MR technique exploits the chemical shift of proton (^{1}H) or phosphorus (^{31}P) containing metabolites in strong magnetic fields. It works on the principle that nuclei with spins are affected by nearby nuclei. Since these affects are limit over distance, these interactions can provide information about the local covalent nature of a molecule, which can be used to unequivocally identify the molecule, providing a standard is known. These interactions also generate shifts in the profile of a nucleus. A chemical shift is defined as a difference between the resonance frequency of a nucleus and a standard is ppm (parts per million), and it provides information about the chemical composition of the tissues. MRS has already been used in the context of both STE and epileptogenesis research.

MRS can identify several metabolite concentration changes in neuro-pathologies. Commonly detected cerebral metabolites are N-acetyle-aspartate (NAA), lactate, glutamic acid (GLU) and glutamine (GLN), gamma-aminobutyric acid (GABA), choline containing compounds and creatine. NAA is often called as neuronal density marker, because it is found predominantly in neurons. Furthermore, a decrease in cerebral NAA is often caused by irreversible neuronal damage, but in some cases this may reflect reversible neurometabolic effect by the insult (Demougeot et al., 2003). For example, due to acute ischemia, decline of NAA identifies the ischemic core (Igarashi et al., 2001). Lactate is the end product of anaerobic glycolysis and may accumulate in tissue after epileptic seizures. GLU is the primary neurotransmitter in the mammalian brain. Increases in GLU and GLN can provide insights into the imbalance of GLU/GLN metabolism, which is a common feature of epileptic

tissue (Simister et al., 2003). GABA is the primary inhibitory neurotransmitter in the brain and decreased concentrations have been reported in many of epilepsies (Kuzniecky and Jackson, 2005). GABA levels behave as a sensitive clinical marker of tissue state, as they may be affected by AED treatment and thus reflect the effectiveness of seizure control (Simister et al., 2003). However, reliable GABA quantification requires dedicated spectral editing techniques (Bielicki et al., 2004). Choline has largely been used as a marker for elevated membrane turnover because it is a component of these structures. Creatine appears to be an index of gliosis, and together with choline their signal can be used to identify regions of metabolic abnormality (van Bruggen and Roberts, 2003; Kuzniecky and Jackson, 2005; Gadian, 1995).

Magnetic resonance spectroscopic imaging (MRSI) is a hybrid technique that takes advantage of both MRS and MRI. MRSI is used to provide information on the spatial distribution of metabolites from multivoxel MRS spectra. It has been proposed that metabolic dysfunction measured by MRSI and hippocampal volume loss detected by MRI volumetry in human epilepsy patients do not have the same neuropathologic basis (Kuzniecky et al., 2001). These findings suggest that MRSI metabolic measurements reflect neuronal and glial dysfunction rather than neuronal cell loss as previously assumed and this can give new insights into disease development and its clinical consequences (van Bruggen and Roberts, 2003; Kuzniecky and Jackson, 2005; Gadian, 1995; Jin, 1999).

2.3.5. Manganese enhanced magnetic resonance imaging

Classical neuroanatomical tracing studies require tissue fixation procedures (and hence the sacrifice of the animal) and are therefore not suitable for application to *in vivo* longitudinal studies. However, in particular, anterograde and retrograde tracing techniques have revealed important insights into neuronal interconnectivity in the nervous systems of many animals (Allegrini and Wiessner, 2003). Manganese enhanced MRI (MEMRI) has gained, increasing interest in respect to both anatomical and functional modeling of neuronal networks. Ca^{2+} is an imperative second messenger for signal transduction in the CNS. Therefore, an accurate, exquisitely balanced intracellular Ca^{2+} homeostasis is required for cellular function. Ca^{2+} can enter cells through voltage-gated Ca^{2+}-channels under neuronal depolarization or through ligand-gated Ca^{2+}-channels regulated by neurotransmitter bindings to specific receptors (Takeda et al., 1998). Activated neurons have active Ca^{2+} turnover. Free bivalent Mn^{2+} ions have a similar ionic radius compared to Ca^{2+}, and therefore can bind to similar sites and is handled similarly to Ca^{2+} in numerous biological systems (Hunter et al., 1980). It is also taken up by neurons (Takeda et al., 1998) and transported both anterogradely and retrogradely

along the axons as well as across synapses (Pautler et al., 1998; Takeda et al., 1998). However, contrary to the common picture, Sloot and the coworkers reported that only minor portion of Mn^{2+} was taken up by the neurons and they suggested that the larger portion was absorbed by glial cells (Sloot and Gramsbergen, 1994).

MRI contrast agents are most molecules with paramagnetic properties that affect proton relaxation rates making them distinguishable from surroundings. Mn^{2+} shortens both the T_1 and T_2 values of water protons, so that the effect on T_1 exceeds that of T_2. T_1-weighed MRI signal enhancement by Mn^{2+} thus reflects functional properties of anatomical neuronal network connections. MEMRI has been demonstrated in several studies to serve as a very powerful method, revealing both network plasticity and regeneration. MEMRI also makes it possible to follow individual animals over time and use them as their own controls (Leergaard et al., 2003). MEMRI has also extra-cerebral applications, such as visualizing the placenta after parenterally administered manganese containing compounds (Kay et al., 1987) and the gastrointenstinal tract after oral administration of Mn^{2+} containing compounds (Cory et al., 1987).

Previous MEMRI studies in animal brain have addressed both the regional distribution and the cerebral toxicity during acute phase (3-22 h) after Mn^{2+} exposure in a rat (London et al., 1989). Systemically administrated Mn^{2+} has been reported to be rapidly cleared from blood and secreted into the bile (Bertinchamps et al., 1966). In attempts to reveal the actions of Mn^{2+} both as a mineral and its toxicity in the brain, several papers have studied Mn^{2+} uptake into the brain suggesting penetration across the BBB (Aschner and Aschner, 1990; Aschner and Gannon, 1994; Murphy et al., 1991; Rabin et al., 1993). However, pronounced Mn^{2+}-induced signal enhancement after systemic administration is first seen in structures devoid a BBB, such as the choroid plexus, the pituitary gland and the pineal gland. The tendency of the globus pallidus to accumulate manganese suggests that there may also be a connection between Mn^{2+} and Fe^{2+} transport, because this brain structure contains more iron than any other(Drayer et al., 1986; Rutledge et al., 1987). T_1 signal intensity in caudate and putamen has been shown to have an intermediate level (Newland et al., 1989). This suggests that selective accumulation of Mn^{2+} occurs in the globus pallidus and the pituitary (Newland et al., 1989). Within the 24 h after the systemic injection of manganese chloride, Mn^{2+} contrast highlights the olfactory bulb, the inferior colliculi, the cerebellum, and the CA3 subfield of the hippocampus (Watanabe et al., 2002). However, the contrast in the CA1 subfield of the latter structure is less conspicuous. Microstructures revealed by MEMRI most likely originate from the mossy fibers and adjacent pyramidal cell layer of the CA3 subfield, as well as from the dentate hilus with parts of the adjacent granule cell layer (Watanabe et al., 2002). Isotope

studies with $^{54}Mn^{2+}$ using infant brains with incompletely developed BBBs confirm high Mn^{2+} concentration in the hippocampal CA3 and dentate gyrus and pons (Takeda et al., 1999). The high affinity of Mn^{2+} to various brain systems suggests neuronal uptake of Mn^{2+} ions from the extracellular space and subsequent axonal transport. Thus, at least part of the MEMRI contrast reflects functional brain response in behaving animals, for example, in the olfactory system (Watanabe et al., 2002; Yu et al., 2005). MEMRI has also been used to image hemodynamic changes in brain, but the effect is intermediate because of the small cerebral blood volume and the low Mn^{2+} concentration inside the vasculature due to its toxicity (Aoki et al., 2002).

Mn^{2+} has pro-oxidant activity that may be linked to its adverse effects in tissues. Direct toxic effects of Mn^{2+} have been observed in dopaminergic neurons (Anantharam et al., 2002; Parenti et al., 1988). At the subcellular level, Mn^{2+} is sequestered by mitochondria where it inhibits oxidative phosphorylation. Exposure of astrocytes to Mn^{2+} results in important functional changes including decreases in glutamate uptake, and increased densities of peripheral-type of benzodiazepine receptor binding sites. This receptor is localized to the mitochondria of astrocytes and is involved in oxidative metabolism, mitochondrial proliferation, and the synthesis of neurosteroids (Hazell, 2002). Manganese also increases gene expression and activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, which is known to be associated with apoptosis. Mn^{2+} also increases the uptake of L-arginine, a precursor of nitric oxide, as well as increasing the expression of the inducible form of nitric oxide synthase gene product. The potential consequences of all of these phenomenon are altered gene expression in astrocytes, failure of energy metabolism, production of reactive oxygen species, increased extracellular glutamate concentration and excitotoxicity, all of which could play a role in manganese-induced neuronal cell death as a result of impaired astrocytic-neuronal interaction (Hazell, 2002). Additionally, Mn^{2+} can interfere neuronal function. Mn^{2+} can suppress and alter several central neurotransmitter systems in the brain (Hong et al., 1984; Seth and Chandra, 1984). It may even modify the morphogenesis of epilepsy, affecting directly epilepsy-related pathogenic mechanisms as well as glutamate uptake (Hazell and Norenberg, 1997).

Taken together, MEMRI can be used to image the anatomical intactness of fiber tracts, increased plasticity due to focal brain injury and the regeneration of descending fiber pathways as well as the functional condition of those networks (Papadopoulos et al., 2002; Chen et al., 2002; Leergaard et al., 2003) in many different experimental animal species including mice (Watanabe et al., 2002; Watanabe et al., 2004), rats (Leergaard et al., 2003; Takeda et al., 1998; Takeda et al., 1999; Watanabe et al., 2001), primates (Saleem et al.,

2002), songbirds (Van der Linden et al., 2002; Van der Linden et al., 2004; Van Meir et al., 2004), frogs (Narita et al., 1990).

2.4. Magnetic resonance imaging studies in experimental epilepsy models

Experimental models of epilepsy have been used to mimic different modalities of disease process and profile experimentally limited pathogenetic tasks and temporal stages of epileptogenesis. Rats have been the most widely exploited experimental animal for modeling epilepsy and epileptogenesis as can be shown from the literature (Nissinen et al., 2000; Pitkanen and Lukasiuk, 2000; Bolanos et al., 1998; Zhong et al., 1995; Wenzel et al., 2000a; Tenney et al., 2003; Suchomelova et al., 2002; Soukupova et al., 1993; Smith and Dudek, 2001; Radley and Jacobs, 2003; Nersesyan et al., 2004; Perez et al., 1996; Kubova et al., 2004; Kubova et al., 2001; Knight et al., 1994; Kubova et al., 2000; Druga et al., 2003; Covolan et al., 2000). A plentiful variety of rat models take advantage of the knowledge and experience collected in other NMR studies (Nersesyan et al., 2004; Dube et al., 2000; Dube et al., 2004; Fabene et al., 2005; Fabene et al., 2003; Tenney et al., 2003; Tenney et al., 2004; van Eijsden et al., 2004; Roch et al., 2002a; Roch et al., 2002b; Zhong et al., 1993) or flurothyl (Zhong et al., 1995; Wolf et al., 2002b; Wolf et al., 2002a; Ebisu et al., 1996; King et al., 1991; Wall et al., 2000; Tokumitsu et al., 1997; Nakasu et al., 1995b). However, since the classification of epilepsy is complicated, as it covers also epilepsy-like syndromes and different brain-derived seizure conditions, the conventional classification system may not be sustainable. It therefore appears to be more appropriate to separate those models from each other according to the used NMR method and animal model describing different level of progression of epilepsy conditions.

NMR methods take advantage of exploring epilepsy in the wide range of different epilepsy models. At a pathology-based point of view, epileptogenesis bears several similarities with ischemia induced neuropathology (Siesjo and Wieloch, 1986). Common NMR bio-markers for the detection of ischemic brain injury are therefore valuable landmarks investigating the early stages of epileptogenesis as well. For example, both epileptic seizures and stroke decreases the ADC of water in the acute phase (Zhong et al., 1993). Similarly, increased T_2 MRI signals were also observed (Zhong et al., 1993; Zhong et al., 1995; Dube et al., 2004). $T_{1\rho}$ MRI has been reported to be very sensitive to ischemia-induced changes in brain tissue showing a high predictive value for the degree of developing neuronal damage (Grohn et al., 1999). $T_{1\rho}$ is based upon T_1 relaxation in the rotating frame. This type of relaxation is sensitive for slow molecular motions and therefore it has similar characteristics to T_2,

however, it has been sugessted to be more specific for the quantification of protein-water interaction (Grohn et al., 1999). This MRI contrast is still relatively new and the comparative data from epilepsy models are still not available (I; II). In NMR epilepsy studies one should also point out the importance of MRS studies (Ebisu et al., 1996; van Eijsden et al., 2004) to assess metabolic changes in epileptic tissue. Further, BOLD fMRI studies have managed to link anatomical injury with functional abnormalities in different seizure and epilepsy models (Nersesyan et al., 2004; Tenney et al., 2004; Tenney et al., 2003).

TEMPORAL PROFILING OF EPILEPTOGENESIS IN NMR STUDIES

Time dependent epilepsy development or epileptogenesis, can be stretched out temporally as an order of different events following the initial insult. Follow-up MRI studies of the initial insult-induced acute brain alterations often involves models, in which chemical agents such as KA (I; II; Ebisu et al., 1994; Covolan et al., 2000; Wenzel et al., 2000a; Wolf et al., 2002a; Nakasu et al., 1995a; Nakasu et al., 1995b; Kondratyev et al., 2001; King et al., 1991), lithium-pilocarpine (III; van Eijsden et al., 2004; Yu et al., 2002; Roch et al., 2002a; Roch et al., 2002b) bicuculline (Zhong et al., 1993; Nersesyan et al., 2004; Wenzel et al., 2000b), fluorothyl (Zhong et al., 1995) or 4-aminopyridine (Fabene et al., 2005) were used. Recently an electrical stimulation model of the amygdala was modified (Nissinen et al., 2000) to be compatible with a MR scanner by using removable deep electrodes for amygdala stimulation (I; II).

The majority of *in vivo* NMR studies have addressed acute changes in the brain following STE, and only few long term follow-up studies have been published (I; II; Roch et al., 2002a; Tokumitsu et al., 1997; Dube et al., 2004; Bhagat et al., 2005; Righini et al., 1994). In acute phase energy metabolism, tissue architecture and neural viability, as probed with DWI, T_2 and ^{1}H-MRS, have been examined. MRS data indicate that anaerobic metabolism similar to hypoxia with lactate accumulation occurs during seizure onset. Further, contrast agent MRI using gadolinium has revealed blood-brain barrier disruption occurs in epilepsy (Roch et al., 2002a). ^{1}H-MRS has shown choline depletion after insults in seizure-sensitive brain areas such as the hippocampus, the amygdala, the piriform cortex and the thalamus (van Eijsden et al., 2004; Fabene et al., 2003; Roch et al., 2002a; King et al., 1991; Ebisu et al., 1996; King et al., 1991; Nakasu et al., 1995a; Nakasu et al., 1995b; Zhong et al., 1993; Zhong et al., 1995). Reduced NAA concentrations and increased DWI signals following excitotoxic injury provide an early warning sign for neuronal injury (Ebisu et al., 1996). EEG-triggered fMRI has revealed positive BOLD effect during spike-and-wave discharges (Nersesyan et al., 2004; Tenney et al., 2004; Tenney et al., 2003). The changes of MRI parameters described above

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are followed by normalization of diffusivity and relaxation times. Minor structural changes and biochemical reactions leading to gliosis, sclerosis and other than epilepsy-related alterations in several brain structures (I; II; Roch et al., 2002b) may not be detectable by *in vivo* NMR methods during clinically silent phase of epileptogenesis. Chronic tissue injury becomes obvious as reflected by decrease in NAA and creatine concentrations (Tokumitsu et al., 1997), as well as increased diffusion and T_1- and T_2-weighted MRI signals (I; II; Roch et al., 2002a). Tissue swelling after STE (Wall et al., 2000; Fabene et al., 2003) and subsequent shrinkage due to sclerosis can be measured by volumetric MRI (Wolf et al., 2002a) in several brain areas.

The small size of rodents may cause problems for their use in NMR methods *in vivo*, since EEG electrodes fixed on skull often cause distortions at MR images. To overcome the size problem, models of epilepsy using large experimental animals have been developed. Bicuculline-induced STE in infant monkeys mimic all common aspects of human TLE (Wenzel et al., 2000b) while a sheep model of TLE produces focal seizures with occasional secondary generalisations (Opdam et al., 2002). In addition, both cats (Tanaka et al., 1993; Wada et al., 1993) and dogs (Mellema et al., 1999) have been used for epilepsy research.

In summary, of the experimental NMR methods, T_1-weighted imaging is used to derive detailed anatomical contrast, sometimes combined with contrast agents, while T_2-weighted imaging reflects well both anatomy and pathological changes. $T_{1\rho}$ can be used to study the interaction of the protein and water pools, whereas DWI contrast looks into translational mobility and behavior of different water pools in tissue. Brain activity changes can be visualized by either fMRI or MEMRI, but the latter demonstrates also neuronal tracts between connected brain structures and cellular level alterations. Finally, MRS and MRSI are valuable for detecting and quantifying several brain metabolites.

2.5. Magnetic resonance imaging of human epilepsy patients

MRI is routinely used in the clinic to image epilepsy patients for several different concerns. According to the Commission on Neuroimaging of the International League against Epilepsy (ILAE) it is good practice to obtain structural (neuro)imaging with T_1-weighed MRI from all epilepsy patients, even within the non-acute phase, except in case of few rare epilepsy syndromes, and from those with contraindications for MRI.

MR techniques were first applied to clinical studies in the late 70's and became clinical routine in the 80's. MRI provided first detailed high resolution images of the brain, showing

the fine structures in the living brain which were, up to then, only obtainable post mortem. The next step of examining epileptic patients by MRI was the localization of brain areas from where the seizures originated, such as very small tumors not reliably detectable by CT. However, the unequivocal detection of a seizure focus is still difficult by conventional neuroimaging methods and ictal EEG, and these are often augmented by either positron-emission tomography (PET) or single-photon-emission computed tomography (SPECT) scans. Some etiological diseases for epilepsy, such as tumors and developmental abnormalities (including hippocampal or cortical dysplasias), are routinely picked up by T_1, T_2, DWI and MRSI detection techniques. In the planning of surgery to remove or resect any of these abnormalities, preoperative MRI scans are known deemed to be crucial (Kuzniecky and Jackson, 2005).

However, in the chronic epileptic brain, functional and anatomical integrity is not always obvious from neuroimaging data. In addition to structural MRI, MRS and fMRI of the brain and the subsequent detection of the epileptic activity generators ("epileptogenic zone") have changed our understanding of this diseases development and made it possible to have more decisive treatments available in the clinic.

The consequences of an epileptogenic process can be detected by different *in vivo* NMR methods in clinical settings. The revealment and quantitative assessments of hippocampal sclerosis are obtained by an increase in T_2-weighted MRI signal and a cooresponding T_1-weighted hypointensity in scans. T_1 MRI can also reveal anatomical details, including atrophy. A very common morphological change in chronic epilepsy is atrophy affecting several brain structures. In the case of the hippocampus, it means a loss in internal architecture as a consequence of neuronal damage and the subsequent replacement of the normal anatomy by gliotic tissue. These changes can be both detected by T_1- and T_2-weighted MRI. Hippocampal MRI volumetry, which is widely used in clinical practices, reveals the extent of the loss in hippocampal volume (Kuzniecky et al., 2001). This is critical because, the reduction in the volume is regarded to be a sensitive and specific marker for hippocampal sclerosis. Furthermore, it can also be compared to the total brain volume, increasing the validity of this method for clinical assessment. Another method, T_2-relaxometry is a quantitative measurement of tissue pathology in hippocampal gray matter and can therefore be used, either to support the findings of other imaging techniques or to determine the extent of the damage itself (Kuzniecky and Jackson, 2005).

3. AIMS OF THE STUDY

The present study was designed to address three main questions.

Firstly, what are the MRI-detectable changes and their temporal profiles in the known seizure-sensitive brain regions such as the amygdala, the piriform cortex, the hippocampus and the thalamus during epileptogenesis and epilepsy? These regions were selected for MRI assessment due to their established involvement in the pathogenesis of human TLE.

Secondly, can one detect atrophy in either the hippocampus, amygdala, or their surrounding cortex as a long-term consequence of animals exposed to STE in the developing brain (at P12), and if so, does the MRI detectable atrophy correlate with the neurodegeneration uncovered by histological methods from the same animals?

Thirdly, can one find surrogate biomarkers, for instance by the use of MEMRI, to reveal the epileptogenic process in the early, asymptomatic phase of the disease before epileptic seizures occur in these animals?

4. MATERIALS AND METHODS

4.1. Animals

The work was accomplished in three parts. In all these studies, the rats were individually housed in a controlled environment (constant temperature, $22 \pm 1°C$, humidity 50-60%, lights on from 07.00 to 19.00). Animals had free access to food and water. All animal procedures were conducted in accordance with the guidelines set by the European Community Council Directive 86/609/EEC.

In the first part of the study (I; II), 20 adult male Harlan Sprague-Dawley rats were used, of which 17 were epileptic and three served as sham controls (i.e they were operated upon but not electrically stimulated). The mean body weight was 333 ± 23 g on the day of surgery and 432 ± 103 g at 153 days after stimulation, when the study was terminated. A further five adult male non-operated age-matched Wistar rats (437 ± 62 grams) were used as naive controls for MRI were used.

In the second part of the study (III), 34 male Wistar rats of postnatal (P) age P12 were used in the experiments. The day of birth was defined as P0. Animals were weaned at P28 and housed under controlled temperature and humidity with a 12/12 hour light-dark cycle as described above. The experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic, as well as by the Committee for the Welfare of Laboratory Animals of the University of Kuopio and the Provincial Government of Kuopio.

In the third part of the study (IV), male Wistar rats were used: 14 epileptic and six control rats, whose weight at the beginning of the study was 303 ± 24 g.

4.1.1. Induction of status epilepticus in the test animals

Three different animal models for epilepsy were used. In the first part of the study (I; II), the amygdala electrical stimulation of TLE, developed by Nissinen et al. was used (Nissinen et al., 2000). First, the electrodes were implanted as described in section 4.1.2. Four weeks after surgery, the baseline EEG was recorded for at least 1 minute in each rat to confirm that the electrodes and plugs were yielding recordable EEG signals. STE was induced by stimulating the amygdala for 20 to 40 min, as determined by the response of the animals to the

stimulation. Briefly, the stimulation consisted of a 100 ms train of 1 ms biphasic square wave pulses (400 µA from peak-to-peak) delivered at 60 Hz every 0.5 s using an A300 Pulsemaster Stimulator connected to two A360 Constant Current Stimulus Isolators (CCSI) (WPI, Sarasota, USA). The animals were connected to the CCSIs with a 6-channel commutator (Plastics One Inc., Roanoke, USA) and shielded cables. Each rat was first stimulated continuously for 20 min. After that, the stimulation was interrupted and the behavioral and electrographic seizure activity of the animal was observed for 60 s. If the behavior of the animal indicated the presence of continuing epileptic activity (head nodding/or limb clonus) and epileptic outbursts in the EEG, observation was continued for up to 5 min. If animal did not meet the criteria of clonic STE (continuous electrographic epileptiform spiking and recurrent clonic seizures) the stimulation was repeated and the behavior was reexamined again for 5 minutes. Once the criteria of STE were obtained, stimulation was not repeated. EEG was monitored continuously for 48 h after stimulation via cortical electrodes using the Nervus EEG Recording System (Nervus, Taugagreining, Iceland) connected with an ISO-1032 amplifier (Braintronics, Almere, Netherlands) SVT-S3000P Hitachi Time Lapse 168 VCR (Hitachi, Tohbu, Japan), and a Panasonic WV-CL350 Video Camera (Panasonic, Tokyo, Japan). A combined video-EEG monitoring system was used for simultaneous screening at EEG and physiological behavior of the animals. The amygdaloid stimulation electrodes were then removed under halothane anesthesia 48 h after stimulation and the electrode guide cannula was sealed with dental acrylate.

Altogether 17 rats were stimulated and a complete six month MRI follow-up regime was available for eight epileptic rats. To control for possible tissue damage produced by the initial electrode insertion, three electrode-implanted, unstimulated controls were imaged by MRI in parallel with the stimulated rats at 73 ± 3 days and at 271 ± 1 days after electrode implantation. In addition, eight age-matched (238 days old, at the end of the study) Wistar rats were included in the study as MRI controls. It should be noted that in previous studies, electrode implantation as such did not induce epileptogenesis (Nissinen et al., 2000).

A prominent feature of the EEG activity during STE was the occurrence of high-amplitude and frequency discharges (HAFDs), which were typically associated with epileptic seizures. HAFD was defined as high-amplitude (>2x baseline) and high-frequency (>8 Hz) discharge in the cortex lasting for at least five seconds. To assess the severity of STE, the number of HAFDs in each animal was counted. Finally, the duration of STE was defined as the time interval between the first and last HAFD.

In the second part of the study (III), 34 rats were intraperitonally (i.p.) injected with an aqueous solution of lithium chloride (3 mmol/kg) 24 h prior to the administration of pilocarpine [n=24, 40 mg/kg dissolved in saline (2 ml/kg), i.p.)]. After pilocarpine injection, the animals were housed in individual cages and their behavioral seizure activity was observed for at least three hours. Both the latency to the beginning of continuous motor seizure activity (motor STE) and the occurrence of generalized tonic-clonic seizures were recorded. After two hours of motor STE, animals were treated with paraldehyde [0.3 ml/kg dissolved in distilled H_2O (3 ml/kg), i.p.]. Four hours after the beginning of STE, rats were injected with saline (up to 2% of their body weight, s.c.) to restore dehydration, and returned back to their mothers (the duration of isolation from mothers in the control and STE groups was the same). The body temperature of the rat pups was kept at 34°C, as it is normally in the nest. Only the rats with motor STE without generalized tonic-clonic seizures were included in the study. Control rats (n=10) were treated with saline instead of pilocarpine or paraldehyde at the corresponding time points. Body weight was measured daily for one week after STE, and thereafter, at 13, 21, 40 days, and three months. These data were used to calculate relative body weight [(body weight at the time of measurement/body weight at P12) * 100%] during the course of the study to investigate the effect of body weight on the MRI and histology data obtained.

In the third part of the study (IV), STE was induced in adult male Wistar rats (14 epileptic and six controls, 303 ± 24 g at the time of the treatment) by injecting KA (11mg/kg, i.p.). The behavior of rats was monitored for four hours to confirm the presence of the criteria for STE.

4.1.2. Electrodes placement and video-EEG monitoring

TECHNICAL ASPECTS

For the first study, both non-magnetic cortical electrodes and removable stimulation electrodes had to be developed. There are many different small, non-magnetic metallic screws available, but all of them have ferric impurities making them impossible to use in MRI experiments. This problem was solved by constructing the electrodes from plastic screws and platinum-iridium wire (diameter = 0.005 mm). These were then fixed to the skull. The rifles of the plastic screws in the scull holes were slashed by ferric screws. Artifacts in MR images, caused by platinum-iridium wire were minimal and occurred in the vicinity of electrodes. Pluggings for the pedestal were made out of platinum-iridium wire and fixed to the pedestal by dental acrylate (Selectaplus CN, Dentsplay DeTrey GmbH, Dreieich, Germany).

Removable stimulation electrodes were also handmade by removing the metallic part of cannula. Then the cap of each cannula was perforated and the electrode was fixed to the cap by dental acrylate. All electrodes were screwed tightly to the cannula before operation. The electrode was placed stereotactically into the brain and the cannulae were fixed to the skull by dentate acrylate. The caps of the cannulae were removed before the first MRI scans.

SURGERY FOR EEG ELECTRODES

For implantation of the stimulation electrode (I; II), the rats were deeply anesthetized with a mixture of sodium pentobarbital (60 mg/kg, i.p.) and chloral hydrate (100 mg/kg i.p.). A bipolar removable stainless steel electrode (diameter 0.127 mm, dorsoventral distance between the tips 0.4 mm; Franco Corradi, Milano, Italy) surrounded by a plastic guide cannula above the skull was implanted into the lateral nucleus of the amygdala of the left hemisphere (3.6 mm posterior to bregma, 5.0 mm lateral to bregma, 6.5 mm ventral to the surface of the brain according to the rat brain atlas of Paxinos and Watson, 1986) (Paxinos and Watson, 1996). The electrode setup was fixed to the skull with dental acrylate.

To record the seizure activity by EEG, Teflon-coated platinum-iridium wire (0.005", A-M Systems Inc., Carlsborg, USA) was fixed with plastic screws (Plastics One Inc., Roanoke, USA). The wire electrode, supported by a plastic screw, was implanted into the skull above the contralateral frontal cortex (coordinates: 3.0 mm anterior and 2.0 mm lateral to the bregma point). Two monopolar electrodes were fixed to the skull symmetrically over the cerebellum and served as ground and reference electrodes.

In the second (III) and third (IV) studies, EEG monitoring was not used because of very complicated technical solutions that would have been needed.

4.1.3. Anesthesia

In the first part of the work (I; II), where electrode implantation was needed, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and chloral hydrate (100 mg/kg i.p.). For the last EEG recordings after all MRI scans, conventional cortical electrodes were placed under halothane/N_2O/O_2 anesthesia. An initial halothane concentration of 2% was rapidly reduced to a surgical concentration of 0.7%.

In the third part of the study (IV), rats were anesthetized for intracerebral $MnCl_2$ injection with sodium pentobarbital and chloral hydrate as described above.

All MRI experiments were carried out in halothane/N_2O/O_2 anesthesia using a halothane concentration of 0.5-1.0%.

4.1.4. Intracerebral injection of manganese chloride

For the third part of the study (IV), 20 rats (14 epileptic and six controls, 290 ± 26g) were operated on two weeks after KA induced status epilepticus. The skull was opened and then all the rats obtained an intracerebral injection of 40nl of manganese chloride (1M) into the entorhinal cortex. Injections were accomplished with self-made calibrated pipettes (diameter = 0.25mm, tip=20μm, graded 50nl/mm) using an air pressure controller (General Valve Corporation, Fairfield, USA). The coordinates of the injection site were chosen according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1996): 3.6 mm posterior to bregma, 5.0 mm lateral to bregma, 6.5 mm ventral to the surface of the brain. Control rats were not injected with KA but were operated on in a similar fashion.

4.1.5. Physiological monitoring

Video-EEG monitoring (I; II) was used to detect the occurrence of spontaneous seizures. The first recording was performed two days after STE, and thereafter at day 28 (continuous video-EEG monitoring (24 h/day) for seven days), 42 (for 11 days), 4 months (for four days), and eight months (for seven days) after STE.

EEG signals were recorded with a Stellate EEG Monitor System (Stellate, Montreal, Canada) with a sampling rate 200 Hz, high pass filter 1 Hz, and low pass filter 100 Hz, that was connected with two ISO-DAM 8 amplifiers (WPI, Sarasota, USA) and to which animals were connected with a 6-channel commutator and shielded cables. This system allows the animals to move freely without twisting the cables. Physiological behavior was imaged using a WV-BP312E Video Camera (Panasonic, Tokyo, Japan) that was positioned above the cages and connected with an SVT-S3000P Time Lapse 168 VCR (Sony, Tokyo, Japan) and PVC-145E Video Monitor (Sony). Recorded data was packed to the video tapes 24 hours in three hours tapes. The video system was connected to the EEG recording system via a time code generator (MUL, TIM Electrode Inc.). A Type 955 Infra Red Light source (Videmech Ltd., Sandhurst, UK) was used at night to allow for continuous video monitoring. A wide angle lens allowed for the video-monitoring of eight to ten animals at the same time. Manifestation of seizure activity was analyzed on both the DDS2-files and video tapes.

All EEG data was analyzed visually by browsing the EEG trace on the computer screen. When an electrographic seizure was observed, its behavioral severity was analyzed from the time-matched video-recording. An electrographic seizure was defined as having high-frequency (> 5 Hz) and high-amplitude (> 2x baseline) discharges in the contralateral frontal cortex, lasting for at least five seconds.

The severity of the behavioral seizures was scored according to a modified Racine's scale (Racine, 1972) as follows. Score 0: electrographic seizure without any detectable motor manifestation; Score 1: mouth and face clonus, head nodding; Score 2: clonic jerks of one forelimb; Score 3: bilateral forelimb clonus; Score 4: forelimb clonus and rearing; Score 5: forelimb clonus and rearing and falling.

The body temperature of animals were maintained during all operations and MRI scans at $37 \pm 1°C$ using a system of circulating water connected to a heater.

4.4. Magnetic resonance techniques

Quantitative MRI measurements (I; II) were performed immediately after the first video-EEG recordings, corresponding to the first two days after STE, followed by 9, 23, 53, 84, 113-115, 152 to 154, and 174 to 176 days after STE. For study (III), the rats were imaged three months after the exposure to STE in childhood. In the MEMRI study (IV) MRI data sets were acquired 3, 5, 7 and 10 days after surgery, with two controls and two epileptic animals being also imaged at 1, 6, 12, 24 and 48 hours after the injection to monitor the transport of manganese ions in the perforant fasciculus.

4.4.1. Hardware

All MRI measurements were performed in a horizontal 4.7 T magnetic field strength (Magnex Scientific Ltd, Abington, UK) equipped with actively shielded imaging gradients (Magnex Scientific Ltd, Abington, UK) interfaced to a Varian ^{UNITY}INOVA console (Varian, Palo Alto, USA).

A linear birdcage volume coil (diameter 40 mm, length 72 mm) was used in transmit-receive mode (I; II; III). Volume coil was chosen to obtain signal also deep regions of the brain like amygdala. A quadrature half-volume coil was used as a transmitter and a receiver (IV), and this coil gave good signal-to-noise ratio (SNR) from regions close to it, however adequate signal was detected also from deeper brain structures.

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To achieve highly reproducible positioning of the rat head inside the magnet and to avoid movement artifacts for all MR experiments, a plastic, MRI compatible, animal holder with ear bars and bite bar was used.

4.4.2. Measurement of T_1 and T_2 using MRI

Cerebral T_2 values were quantified from a coronal 1 mm slice (field of view 35 mm, matrix size of 128*256) set according to pilot images so that the centre of the slice was 2.8 mm from bregma (I; II). The same slice was selected also for diffusion- and $T_{1\rho}$ MRI. The acquisition parameters for T_2 quantification were as follows: TR=1.5s, TE=20/40/60 ms and 4 averages/line using a multi-echo sequence. MRI was performed at days 2, 9, 23, 53, 84, 113-115, 152-154 and 174-176 after STE and all quantitative parameters were determined.

Anatomical T_2 -weighted imaging was performed three months after STE, using MRI parameters as follows: TR=2s, TE=55ms, FOV=35mm, data matrix 128*256, slice thickness 1 mm, 8 slices (III). Two of these slices containing the region of interest (*i.e.*, the hippocampus, the amygdala, the piriform and the perirhinal cortex) were chosen for quantitative anatomical measurements. Atrophy in the septal hippocampus and the perirhinal cortex was assessed in the section corresponding to a level, -3.8mm, from the bregma by the rat brain atlas of Paxinos and Watson, 1996 (Paxinos and Watson, 1996). For the assessment of hippocampal atrophy in T_2 MR images, three lines were drawn to divide the hippocampus into four equal segments, matching those used for histology. To assess atrophy of the perirhinal cortex, a line connecting the pial surface and the capsula externa in the fundus of the perirhinal cortex was drawn. Volumetric amygdala measurements were performed from a more caudal section, which also included the piriform cortex, because there were no landmarks in MR images that would reveal the border between these two regions. Finally, to measure atrophy in the amygdala, a line was drawn between the ventricular surface (corresponding to the medial corner of the border between the lateral and basal nuclei of this organ in histology sections) and brain surface corresponding to the most ventrolateral corner of the piriform cortex.

MRI data was obtained 3, 5, 7 and 10 days after surgery (IV). In addition, two controls and two epileptic animals were imaged 1, 6, 12, 24 and 48 hours after injection to monitor transport of Mn^{2+} ions in the perforant fasciculus. T_1-weighted (TE=2.7ms, TR=120ms) 3D gradient echo imaging was performed using an adiabatic 70° BIR-4 excitation pulse to minimize flip angle dependent contrast variations that occur due to B_1 field inhomogeneities

when a local transmitter coil was used (Staewen et al., 1990). A volume of 25 x 25 x 35 mm was covered with 192 x 64 x 256 points, with two averages per phase encoding step, leading to a total acquisition time of 49 minutes/animal 3D image set. Signal intensities were measured and normalized with adjacent muscle tissue. The inhomogeneity correction factor for the coil receptive sensitivity profile was performed using a 3D data set derived from a measurement of gelatin. The semiautomatic analysis method was created using Matlab 6.1 software. First, the slice was selected -3.8mm from bregma. Then, the reference area was drawn on to the brain cortex, adjacent to the hippocampus. The pixels in the selected two-dimensional image, with 1.3 times stronger signal intensity in the ipsilateral side and 1.2 times stronger signal in contralateral side were counted. Enhancements were localized in both the hippocampal CA3 +dentate gyrus and the CA1+CA2 and the dorsal thalamus. Constants of 1.2 and 1.3 (=1.3*ka+1*sd) were established empirically, so that the brain substructures of interest were very clearly resolvable from the surrounding tissue.

4.4.3. $T_{1\rho}$ MRI

$T_{1\rho}$ was quantified using four variable length (10-70 ms) adiabatic spin-lock (SL) pulses with B_1 of 0.7G followed by a crusher gradient in front of a fast spin echo imaging sequence (TR=2.5 s, echo spacing =10 ms, 16 echoes/excitation, four averages) (I; II). The on-resonance spin-lock pulse consisted of a hyperbolic secant adiabatic half passage (AHP) pulse, followed immediately by the spin-lock period and a second AHP pulse which was applied to return the magnetization back to the z-axis.

4.4.4. Diffusion MRI

The trace of the diffusion tensor (D_{av}) images were obtained using the method of Mori and van Zijl (Mori and van Zijl, 1995), incorporated into a spin-echo sequence (TR=1.5 s, TE=55 ms, b-values: 0, 470, 856 s/mm^2) (I; II). The method uses four pairs of bipolar gradients with a diffusion time of 4.8 ms, yielding the trace images (D_{av} = 1/3TrD) in a single scan. Both tissue thickness and the size of any given anatomic area were determined from the diffusion weighted (TE=55 ms, b= 856 s/mm^2) images, by measuring either the width of a given region from a slice (amygdala) or outlining the borders of the given region (hippocampus) based on visual judgement.

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4.5. Histological methods

BRAIN PERFUSION AND FIXATION

Rats were perfused transcardially for histological analysis after the follow-up period (I-IV). Under terminal anesthesia with sodium-pentobarbital (60 mg/kg, i.p.) and chloral hydrate (100 mg/kg, i.p.) rats were perfused according to the Timm fixation protocol: 0.37% sulphide solution (30 ml/min) for 10 min followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (30 ml/min), +4°C, for 10 min. The brains were then removed from the skull mechanically and postfixed in a buffered 4% paraformaldehyde solution for 4 h. This was then followed by cryoprotection in a solution containing 20% glycerol in 0.02 M potassium phosphate buffered saline at pH 7.4 for 24 h. The brains were then blocked, frozen in dry ice, and stored at -70°C until cut. The brains were sectioned in coronal plane (50 μm, 1-in 5 series) with a sliding microtome. The sections were stored in a tissue-collecting solution with cryoprotectant properties (30% ethylene glycol and 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until further processed. Adjacent sections in each series were used for Nissl, Timm, and Fluoro-Jade staining, respectively.

TIMM STAINING

Mossy fiber sprouting was analyzed from sections stained with the Timm sulfide/silver method (Sloviter, 1982). For staining, all coronal sections (30 μm, every fifth section) including the hippocampus were mounted on gelatin-coated slides and dried at +37°C. Staining was performed in darkness according to the following procedure: the working solution containing gum arabic (300 g/l), sodium citrate buffer (25.5 g/l citric acid monohydrate and 23.4 g/l sodium citrate), hydroquinone (16.9 g/l), and silver nitrate (84.5 mg/l) was poured into a staining dish. The sections were then developed until an appropriate staining intensity was attained (60-75 min). Slides were then rinsed under tap water for 30 min and placed in a 5% sodium thiosulfate solution for 12 min. Finally, sections were dehydrated through an ascending series of ethanol, cleared in xylene, and cover-slipped with DePeX mounting medium (BDH Laboratory Supplies, Dorset, UK).

Mossy fiber sprouting was analyzed from both the septal and temporal ends of the hippocampus. At the septal pole of the hippocampus, the tip, mid and crest portions of the granule cell layer, which corresponds to the region from which MRI data were obtained, were analyzed separately. The density of mossy fiber sprouting was scored according to Cavazos and colleagues (Cavazos et al., 1991) as follows: Score 0 = no granules, Score 1 = sparse

granules in the supragranular region and in the inner molecular layer, Score 2 = granules evenly distributed throughout the supragranular region and the inner molecular layer, Score 3 = almost a continuous band of granules in the supragranular region and inner molecular layer, Score 4 = continuous bands of granules in the supragranular region and the inner molecular layer, Score 5 = confluent and dense laminar bands of granules that covers most of inner molecular layer, in addition to the supragranular region. For each section, mean sprouting scores in the tip and mid portions were calculated. Thereafter, mean Timm scores obtained in the tip, mid, and crest potions of all sections from the septal, dorsal-mid, or ventral-mid portions of the hippocampus were calculated separately.

NISSL STAINING

Nissl staining was used to identify cytoarchitectonic boundaries, as distribution and degree of neuronal damage and position of the injection pipette. The first series of 1-in-5 sections was stained for thionin. The method to score the severity of neuronal damage in the various temporal lobe structures has been described previously by Nissinen and colleagues (Nissinen et al., 2004). In brief, Score 0 = no damage, Score 1 = ≤ 20% neuronal loss, Score 2 = neuronal loss of 20-50%, Score 3 = ≥ 50% neuronal loss.

Cresyl violet staining was used for the histological confirmation of the findings that were observed in the MR imaging experiments. A stack of six consequtive sections was chosen (150 μm apart, total "slice thickness" 900 μm) matching the coronal slice that was used to assess hippocampal atrophy in the MR derived images. Shortly, cresyl violet stained sections were viewed under brightfield illumination using Olympus AX70 microscope equipped with Olympus DP70 digital camera and Olympus Micro Image Analysis Software (Media Cybernetics,L.P., Newburyport, USA). Thickness measurements were started at the level, at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells, -3.30 from bregma (Paxinos and Watson, 1996). Three lines were drawn between the alveus and the ventral surface of the infrapyramidal molecular layer that resulted in the partitioning of the granule cell layer into four equal parts. Subsequently, the length of the three lines (*i.e.*, thickness of the hippocampus) was measured in all six consecutive sections and the mean of all 18 measurements per animal was calculated. The mean values of left and right hippocampus were compared for each animal and this comparison formed the basis of the subsequent statistical analysis.

The thickness of the CA1 was measured as a distance between the alveus and the hippocampal fissure. The positioning of lines used for the measurements was adjacent to that

used for the hippocampal thickness measurements. Mean values from both CA1 hippocampal subregions from the left and right hemispheres were used for statistical analysis.

Amygdala thickness was measured at the level corresponding to the antero-posterior level, – 3.8 relative to the Bregma point (Paxinos and Watson, 1996). A line was drawn between the medial corner of the border between the lateral and basal nuclei and the most ventrolateral corner of the piriform cortex. The mean of measurements in two consequtive sections (150 μm apart) was calculated for each animal. The means of the left and right amygdala measurements were used for statistical analyze.

Thickness of the perirhinal cortex was measured at the level corresponding to antero-posterior level, –3.8 relative to the Bregma point (Paxinos and Watson, 1996) in two consecutive sections (150 μm apart). A line connecting the pial surface and the capsula externa in the fundus of the perirhinal cortex was drawn. The mean of left and right hemisphere was calculated for each animal.

FLUORO-JADE B AND SILVER HISTOCHEMISTRY

Fluoro-Jade B and silver staining were used to confirm the occurrence of acute STE-induced neurodegeneration in regions that were found to be atrophied in chronic MR imaged animals. The brains were postfixed and cryoprotected as descibed above. The tissue was cut in the coronal plane (50 μm) with a Leica CM 1900 cryocut, and sections were stored in a cryoprotectant tissue-collecting solution until stained. The sections were collected in 5 series with every fifth section belonging to the same group. To detect degenerating neurons, adjacent series of sections were processed for cresyl violet, Fluoro-Jade B, and silver stainings.

Fluoro-Jade B histochemistry was done according to the method described by Schmued and colleagues (Schmued et al., 1997). To illustrate the distribution of neuronal damage in different brain areas, Fluoro Jade B–labeled cells were plotted from selected sections with a computer-aided digitizing system (MicroBrightfield, Minnesota Datametrics, St. Paul, MN). Anatomical boundaries were drawn from adjacent cresyl violet-stained sections using a stereomicroscope equipped with a drawing tool, and then superimposed on scanned plots by using Corel Draw software (version 11).

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The silver impregnation technique described by Gallyas and colleagues (Gallyas et al., 1980) was used to independently confirm degenerating neurons.

4.6. Data analysis

The data generated in this work was analyzed using the following programs: Microsoft Office Excel (Microsoft Inc, Redmond, USA), SPSS for Windows and SigmaStat® (SPSS Inc., Chicago, USA), where indicated. Slices from MRI and histology were plotted according to reliable landmarks to position the ventricles and hippocampus. Correlations were discovered using a Pearson Chi-Square-test. Changes in seizure numbers between different animal groups [mild epilepsy (<1 seizure/day) and severe epilepsy (>1 seizure/day)] were analyzed using the Mann-Whitney U-test. A P value of less than 0.05 was considered statistically significant. The comparison of measurements between the control and STE rats was done by using unpaired Student's t-test. The correlation of MR imaging and histological data or body weight and brain atrophy from the same animals was done using the Spearman test. Again, P values of less than 0.05 were considered significant. All values are expressed as mean ± SEM.

5. RESULTS

Follow-up study assessing the progression of brain damage after status epilepticus and its association with epileptogenesis in a rat model of temporal lobe epilepsy (I, II).

Altogether 25 rats were included in the study: five normal controls without any treatment, three unstimulated controls with electrodes implanted in the amygdala, and 17 stimulated animals with electrodes. Four of the 17 stimulated rats died within 24 to 48 hours after STE and were therefore excluded from the study. The progression of the structural changes was observed with MR imaging in 10 of 13 rats. Three rats died before spontaneous seizures were detected in video-EEG recordings and the confirmation of epilepsy diagnosis was deficient. Eight rats of the 13 rats survived the entire eight month follow-up period and were also used for histologic analysis.

Ten rats were examined for the spontaneous seizures in video-EEG recordings: six of them expressed spontaneous seizures during the first video-EEG recordings (performed between days 28-35) after STE and all 10 rats had spontaneous seizures during the second session of video-EEG measurements (followed at days 42-52). During the first video-EEG follow-up, the number of daily spontaneous seizures varied between 0.5 and 25.7 and in second one between 0.1 - 32 seizures per day. The difference between mild and severe disease was defined by seizure activity during a 24 hour period: less than one seizure per day was considered as a mild disease and more than one seizure was considered as a severe disease. Based on this criterion, fifty-two days after STE, four rats displayed a mild disease and the remaining six were suffering from severe disease. One rat with a mild disease at this time point, later developed several daily seizures, and was therefore included in the group of animals with severe disease in the final data analysis. The mean score indicating the behavioral severity of spontaneous seizures was 3.9 ± 1.1 in the first video-EEG follow-up and 2.9 ± 1.9 during the second one.

Ten rats were scanned for quantitative MRI. Two rats died during the follow-up period (one on day 53 and another on day 84) and were not included in the relaxation time or diffusion MRI results.

Brain structures in both hemispheres, including the amygdala, the piriform cortex, and the thalamus, showed a consistent remarkable increase in both T_2 and $T_{1\rho}$ relaxation times by more than >26% ($p<0.001$), by two days after stimulation. At the first MRI measurement time

point, D_{av} was elevated in both ipsilateral and contralateral amygdalae (p<0.05), but showed normal values in the other brain structures examined. In the hippocampus, the only MRI abnormality was elevated $T_{1\rho}$ in the ipsilateral side (p<0.05) during first imaging session. MRI parameters measured from brain regions two days after STE returned to control levels, except for $T_{1\rho}$ (p<0.05) which remained slightly elevated (114% ± 2% of control) in the contralateral piriform cortex until day 53. Assessment of the time-dependent changes in the MRI variables revealed secondary changes in several brain regions. These started on days 23 and 53 after stimulation, which coincided with the beginning of spontaneous seizures in the animals. In the ipsilateral amygdala, D_{av} was elevated in scans acquired on days 23 and 84 (p<0.05), while in the contralateral side, $T_{1\rho}$ was increased in the images after day 53 (p<0.05). In the ipsilateral hippocampus, $T_{1\rho}$ was prolonged on 23 and 53 days after stimulation (p<0.05). Also in the latter time points, D_{av} was abnormally high in the hippocampal formation. In the thalamus, the only MRI abnormality after STE was a decreased T_2 on day 114 (p<0.01). In the piriform cortex, no MRI changes were detected after day 53, except a decrease in T_2 at day 114 (p<0.01). Due to severe atrophy in the piriform cortex and amygdala, the last few data points could have been influenced by an increased partial voluming effect from cerebrospinal fluid (CSF) in the adjacent enlarged ventricles. The absolute values of T_2, $T_{1\rho}$, or D_{av} MRI did not significantly correlate with either severity of the epilepsy (daily seizure frequency), or neuronal damage in the amygdala or the hippocampus, or mossy fiber sprouting, as determined by histology, eight months after STE. The thickness of the amygdala and the piriform cortex in the ipsilateral side was decreased in the MR images aquired nine days after STE, relative to the control Sprague-Dawley and Wistar rats. Additionally, no strain differences were observed in the control animals. However, large, animal to animal, variations in the volumes of the hippocampi and parietal cortices within the epileptic rat group were observed. These changes were not consistently statistically significant during the whole follow-up period.

Nissl staining demonstrated severe damage (score > 2.0) in all of the epileptic animals occurred in the amygdale, the piriform cortex and the thalamus at sites that corresponded to those areas, which were discovered during the MRI, analyzes. In the septal hippocampus, the granule cell layer was mildly damaged in one of eight rats included to the study. The hilus showed severe neuronal loss (score >2.5) in four and mild (score <0.5) in two of these eight animals. In two rats, no hilar damage was observed. The CA3 pyramidal cell layer was damaged in five of the eight rats (score ≤ 2.0) and the CA1 pyramidal cell layer in two of the eight rats. There was a high correlation between neuronal cell damage and the mossy fiber sprouting in the dentate gyrus, as assessed by histology, 265 days after STE. MRI parameters

did not correlate with hippocampal histopathology. The lack of correlation might be explained by the fact that the damage was limited to selective subfields of the hippocampus and the coarse resolution of *in vivo* MRI did not allow for the separation of those subfields and the detection of the changes evident at microscopic level.

Fluoro-Jade B staining revealed a large number of Fluoro-Jade B -positive cells with neuronal morphology in the granule cell layer as well as in the CA3a and CA3c regions in one of the eight epileptic animals. The labeled cells were located in the hippocampus at the level where the septal and temporal ends become fused in the coronal sections that corresponds to the caudal two-third of the hippocampus (and caudal to the slice imaged with MRI). In the remaining seven rats, labeling in cells with a glial appearance was observed. This was typically located in the layer II of the piriform cortex, as well as in the ventral and dorsal intermediate subfields of the entorhinal cortex.

Mossy fiber sprouting was present in all dentate gyrus subfields. At the level of the septal hippocampus, matching the MRI analysis, the mean density of mossy fiber sprouting in the ipsilateral tip portion of the granule cell layer was 3.7 ± 1.4, in the mid portion 3.2 ± 1.9, and in the crest 3.4 ± 1.5. The mean sprouting from those portions was 3.5. Analysis of the data from each individual rat indicated that the density of the mean mossy fiber sprouting was increased in all epileptic animals relative to controls ($p<0.05$). The density of the sprouting showed no difference between the ipsilateral and contralateral hemispheres. No differences between the animals with rare or frequent seizures were observed. The correlation between the severity of hilar cell loss and the density of mossy fiber sprouting was evident in both ipsilateral and contralateral hippocampus.

Study of early-life status epilepticus induced alterations in the amygdala and hippocampus (III)

STE developed in all rats (24/24) that received a systemic administration of pilocarpine. The mean latency to motor STE was 798 ± 59 s (mean $\pm$ SEM), which was characterized by twitching of facial muscles, chewing, head bobbing, forelimb clonus, tail erection and "swimming" movements. A single dose of paraldehyde (0.3 ml/kg) suppressed the clonic seizures. Brief periods of swimming movements and head bobbing, however, occurred occasionally during the next 12 hours. In the STE group mortality rate during the first 24 hours after STE was 42%, but no mortality was evident after this time point. Rats with STE had a decrease in the body weight at 24 h after STE (loss of 2% *vs*. 18% gain in controls compared to weight at P12, $p<0.001$). In the STE group relative body weight stayed low until

P40 (p<0.05). However by three months after STE, at the time of the MRI, there were no differences in absolute (467 ± 12 vs. 468 ± 15g, p=0.815) or relative (1645 ± 218 vs. 1545 ± 192%, p=0.265) body weights between the control and epileptic rats. Finally, no correlation between the body weight and thickness of the hippocampus, the amygdala, or the perirhinal cortex was observed in MRI scans.

MRI was performed three months after STE to measure the size of the hippocampus, the amygdala and the perirhinal cortex. No atrophy of the septal hippocampus was detected relative to the control animals (1.27 ± 0.14 vs. 1.35 ± 0.08 units, size of interpolated pixels, p=0.135). Data analysis from individual animals showed that three of the 13 rats (23%) had reduction in the hippocampal thickness exceeding 2 standard deviations (2SD) from the mean observed in the control group. Atrophy in the region containing the amygdala and the piriform cortex was evident from control animals (2.79 ± 0.03 vs. 2.91 ± 0.04 units, p=0.023). Data from individual animals showed that two of the 13 rats (15%) had a reduction in the thickness of the amygdaloid region of more than 2SD from the control animal mean. The perirhinal cortex was atrophic in rats with STE relative to the controls (1.92 ± 0.03 vs. 2.06 ± 0.03 units, p=0.003). Four of the 13 rats (31%) showed a decline in the thickness of the perirhinal cortex by more than 2SD from the control mean.

Morphometric analysis of the atrophy in histological sections was assessed three months after STE. A tendency towards reduced thickness in the septal hippocampus was found in rats that recieved STE at P12 compared to that in controls (-3.7% in histology, -6.2% in MR imaging). Two of the 13 rats (15%) showed a reduction in the hippocampal thickness by more than 2SD from the control mean. The thickness of the CA1 was reduced in rats with STE compared to the control siblings (0.706 ± 0.005 mm vs. 0.736 ± 0.012 mm, p=0.015). Rats exposed to STE had atrophy in the region containing the amygdala and the piriform cortex, compared to controls (2.042 ± 0.030 vs. 2.175 ± 0.09 mm, p=0.006). Data from individual animals showed that four of the 13 rats (31%) had decreases in the thickness of amygdaloid region by more than 2SD from the mean of the control animals. The perirhinal cortex was atrophic in rats with STE compared to controls (1.917 ± 0.028 vs. 2.056 ± 0.027 units, p=0.003). Two of the 13 rats (15%) had a reduction in the thickness of perirhinal cortex by more than 2SD from the control group mean. A positive correlation between the MRI and the histology measurements in the hippocampus was found in both the amygdala-piriform and perirhinal cortical regions.

Acute neuronal damage in the medial temporal lobe in the P12 STE rats was observed by histology. All animals with STE showed degenerating neurons eight hours after STE, whereas no positive labeling for neuronal degeneration was found in control animals. The

distribution and severity of damage was similar in Fluoro-Jade B and silver preparations. In the hippocampus, in either the Fluoro-Jade B or silver-stained preparations that were sampled eight hours after STE, a large number of positive degenerating neurons were found. These were commonly localized in the pyramidal cell layer of the CA1 subfield. We also found positive labeling in the granule cell layer. Only occasional were degenerating neurons detected in the hilus (2-5 labeled neurons per section). In the amygdala, the highest density of Fluoro-Jade B positive cells was found in the rostral division of the medial nucleus. Scattered labeling was also found in the lateral nucleus, the parvicellular division of the basal nucleus, the parvicellular division of the accessory basal nucleus, the periamygdaloid cortex, the anterior cortical nucleus, the other divisions of the medial nucleus, the lateral division of the amygdalohippocampal area, the posterior cortical nucleus and the nucleus of the lateral olfactory tract. Scattered Fluoro-Jade B positive neurons were also found in the adjacent piriform cortex (predominantly in layer III) that was part of the amygdala MR images. Sporadic Fluoro-Jade B and silver positive neurons were found in layers II-VI of the perirhinal cortex as well. However, despite the rather similar STE expression, the severity and distribution of histologic damage showed substantial variability between animals.

MEMRI study of mossy fiber plasticity in vivo (IV)

Epileptic rats typically developed spontaneous seizures within 2-3 weeks after KA injection and the animals showed spontaneous epileptic seizures even during general handling of the animals. T_1-weighted MRI detected severe alterations in different brain areas. These included a decrease in the thickness of temporal lobe structures (amygdala, piriform cortex, entorhinal cortex) and an enlargement of the lateral ventricles bilaterally in epileptic animals (p=0.001) due to the atrophy of surrounding tissue. Decrease in thalamic volume was not detected, however.

Timm histochemistry showed mild to moderate levels of abnormal axonal growth from the granule cell layer to the inner molecular layer of the hippocampus in epileptic animals. Significant changes between animal groups were found, both in the temporal (p=0.001 left and p=0.01 right side) and the septal end (mid, tip and crest altogether p=0.05, bilaterally).

All measured hippocampal subregions expressed obvious cell loss in all epileptic animals, and the mean neuronal damage level varied from 20-50%. Cell loss, as assessed by Nissl staining, had mean scores in hippocampal CA1 area, 1.0 in the left and 1.1 in the right side, in the hilus region 1.1 in the left and 0.9 in the right side. Hippocampal CA3a, CA3b and CA3c

subregions were measured separately and the cell loss in CA3 region in the 0-5-graded scale was 1.4 in the left and 1.5 in the right hemisphere. No correlation was seen between granule cell loss and mossy fiber sprouting.

No signs indicating the spreading of the Mn^{2+} ions were detected in MRI, one hour after the injection. However, threshold analysis in Matlab showed significant enhancement of T_1-weighted signal in the dorsal thalamus bilaterally on 3, 5 and 7 days after intracerebral $MnCl_2$ injection. Additionally, T_1-weighted MRI signal was elevated in hippocampal CA3+DG areas bilaterally on day five and only ipsilaterally on days three and seven. The CA1 area showed no significant MRI changes between the groups of epileptic and control animals. A positive correlation between the cell damage and the MRI signal intensity was observed on days 3, 5 and 7 in the ipsilateral side and on day five on contralateral side in epileptic animals. The correlation between the mossy fiber sprouting and the T_1-weighted MRI signal on days three and five was observed in the ipsilateral side and on days five and seven, in the contralateral side. Ten days after the injection there were only traces of the T_1 signal increase due to manganese, with no differences between two animal groups.

In addition, two controls and two epileptic rats were imaged also 1, 6, 12, 24 and 48 hours after $MnCl_2$ injection and they showed similar enhancements of hippocampal structures 6, 12, 24 and 48 hours later.

6. DISCUSSION

6.1. Methodological considerations

There are only a few recent animal studies described in literature combining MRI and the follow-up of the epileptogenic process in animals straight after STE (I; II; Roch et al., 2002a; Tokumitsu et al., 1997; Dube et al., 2004; Bhagat et al., 2005; Righini et al., 1994). Several established MRI methods were chosen to make our studies comparable with the others, as well as taking full advantage of the MR expertise of the A.I.Virtanen Institute. Because of this conventional MRI methods (T_2, D_{av}) were combined with new ones ($T_{1\rho}$) for imaging of the epileptic rats. Imaging time should be shortened as much as possible to reduce the possible confounding effects of anesthesia on epileptogenesis. In the present studies, total anesthesia time remained less than one hour for each animal for each MRI session.

One crucial way of determining the different phases in epileptogenesis process is obtained from the electrical activity of the brain. Simultaneous recording of EEG and MRI is extremely complicated, because there are no commercially available non-magnetic EEG electrodes for small laboratory animals (rats or mice). This is one of the main reasons why most studies using MRI do not confirm epilepsy diagnosis with EEG recordings. A common solution for this problem is the visual observation of animals on a regular basis. In the present study animals with mild and severe epilepsy were unambiguously separated. Mild disease animals can have only few seizures during a week, and it is impossible to pick those rare seizures without long-lasting EEG monitoring. We solved this issue by using slightly magnetic electrodes of tenuous platinum-iridium wire with plastic screw fixations. Using these electrodes, readable EEG signals can be obtained with only modest distortions in the MRI data. Otherwise self-made sockets cannot be used repeatedly in general, and the number of leads to animals for EEG recording system is limited.

6.2. Animal models

Experimental models of STE make an effort to mimic human STE as much as possible in both clinical and pathophysiology aspects. In addition, a study population of inbred animals can be made homogeneous with regards to genetic background, age, status epilepticus duration, follow-up and outcome measures. However, there is still substantial variability in severity and distribution of degenerating cells between animals as supported by the data of the present studies. Previous studies have reported observations that are consistent with ours

(Dube et al., 2000) even though such conclusions are often hidden in the data presented as mean values of the entire animal group. Individual variation in pathology is rarely discussed, while it may be a factor explaining some of the heterogeneity of functional outcome after STE. For example, the duration of epileptogenic period or the seizure frequency and type in animals that develop epilepsy after STE may be very different (Sankar et al., 1998; Kubova et al., 2004). The association of structural variability with the variation in functional outcome needs to be explored in future studies, by combining MRI and histology results with a long-term functional follow-up in individual animals, not only within animal groups.

It should be constant question that we should ask ourselves when we use animals to model human disease: does the model represent its human equivalent? If an animal model is comparable with a human disease, what are the limitations to translate the results and conclusions to the human disease? MRI is one of the few methods to provide data from anatomy and physiology *in vivo* from both animals and humans non-invasively. Previously, experimental animals had to be sacrificed for histology at each time point for analyzing the ongoing disease morphogenesis. MRI makes it possible to follow-up all the individuals and monitors the morphogenesis of the diseases, such as changes in the behavior of spontaneous seizures, which are characteristics of fully developed epilepsy. Chemical agents, such as KA, domoic acid and pilocarpine, model the STE and epileptogenetic changes in brain in an "unnatural way", because the pathogenic factors leading to human STE and epilepsy are totally different. Different rat strains were used in our studies because of the availibility of animals of appropriate size and age. We did not observe differences in epileptogenesis between the strains used and therefore, we are confident that the use of these rat strains did not bias our observations.

6.3. Histologically detectable changes during epileptogenesis

Severe atrophy was revealed in the amygdala, piriform cortex and some hippocampal areas in the present study. Mossy fiber sprouting was also evident in a majority of the epileptic animals (I; II). Body weight monitoring demonstrated that epileptic animals did not gain weight after STE, but at later time point, measurements confirmed that epileptic animals weighed as much as controls showing that they did not suffer from malnutrition. This is very important in the light of the observed atrophy associated with epileptogenesis, which is believed to be due to the disease process, not by trivial reasons such as feeding behavioural changes.

MRI abnormalities were correlated with tissue micro-structural alterations as assessed by histology. One of the key observations was that, in the acute phase, the extent of STE-related edema in the amygdala does not predict the long-term progression and severity of epilepsy, as defined by seizure frequency. Further, there was no correlation between MRI -detected edema on day three and neuronal damage or mossy fiber sprouting assessed eight months after STE. Recently Roch and coworkers (Roch et al., 2002b) reported that T_2-weighted MRI signal increases in both the piriform and entorhinal cortices by 24 hours, predicted the occurrence of epilepsy after pilocarpine-induced STE in 21 day old (P21)rats. However, they did not correlate the degree of tissue damage with severity of epilepsy. In the present study only animals with long-lasting STE were included in the analyses and therefore, an association of milder damage with the development of epilepsy was not observed.

The temporal profile of damage in different brain structures can reveal both characteristics of the epilepsy model in question as well as the behavior of the epileptogenic process. The amygdala was the site for the induction of STE by electrical stimulation, and because of this it became damaged bilaterally soon after STE. Furthermore, various nuclei of the amygdala have been shown to have different sensitivities to STE and the damage progress in the amygdala structures advances at various rates (Lukasiuk and Pitkanen, 2000; Jutila et al., 2002). Therefore, elevated D_{av} values in the amygdala, 2.5 months after STE, was unexpected even if the atrophy of the amygdala continued to progress during the four month follow-up period. Interestingly, hippocampal D_{av} levels recovered to normal, probably as a result of the continuing reorganization of the neuronal networks, guided by cell loss and the subsequent neurogenesis, gliosis and axonal sprouting (Jutila et al., 2002). It has been suggested that the dentate gyrus of hippocampus acts as a filtering site in the entorhinal cortex-hippocampus neuronal circuitry and its dysfunction causes damage to the hippocampus itself. This process may also contribute to development of temporal lobe epilepsy (Zhang et al., 2001).

The MRI parameters used here are dependent both on the long-lasting neuronal changes and acute ictal activity (Fabene et al., 2005; Roch et al., 2002a; Tokumitsu et al., 1997; Dube et al., 2004; Bhagat et al., 2005). To obtain reliable MRI data from tissue status, seizure activity should be avoided, at least during the hour before imaging, to differentiate tissue damage from changes brought about by seizure induced hypermetabolism and excitotoxicity. This might not be possible, because some epileptic animals have extremely high seizure activity, even up to or more than one seizure per hour. By this criterion, these animals suffering from very frequent seizures should be disqualified from the entire study, because the resulting MR parameters are not comparable with animals having lower seizure frequencies. To reduce the influence of seizures to MRI parameters we monitored animals visually before MR-imaging

at least for one hour, and if animal had generalized seizures, the imaging session was postponed. However this might not be enough because visual detection in partial seizures and video-EEG recordings should be used to explicitly exclude the possibility of ictal activity.

6.3.1. Mossy fiber sprouting

There are several hypotheses that describe a possible relationship between epileptogenesis and the phenomenon of mossy fiber sprouting. Firstly, normally mossy fibers do not project to the granule cells, but the sprouted mossy fibers make excitatory synapses to the granule cells. This process has been thought to serve as a leading anatomical substrate for the creation of a new short-circuiting network in the hippocampus, as mossy cell loss triggers the mossy fiber sprouting (Gutierrez and Heinemann, 1999; Wenzel et al., 2000a). Other scientists emphasize the role of sprouting very differently. It is claimed that surviving mossy cells become hyperexcitable and they amplify this hyperexcitable activity pattern to the granule cells (Santhakumar et al., 2000). One possibly related property is the formation of "basal dendrites", when granule cells start to grow dendrites to the opposite pole of cells (Spigelman et al., 1998; Ribak et al., 2000), receiving similar synaptic inputs as normal dendrites. Mossy cell loss removes excitatory synapses from basket cells and this may render these inhibitory interneurons hypoactive (Sloviter, 1991) and thus, modify the function of the intrahippocampal networks to which they belong (Sloviter, 1991; Bekenstein and Lothman, 1993; Bernard et al., 1998; Jefferys and Traub, 1998; Prince and Jacobs, 1998). Increased densities of sprouted mossy fibers have also been seen in electrically stimulated rats without seizures, and thus the presence of sprouting is not necessarily associated with the occurrence of spontaneous seizures but may correlate with neuronal loss (Nissinen et al., 2001). In addition, neurogenesis has thought to present a link between spontaneous seizures and the reorganization of the hippocampal network. However this might not be true since seizure-induced neurogenesis does not always lead to mossy fiber sprouting (Radley and Jacobs, 2003; Covolan et al., 2000).

Another interesting aspect of the underlying plasticity that has been recently pointed out is the so-called secondary epileptogenesis phenomenon (Morrell and de Toledo-Morrell, 1999; Sutula, 2001; Teyler et al., 2001). This concept claims that surgical removal of the epileptic focus is not enough to abrogate seizures because other brain areas can assume control for the triggering of seizures. Mossy fiber sprouting can influence the seizure genesis, not only at the cellular but also at the network level. Secondary epileptogenesis requires further scientific evidence. Our study employing the MEMRI technique may also provide functional data from the dentate gyrus besides imaging merely anatomical connections. These results can be

considered indicative, since the animal group was relatively small and heterogeneous in outcome, and we did not combine MEMRI with electrophysiological methods to detect changes in the circuitry currents in the hippocampus *in vivo*.

6.3.2. Neuronal loss

All animals (I; II) expressed spontaneous seizures by day 53 and at this time point quantitative MRI revealed hippocampal $T_{1\rho}$ and D_{av} abnormalities. There are two obvious explanations for the MRI changes in the hippocampus. Firstly, MRI changes can be associated with irreversible structural changes caused by damage to the tissue in a similar manner to the KA (Wolf et al., 2002a) or the pilocarpine (Wall et al., 2000) models. Secondly, MRI abnormalities could be caused by partially reversible changes in water homeostasis due to recurrent spontaneous seizures. To exclude the latter, all animals should have been monitored by video-EEG before MRI sessions, which is an extremely demanding exercise. Since some animals had more than one seizure per hour, the latter concern may be substantiated. In case of these animals it could also used to explain the remarkable individual variability of MRI parameters observed between the animals. Assessment of neuronal damage, eight months after STE, showed Fluoro-Jade-B -positive cells in the CA3 of the ventral hippocampus in only one of the eight rats, even though all animals had recurrent seizures. Therefore, consistent with a previous pilot study (Pitkanen, 2002), the present data supports the view that the recurrent seizures and the progressive tissue damage, with continuing loss of neurons, are not linked.

6.3.3. Epileptogenesis in the developing brain

When immature animals in experiments, an additional question arises - what is the developmental stage of animals in comparison with humans and how will it influence epileptogenesis. The data suggest that P12 rats can be used as models for epilepsy in early infancy (III, Druga et al., 2003, Kubova et al., 2002). It is, indeed, very difficult to correlate levels of maturation in rodents with those in humans. Based on timing of the "growth spurt" occurring in human babies during the last few weeks of gestation and the first few months of life, Dobbing correlated human newborns with P7-P12 rats (Dobbing, 1970). Data published describing the development of electrical activity of rat brain demonstrate that EEG activity up to P10 is interrupted by periods of electrical silence akin to the "tracé alternant" described in preterm newborns, but never seen in full-term human newborns (Ellingson, 1964). However these classifications can vary depending on the type of experimental evidence used to support the claims. For example, based on anatomical data, rat hippocampal development during the

second postnatal week seems to correspond to that evident in human infancy or early childhood (Avishai-Eliner et al., 2002). This data suggests that the P12 rats used in the present study correspond to infancy in humans. Despite the technical difficulties dealing with newborn animals, quantification of STE in P12 rats could provide valuable information from the associations of duration and severity of STE, supported by counting neurodegeneration and functional outcome follow-up (Kubova et al., 2000). Inter animal variability seen in the present study could provide a valuable "spectrum" of changes that make it possible to assess the association between the severity of damage in histology and MRI results. Comparison of the temporal profile in the development of epileptogenic alterations is still hampered by the fact of inherent differences in the life span of human and rodents.

6.4. MRI methods

6.4.1. T_2, $T_{1\rho}$ and D_{av} magnetic resonance imaging of epileptogenesis

Acute ischemic stroke is perhaps the best understood disease model in terms of the cellular damage described by MRI. Using this information, the dynamics of cellular changes in STE-induced alterations detected by MRI are being made in comparison to the alterations in acute stroke.

MRI showed increases in T_2, $T_{1\rho}$ and D_{av} values in the acute phase, two days after STE, followed by the normalization of these MRI parameters. Within the next few weeks there was a secondary increase in D_{av} and $T_{1\rho}$ values in several brain areas connected to the amygdala by at least one known pathway. Interestingly, reversal with a much shorter time constant is reported after a transient ischemic event, and an initial MRI diffusion failure is followed by normalization during the reperfusion phase and a further, secondary increase. The reversible behavior of the MRI diffusion changes after STE was unexpected, because increased diffusion is generally observed in the late irreversible phase of a stroke. The increase in the $T_{1\rho}$ value was reversible after STE as well, while in stroke, elevated $T_{1\rho}$ values have been found to correlate with irreversible damage (Grohn et al., 2000). These data suggest that the interpretation of MRI parameters in terms of the shrinkage of extracellular space and edema after STE cannot be directly adopted from stroke studies. The increase in the T_2 value in the amygdala, thalamus, and piriform cortex, by two days after STE, is consistent with previous reports of hyperintensity in T_2-weighted MRI during bicuculline (Zhong et al., 1993) or flurothyl (Zhong et al., 1995)-induced prolonged seizures, as well as days after KA (Ebisu et al., 1994) or pilocarpine-induced STE (Wall et al., 2000). In addition, prolonged hippocampal

T_2 values remained unchanged throughout the follow-up in the present study. In the KA and pilocarpine models, the increase in the hippocampal T_2–weighted signal has been detected from three to 24 hours after STE (Nakasu et al., 1995a). One possible explanation can be difference in the damage inflicted on the hippocampus in the present model relative to the KA and pilocarpine models of epilepsy. T_2 hyperintensity may reflect predominantly tissue water content (i.e. edema) in the brain structures after prolonged STE. Edema could well be a key factor explaining the MRI signal changes, both in $T_{1\rho}$ and T_2 images. $T_{1\rho}$ changes in the subacute stage, after STE, can be explained by a post-status edema together with a reorganization of the extracellular matrix. While edema formation influences both relaxation times, $T_{1\rho}$ MRI is thought to be more sensitive to factors related to the macromolecular pool (Brown and Koenig, 1992), and alterations in the macromolecular content of the tissues affected might contribute to the subacute changes in $T_{1\rho}$ in epilepsy as well.

Several previous studies have reported reduced water diffusion during the first few hours after STE induction (Zhong et al., 1993; Nakasu et al., 1995a) in brain areas including the amygdala, piriform cortex or thalamus. There are MRI findings similar to that of diffusion during acute stroke and they are likely to be associated with a change in water compartmentalization and energy imbalance (Hrabetova et al., 2002; Kuzniecky and Jackson, 2005). Rats were imaged, starting on day two after STE, while D_{av} was already elevated in the stimulated amygdala, but not in brain areas connected to this organ. Increased ADC at this time point is opposite in sign to the one reported from acute seizures (Righini et al., 1994) or spreading depression showing decreased diffusion (de Crespigny et al., 1998). A diffusion increase was observed only in brain structures developing the most severe damage over time. It may be that during acute STE these structures undergo severe acute energy failure, a tissue status resembling that in cerebral ischemia (Knight et al., 1994).

It is important to note that the normalization of either diffusion or relaxation times in MR images should not be interpreted as signs for good long-term prognosis of the tissue after seizures. Recovery of MRI parameters might indicate different biochemical and cellular events which precede inevitable tissue damage, such as gliosis and other epilepsy related cellular alterations (Bouilleret et al., 2000; Roch et al., 2002b).

In the present models, a massive amygdaloid atrophy was associated with robust changes of several MRI parameters in the early phase of epileptogenesis. Conversely, hippocampal D_{av} values were normal during the subacute phase, but elevated bilaterally approximately two months after STE. Hippocampal $T_{1\rho}$ values remained increased for about two month's

duration of the follow-up. Recently, Roch and colleagues (Roch et al., 2002b) reported a progressive increase in the hippocampal T_2 value, starting 2 days after pilocarpine-induced STE and lasting for up to nine weeks. Despite of differences in the models used, the changes in the hippocampal MRI were both delayed and progressive. In the present model, pyramidal cell loss in hippocampus was mild during the acute phase, and continued to progress for approximately two months (Lukasiuk and Pitkanen, 2000), an observation consistent with the present MRI data. As the present study indicates, hippocampal degeneration can last for up to eight months and may be longer in rat. This process could translate to tens of years of decay in human epilepsy patients.

6.4.2. MEMRI of mossy fiber sprouting

Several researchers have studied Mn^{2+} uptake into the brain and suggested that Mn^{2+} cannot move across the BBB (Aschner and Aschner, 1990; Aschner and Gannon, 1994; Murphy et al., 1991; Rabin et al., 1993; Lee et al., 2005). To avoid the need for chemical disruption of blood-brain barrier and to obtain more detailed information from perforant pathway alterations, intracerebral injections into the entorhinal cortex appears to give a better contrast despite surgery-related minor focal brain injury. Sprouting is histologically demonstrable owing to the fact that mossy fibers contain high concentrations of zinc in hippocampus, so that stains for heavy metals, such as the Timm method, can distinguish the granule cell axons well (Scharfman, 2002). We observed that all areas showed mossy fiber sprouting, including the dentate gyrus, CA3 and CA1 area (Scharfman, 2002). Sprouted axons take up Mn^{2+} and sprouting fibers can be labeled also in other brain structures such as neocortex (Scharfman, 2002), which is easy to visualize by MEMRI. MEMRI highlighted hippocampus in the scans was acquired six hours after surgery but one hour after the operation, MEMRI contrast was presented only in the injection site and not yet in the hippocampus. It has been previously estimated that Mn^{2+} ions travel at the speed of 2.8mm/h in the visual cortex pathway (Watanabe et al., 2001). Our observations agree with this estimate for the transport rate in a pathway between the entorhinal cortex and the hippocampus. The present data also show a correlation between the MEMRI signal and the histologically detected mossy fiber sprouting earlier (by three and five days) in the ipsilateral versus the contralateral hippocampus (by five and seven days). These results can be explained by the shorter distance the Mn^{2+} ions have to travel. By ten days after the $MnCl_2$ injection MEMRI signal was hardly noticeable in any parts of the brain.

We observed strong enhancement of MEMRI contrast in the dorsal thalamus in the KA treated rats. There is no present evidence for direct network connection between thalamus and

entorhinal cortex. Therefore, we believe that the thalamus MEMRI signal is due to the diffusion of Mn^{2+} directly after the injection, reflecting the activation of the postsubiculum-thalamus pathway (van Groen and Wyss, 1990; Shibata, 1996). Some nuclei of thalamus have been shown to be very sensitive to STE, expressing immediate breakdown and leakage of the blood-brain barrier (Roch et al., 2002a). Here we observed a substantial loss of volume in several temporal lobe structures such as the amygdala and the piriform- and entorhinal cortices, but no evidence for a decrease in thalamic volumes (Dreifuss et al., 2001) during the three weeks after KA induced STE. It may be that the thalamus should be a subject to research in epilepsy. It has been suggested that increased thalamic synaptic activities could mediate antiseizure effects (Henry et al., 1999). For example, in one form of epilepsy therapy, namely vagal nerve stimulation, seizure frequency decreased enormously during the nerve stimulation, and it increased right and left thalamic CBF, correlating with a decline in seizures (Henry et al., 1999). Neuronal affinity for manganese uptake can be affected by altered tissue metabolism, as epilepsy patients suffering from seizures usually have lower Mn^{2+} concentrations in their blood, compared to the healthy population (Dupont and Tanaka, 1985; Papavasilious et al., 1979) and this has not been taken into account in experimental epilepsy models using MEMRI. Thalamus enhancement may reflect the activation of individual thalamic nuclei during epileptogenesis. Therefore, uptake of Mn^{2+} by the thalamus after STE make it a new target for MEMRI and this phenomenon should be studied further.

7. SUMMARY AND CONCLUSIONS

The present MRI study demonstrates that the neurodegeneration process continues along with plasticity changes long after the STE-associated seizure activity. Cellular and network reorganization, exemplified by mossy fiber sprouting, gliosis and neurogenesis in the subacute and chronic phase of epileptogenesis, interfaced with a poor outcome in histology, are not correlated with multiparametric MRI changes observed in the acute phase.

The temporal profiles of MRI changes in different brain structures indicate that some MRI parameters of amygdala can recover from the initial insult, and be retained progressive atrophy development over the time. However, increased $T_{1\rho}$ and D_{av} values at subsequent time points indicate a progression in the tissue damage. Epileptogenesis involves the hippocampus later on in the disease process. In fact, the MRI data suggest that cellular alterations in these structures do not precede parallel to clinical manifestations of TLE and tissue alterations detected by MRI during epileptogenesis might not predict the severity of the tissue outcome in fully expressed epilepsy.

STE at P12 in rats leads directly to neurodegeneration in the medial temporal lobe structures. Hippocampi and amygdaloid complexes possess a focal nature in the alterations, not a cell layer specific manner as could be expected from histology analysis of adult epileptic human tissues. The present data strengthen the view that even though STE induced in P12 animals leads to neurodegeneration, factors other than neuronal cell death may play a significant role in the development of brain atrophy and epileptogenesis.

Neuronal tract tracing methods, such as MEMRI, are able to detect mossy fiber sprouting in epilepsy, including the reorganization of mossy fiber pathways in the hippocampus before spontaneous seizures appeared. Furthermore, MEMRI can be regarded as a method revealing surrogate marker for ongoing epileptogenesis in experimental animal models of epilepsy. Our data provide the first *in vivo* evidence arguing that target cells of the postsubiculum–thalamus pathway may undergo reorganization during epileptogenesis. MEMRI as a tract tracing method should be viewed as a promising tool to be employed for microimaging studies dealing with neuronal network connections in epilepsy animal models.

Different NMR methods have been shown to reflect sensitively different aspects of tissue water environment changes and their association with both STE and chronic epilepsy. Thus,

studying epileptogenesis and epilepsy with animal models by using NMR imaging techniques should be encouraged in the future.

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