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Long-term studies of epileptogenesis after focal cerebral ischemia in rats

Doctoral dissertation

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ABSTRACT

Stroke represents one major cause of epilepsy. The underlying mechanisms of epileptogenesis, however, are not known. Valid animal models are needed if one wishes to clarify which factors associate with epileptogenesis after focal cerebral ischemia. The present study aimed to investigate the occurrence of post-stroke epilepsy in rats using the transient intraluminal filament model of middle cerebral artery occlusion (filament model of MCAo), the endothelin-1 induced middle cerebral artery occlusion (ET-induced MCAo) and the cortical photothrombosis with Rose Bengal (photothrombosis). Video-EEG was intermittently recorded up to 12 months after ischemia induction to monitor the occurrence of seizures. In addition, sensorimotor and learning and memory performance of the rats were assessed. At the end of the follow-ups, the association between late seizures and hippocampal cell loss and aberrant mossy fiber sprouting was investigated. No electrographic seizures were observed after the filament model of MCAo, but after ET-induced MCAo one rat out of 26 had seizures and after the photothrombosis 7 rats out of 36 experienced convulsions. After the ET-induced MCAo, the epileptic rat did not develop aberrant mossy fiber sprouting in the dentate gyrus of the hippocampus. After the photothrombosis, the hilar cell number did not differ between rats with and without seizures, but the occurrence of seizures was associated with slightly denser mossy fiber sprouting when compared to non-epileptic animals. The behavioral data could not be compared between rats with and without late seizures due to the small number of rats with epilepsy in the groups. In summary, the development of epilepsy was a rare occurrence in two models of large artery occlusion. In contrast, cortical thrombotic small vessel occlusion produced the highest percentage of rats with epilepsy. Following the cortical photothrombosis, the epileptogenesis was associated with changes in the hippocampus in addition to changes in the primary lesion site. In conclusion, the cortical photothrombosis with Rose Bengal dye in rats seems to be suitable for use in studies of epileptogenesis after small cortical thrombotic lesions.

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ABBREVIATIONS

ACA	anterior cerebral artery
AED	antiepileptic drug
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CCA	common carotid artery
CA1	CA1 subfield of the hippocampus
CA2	CA2 subfield of the hippocampus
CA3	CA3 subfield of the hippocampus
ECA	external carotid artery
EEG	electroencephalography
ET	endothelin-1
GABA	gamma-aminobutyric acid
ICA	internal carotid artery
MCA	middle cerebral artery
MCAo	occlusion of the middle cerebral artery
NMDA	N-methyl-D-aspartate
video-EEG	video-electroencephalography

LIST OF ORIGINAL PUBLICATIONS

This work is based on four original articles which are referred to by Roman numerals I-IV in the following text.

I **Karhunen H**, Pitkänen A, Virtanen T, Gureviciene I, Pussinen R, Ylinen A, Sivenius J, Nissinen J, Jolkkonen J. Long-term functional consequences of transient occlusion of the middle cerebral artery in rats - A 1-Y follow-up of the development of epileptogenesis and memory impairment. Epilepsy Research 2003;54(1):1-10.

II **Karhunen H**, Nissinen J, Sivenius J, Jolkkonen J, Pitkänen A. A long-term video-EEG and behavioral follow-up after endothelin-1 induced middle cerebral artery occlusion in rats. Epilepsy Research 2006;72(1):25-38.

III **Karhunen H**, Bezvenyuk Z, Nissinen J, Sivenius J, Jolkkonen J, Pitkänen A. Epileptogenesis after cortical photothrombotic brain lesion in rats. Neuroscience, in press.

IV **Karhunen H**, Sivenius J, Jolkkonen J, Pitkänen A. Epileptogenesis after experimental focal cerebral ischemia. Neurochemical Research 2005;30(12): 1529-1542.

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1. INTRODUCTION

Stroke represents an underlying cause of recurrent seizures in 30% of the symptomatic epilepsy cases (Hauser, 1992). Furthermore, the incidence of post-stroke epilepsy is on the increase as the population ages. It is believed that the major factors associated with the development of post-stroke seizures and epilepsy are the size and the cortical location of the lesion and the hemorrhagic involvement (Gupta et al., 1988; Faught et al., 1989; Ryglewicz et al., 1990; Kotila and Waltimo, 1992; Giroud et al., 1994; So et al., 1996; Arboix et al., 1997; Burn et al., 1997; Paolucci et al., 1997; Bladin et al., 2000; Bentes et al., 2001; Labovitz et al., 2001; Cheung et al., 2003; Lamy et al., 2003; Vespa et al, 2003; De Reuck et al., 2006a). Although stroke can be viewed as a precipitating insult for epilepsy, the underlying mechanisms are still far from clear.

Valid animal models are needed if one wishes to investigate the factors that have effect on epileptogenesis after stroke. Confounding factors that are commonly observed in stroke patients, such as medication, can be eliminated when using animal models (Zorowitz et al., 2005a; Zorowitz et al., 2005b; Zorowitz et al., 2005c). In addition, the underlying mechanisms of epileptogenesis after cerebral ischemia can be investigated from the initial insult to the epileptogenesis phase and ultimately to recurrent seizures (Pitkänen and Sutula, 2002). Further, new treatment options for post-stroke seizures can be tested before clinical trials and the effect of antiepileptic drug (AED) treatment on the recovery process can be studied.

Previous experimental trials have shown that early onset seizures can be observed after both global and focal cerebral ischemia (Kudo et al., 1982; Truong et al., 1994; Reid et al., 1996; Uchino et al., 1996; Krugers et al., 2000; Lu et al., 2001; Wang et al., 2001; Shuaib et al., 2002; Williams and Tortella, 2002; Hartings et al., 2003; Williams et al., 2004a). Further, half of the young adult animals may develop epilepsy after a cortical photothrombotic lesion (Kelly et al., 2001; Kharlamov et al., 2003). In contrast, it seems that the development of epilepsy is absent or at least rare following the combined occlusion of the middle cerebral and common carotid arteries (Kelly et al., 2006). Despite the available animal models of stroke, it still remains to be elucidated how the development of epilepsy associates with the brain pathology and behavioral recovery after cerebral ischemia.

The present study aimed to investigate the occurrence of post-stroke epilepsy in rats using the transient intraluminal filament model of the middle cerebral artery occlusion (MCAo), endothelin-1 (ET) induced MCAo and cortical photothrombosis with Rose Bengal dye. The occurrence of seizures was monitored intermittently with video-electroencephalography (video-EEG) for up to 12 months. The association with brain pathology and spontaneous late seizures was investigated. Furthermore, one aim was to study whether sensorimotor or learning and memory performance associates with epileptogenesis after focal cerebral ischemia.

2. REVIEW OF THE LITERATURE

2.1 Cerebral stroke - the initial insult

It is estimated that 1.3% of the population in Western countries has experienced a cerebral stroke, i.e. a sudden loss of neurologic function due to a vascular incident (Wolf et al., 1998). Stroke imposes a major burden on society; it accounts for 2-4% of health care costs (Bergman et al., 1995; Evers et al., 1997). The major components of the life-time costs include the expenses of acute hospitalization, rehabilitation and nursing home care (Dewey et al., 2001). This is due to the fact that even though about two thirds of the surviving stroke patients recover to be independent in their daily activities, the rest will need either assistance or institutional care (Miyai and Reding, 1998). In addition to the disabling state, stroke is often accompanied by other sequelae such as epileptic seizures, fractures, cognitive impairment and depression (Kotila and Waltimo, 1992; Censori et al., 1996; So et al., 1996; Bladin et al., 2000; Lossius et al., 2002; Cheung et al., 2003; Whitson et al., 2006).

2.2 Post-stroke seizures and epilepsy

An epileptic seizure is an unpredictable and transient interruption of normal brain function due to an abnormally synchronous and excessive firing of neurons (Fisher et al., 2005). Seizures can be divided into partial seizures and generalized seizures according to whether they are restricted to one hemisphere or whether they involve both hemispheres from the onset, respectively (Engel et al., 2001). Seizures occurring within the first day after the brain insult are considered provoked, irrespective of whether the patient develops epilepsy or not.

Epilepsy is the collective name for a diverse set of disorders of the brain that have an abnormal predisposition to epileptic seizures (Fisher et al., 2005). The definition of epilepsy includes the requirement of at least one seizure and the presence of an enduring alteration in the brain (Fisher et al., 2005). Epilepsy syndromes or types of epilepsy can be classified into three groups. In the idiopathic epilepsy syndromes, the epilepsy exists without an underlying lesion or without any known brain disorder (Engel et al., 2001). They are believed to be genetically determined. The present study aimed to model symptomatic epilepsy which is due to a disorder or a lesion in the brain (Engel et al., 2001). In the elderly, the most commonly identified cause of symptomatic epilepsy is cerebrovascular disease (Hauser, 1992). In

presumed symptomatic (cryptogenic) epilepsy, some acquired cause is thought to underlie the epilepsy, but this cannot be identified with current methods (Engel et al., 2001).

2.2.1 Occurrence of seizures after stroke

After an ischemic stroke, seizure occurrence exhibits a bimodal distribution (Sung and Chu, 1990; Bladin et al., 2000). The first peak in seizure occurrence is within 2 weeks and the second peak is between 6 to 12 months after the stroke (Sung and Chu, 1990; Bladin et al., 2000). About 40% of all epileptic seizures that are observed during the mean follow-up time of 9 months occur within the first day after the ischemic stroke and more than 20% of all seizures between 6 to 12 months (Bladin et al., 2000). In contrast, after intracerebral haemorrhage, 60% of the seizures occur during the first 24 hours and after that time, the seizure occurrence declines (Bladin et al., 2000).

The percentages of patients suffering seizures and epilepsy have varied due to differences in data collection, patient inclusion criteria and follow-up time (Table 1). To briefly summarize, seizures have been reported to occur from 3% to 15% and epilepsy from 3% to 14% of the patients with ischemic stroke in those studies with long follow-up times (Kotila and Waltimo, 1992; Paolucci et al., 1997; Bladin et al., 2000; Cheung et al., 2003). After hemorrhage, between 4% to 29% of the patients have been described as experiencing seizures and from 3% to 15% develop epilepsy (Kotila and Waltimo, 1992; Paolucci et al., 1997; Bladin et al., 2000; Cheung et al., 2003).

2.2.2 Factors associated with the occurrence of post-stroke seizures and epilepsy

An almost two-fold increased risk of seizures is observed in patients with hemorrhagic stroke when compared with ischemic stroke patients (Bladin et al., 2000). In addition, it is believed that the two most important factors associated with seizures and epilepsy after stroke are the large size and cortical location of the lesion (Table 2; Gupta et al., 1988; Faught et al., 1989; Ryglewicz et al., 1990; Giroud et al., 1994; So et al., 1996; Arboix et al., 1997; Burn et al., 1997; Bladin et al., 2000; Bentes et al., 2001; Labovitz et al., 2001; Cheung et al., 2003; Lamy et al., 2003; Vespa et al, 2003; De Reuck et al., 2006a). The majority of patients with post-stroke seizures have a lesion in the cerebral cortex with or without the involvement of the subcortical regions (Bladin et al., 2000, Dhanuka et al., 2001; Lossius et al., 2002).

TABLE 1. Clinical studies investigating post-stroke seizures and epilepsy.

Ref.	Data collection	Patient Inclusion	Follow-up	Patients (n)	Mean age (y)	Males	Seizures	Epilepsy
Faught et al., 1989	One hospital	First-ever ICH	mean 4.6 y	123	61.8	46%	25.2%	-
Sung and Chu, 1990	One hospital	IBI (cerebral thrombosis)	20 mo for early sz and 22 mo for late sz	118 (76 with complete data)	63.0	61%	5% prior stroke; 28% within 1 mo; 68% within 1 y; 86% within 2 y	-
Kotila and Waltimo, 1992	One department	IBI, ICH or SAH and ambulatory rehabilitation needs	2.7 - 52.9 mo (mean 39.9 mo)	200 total; 157 IBI; 20 ICH; 23 SAH	52.3	74%	-	17% total; 14% IBI; 15% ICH; 35% SAH
Giroud et al., 1994	Population-based stroke registry	IBI, ICH, SAH or TIA with CT scan	0-15 d	1640 total; 1213 IBI; 168 ICH; 259 TIA	73.0	53%	5.5% total; 4.9% IBI; 14.8% ICH; 2% TIA	-
So et al., 1996	Population-based medical registry	IBI	5.5 y	535	71.6	52%	6.2% had sz on 0-7 d; 6.2% had sz after >7d	4.1 %
Arboix et al., 1997	Population-based stroke registry	First-ever IBI, ICH, SAH, TIA or sub-/epidural hematomas	0-48 h	1220 total; 1012 IBI; 81 ICH; 127 TIA	69.5	52%	2.4% total; 2% IBI; 4% ICH 1.6% TIA	-
Burn et al., 1997	Prospective, community-based stroke registry	First-ever IBI, ICH or SAH	> 2 y	675 total; 545 IBI; 66 ICH; 33 SAH; 31 ?	72.2	47%	7.7% total; 6% IBI; 11% ICH; 18% SAH; 0% ?	-
Paolucci et al., 1997	Rehabilitation unit	First-ever IBI or ICH with physical rehabilitation needs and age ≤79 y	1.0 y	306 total; 247 IBI; 59 ICH	63.6	50%	15.0% total; 14.6% IBI; 28.6%ICH	-
Reith et al., 1997	Prospective, community-based (one department)	IBI or ICH	0-14 d	1195 total; 900 IBI 75 ICH 220?	76.6 with sz; 74.2 without sz	46%	4.2% total; 11% IBI on CT; 17% ICH on CT	-
Bladin et al., 2000	Prospective, multicenter, international	IBI or ICH	mean 9 mo	1897 total; 1632 IBI; 265 ICH	72.0	58%	9% total; 8.6% IBI; 10.6% ICH	2.5% total; 2.5% IBI; 2.6% ICH
Bentes et al., 2001	Hospital-based stroke registry	First-ever subcortical IBI	> 1 y	113	57.0	73%	3.5%	-
Dhanuka et al., 2001	Prospective, one department	IBI or ICH and diagnosis of post-stroke sz	3-60 mo (mean 15.9 mo)	35 total; 20 IBI 15 ICH	45.4	57%	77% had early sz; 23% had late sz	11% total; - IBI; - ICH

TABLE 1. Clinical studies investigating post-stroke seizures and epilepsy (continued).

Ref.	Data collection	Patient inclusion	Follow-up	Patients (n)	Mean age (y)	Males	Seizures	Epilepsy
Labovitz et al., 2001	Population-based stroke registry	IBI, ICH or SAH	sz before and/or 0-60 d	904 total; 704 IBI; 150 ICH; 50 SAH	68.3	44%	4.1% total; 3.1% IBI; 7.3% ICH; 8.0% SAH	-
Velioglu et al., 2001	One hospital	First-ever IBI or ICH	mean 3.7 y (with sz)	1174 total; - IBI; - ICH	59.7	58%	15.3% total; - IBI; - ICH	-
Leys et al., 2002	Prospective, one stroke unit	IBI or IBI + h. and age 15 - 45 y	median 3 y	287 total; 282 IBI; 5 IBI + h.	36.0	55%	6.6% total; - IBI; - IBI + h.	-
Lossius et al., 2002	One hospital	IBI or ICH and age ≥60 y and in hospital within 24 h of onset of symptoms	0-12 mo	472 (1.5% with prior epilepsy)	75.0 with PSE; 76.0 without PSE	51%	3.4% total; - IBI; - ICH	2.5% total; - IBI; - ICH
Cheung et al., 2003	Population-based stroke registry	IBI, ICH or SAH	1 y	994 total; 752 IBI; 242 ICH+SAH	70.7	54%	3.4% total; 3.3% IBI; 3.7% ICH+SAH	0.7% total; - IBI; - ICH+SAH
Lamy et al., 2003	Prospective, multicenter	IBI (≤ 3 mo) and age 18 - 55 y	37.8 ± 9.7 mo	581 (1.2% with prior epilepsy)	42.5	57%	2.4% had sz on 0-7 d; 3.4% had sz after >7d	1.9% total; 55% of patients with sz after >7d
Varona et al., 2004	Stroke registry	First-ever IBI and age 15-45 y	11.7 y	272	36.6	65%	10%	-
Lossius et al., 2005	One hospital	IBI and age ≥60 y and in hospital within 24 h of onset of symptoms	range 14-96 mo	484	74.3 with PSE; 76.3 without PSE	52%	5.7%	3.1%
Benbir et al., 2006	Stroke unit	IBI or ICH and no prior epilepsy	mean 5.5 ± 2.4 y	1428 total; 1327 IBI; 86 ICH; 15 VI and IDST	62.8	59%	-	3.6% total; 2.7% IBI; 12.8% ICH; 26.6% VI and IDST
Sylaja et al., 2006	One hospital	IBI and CT within 24 h	90 d	326	- (median 73.0)	-	1.5% had sz at onset of stroke	-

Abbreviations: -, data not available/shown; ?, unknown cause; h, hemorrhagic changes; IBI, ischemic brain infarct; ICH, intracerebral hemorrhage; IDST, intracranial dural sinus thrombosis; PSE, post-stroke epilepsy; SAH, subarachnoid hemorrhage; sz, seizure; TIA, transient ischemic attack; total, all cases included; VI, venous infarction.

TABLE 2. Factors associated with post-stroke seizures and epilepsy in clinical studies.

Reference	Early seizures	Late seizures	Seizures	Epilepsy
Ischemic	-	-	-	lesion in ctx and subcortical structures
Gupta et al., 1988				
Giroud et al., 1994	emboligenic cardiac condition	-	-	-
So et al., 1996	anterior lesion location*; embolus	recurrent stroke*; early onset of seizures*	-	recurrent stroke*; early onset of seizures*
Arboix et al., 1997	lesion size; lesion in ctx*; hemorrhagic involvement; younger age; acute confusional state*	-	-	-
Burn et al., 1997	-	-	anterior lesion location	-
Bladin et al., 2000	-	-	lesion in ctx* or striatum; disability*	late onset of seizures*
Bentes et al., 2001	lesion in the striatum; emboligenic cardiac condition	-	-	-
Lamy et al., 2003	lesion in ctx*	lesion size*, lesion in ctx*; hemorrhagic involvement; disability*; loss of consciousness; early onset of seizures*	-	-
Cheoung et al., 2003	-	-	lesion in ctx*; anterior lesion location	-
De Reuck et al., 2006a	-	lesion size	-	-
Hemorrhagic	-	-	lesion in ctx or caudate nucleus	-
Faught et al., 1989				
Giroud et al., 1994	lesion in ctx	-	-	-
Bladin et al., 2000	-	-	lesion in ctx*	-
Cheoung et al., 2003	-	-	lesion in ctx*	-
Vespa et al., 2003	lesion in ctx	-	-	-

TABLE 2. Factors associated with post-stroke seizures and epilepsy in clinical studies (continued).

Ref.	Early seizures	Late seizures	Seizures	Epilepsy
All cases Ryglewicz et al., 1990	-	-	-	lesion size; lesion in ctx
Kotila & Waltimo, 1992	-	-	-	hemorrhage; female gender
Giroud et al., 1994	lesion in ctx; hemorrhage; male gender; loss of consciousness	-	-	-
Burn et al., 1997	-	-	hemorrhage; early seizures	-
Paolucci et al., 1997	hemorrhage*; younger age*; disability*	hemorrhage*, disability		
Reith et al., 1997	emboligenic cardiac condition; disability*	-	-	-
Bladin et al., 2000			hemorrhage*	late seizures*
Dhanuka et al., 2001	-	-	-	late seizures
Labovitz et al., 2001	disability	-	-	-
Lossius et al., 2002	-	-	-	disability*
Cheoung et al., 2003	-	-	lesion size; lesion in ctx; age >65 y; male gender*	-
Cordonnier et al., 2005	disability*; pre-existing dementia	-	-	-
Benbir et al., 2006	-	-	-	hemorrhage, venous infarctions

Abbreviations: *, result from multivariate analysis; anterior, lesion location in the anterior circulation territory; all cases, ischemic and hemorrhagic stroke cases combined; ctx, cortex; early seizures, seizures that occur 0-2 wk after stroke; late seizures, seizures that occur more than 2 wk after stroke; seizures, early and late seizures combined.

2.2.3 Neurological deficits and post-stroke seizures and epilepsy

The clinical severity of stroke at admission to hospital can predict the occurrence of early onset seizures, late onset seizures and the development of epilepsy (Paolucci et al., 1997; Reith et al., 1997; Lossius et al., 2002; Lamy et al., 2003). Further, seizures are observed in 6% of young ischemic stroke patients, who do not need help in their daily activities (Leys et al., 2002). In comparison, more than 13% of the patients develop seizures when they are dependent in their daily activities (Leys et al., 2002). Poor functional ability is

also an identified predictor of the appearance of status epilepticus after stroke (Velioglu et al., 2001).

A seizure at stroke onset need not have any influence on the ultimate outcome of the patients (Burn et al., 1997; Reith et al., 1997). In contrast, when the initial post-stroke seizure appears more than 2 wk after the initial insult, this may be followed by a transient or even a permanent worsening of the neurological deficit (Bogousslavsky et al., 1992; Lamy et al., 2003; Vespa et al., 2003; De Reuck et al., 2006a). After status epilepticus, a neurological deterioration is observed in 48% of the stroke patients and further, this deterioration is permanent in 6% of these cases (Rumbach et al., 2000). However, negative results have also been obtained in a study with patients needing rehabilitation since the occurrence of seizures more than 2 weeks after the stroke did not have any effect on the rehabilitation or functional outcome (Paolucci et al., 1997).

The worsening of the neurological deficit after late post-stroke seizures may be associated with partial seizures of longer duration or multiple partial seizures with secondary generalization (Bogousslavsky et al., 1992; Lamy et al., 2003). A new cerebral lesion or an extension of the existing lesion might underlie the phenomenon of a worsening of the neurological deficit after late post-stroke seizures (De Reuck et al., 2006a), although not all studies have found this association (Bogousslavsky et al., 1992; Lamy et al., 2003). The question still remains whether the extension of a lesion is due to a new vascular incident or whether it is secondary to a seizure-induced damage (De Reuck et al., 2006a).

In addition to seizures, AED treatment after stroke can also have an influence on the recovery (Goldstein, 1993; Goldstein, 1998). Interestingly, AEDs are prescribed for stroke patients for other causes in addition to seizure therapy. About 68% of stroke patients receive pain medication and more than 98% of them are treated with AEDs, such as gabapentin, clonazepam, carbamazepine and levetiracetam (Zorowitz et al., 2005a).

2.3 Seizures, epilepsy and recovery after brain ischemia in rats

2.3.1 Seizures and epilepsy after large artery occlusions

The occurrence of early and late seizures has been described after the intraluminal filament model of MCAo and after the combined occlusion of MCA and common carotid artery (CCA). Briefly, in the intraluminal filament model of MCAo, a filament is introduced into the internal carotid artery (ICA) and advanced until it blocks the blood flow to the MCA

(Longa et al., 1989). In permanent occlusion, the filament is left permanently in the artery. In transient occlusion, the filament is retracted to permit the reperfusion. In another large artery occlusion model, the blood flow is blocked in MCA distally to the lenticulostriate arteries through a burr hole made in the skull. A concomitant occlusion of the ipsilateral common carotid artery (CCA) is induced via a neck incision (Aronowski et al., 1997).

Early seizures occur after transient and permanent MCAo with the intraluminal filament model in rats (Lu et al., 2001; Hartings et al., 2003; Williams and Tortella 2002; Williams et al., 2004a). After transient MCAo, about 80% of the rats demonstrate non-convulsive seizures, which occur approximately 25 min following the occlusion and last about 2 minutes (Williams and Tortella, 2002). After the permanent MCAo, about 90% of the rats demonstrate non-convulsive seizures, which appear approximately 30 to 60 minutes following the occlusion and last, on average, for 50 to 70 seconds (Williams et al., 2004a; Williams et al., 2006). The majority of the early seizures occur during the first 2 hours after the MCA occlusion in rats (Hartings et al., 2003; Williams et al., 2004a).

The occurrence of late seizures after large artery occlusions seems to depend on the age of the rats at the ischemic induction. In 2.5-months old Long-Evans rats, the combined occlusion of the MCA and CCA does not result in late epileptic seizures during the 6-month long follow-up period (Kelly et al., 2006). In contrast, 25% of 4-month old rats and 100% of 20-month old F344 rats exhibit epileptic seizures (Kelly, 2006). An estimate for the latency period between ischemia induction and seizure occurrence is between 2 to 4 weeks (Kelly, 2006). The duration varies according to the seizure type; i.e. forelimb clonus lasts from 5 to 20 seconds and forelimb clonus and rearing for about 1 minute (Kelly, 2006). There is one estimate of the seizure frequency in 20-month old rats of about one to two forelimb clonus and rearing-type seizures per week (Kelly, 2006).

2.3.2 Seizures and epilepsy after cortical small vessel occlusion

In the photothrombosis model, intravenously injected Rose Bengal dye is activated through the skull by a light to produce a cortical lesion in the desired location (Watson et al., 1985). Although the occurrence of early seizures has not been described in the cortical photothrombosis model, the occurrence of late seizures has been studied in more detail. In 2-month old rats, photothrombosis evoked late seizures in about 50% of the animals (Kharlamov et al., 2003). The duration of late seizures was reported to range from 2 to 3

seconds (Kharlamov et al., 2003). The most common behavioral change seen during a seizure was motor arrest (Kharlamov et al., 2003). Late seizures were observed as early as the first recording day, which was 26 days after the cortical lesion had been made with photothrombosis (Kharlamov et al., 2003). The latency time between photothrombosis and seizure, however, can also be greatly delayed, these workers observed seizures occurring 181 days after the operation (Kharlamov et al., 2003).

2.3.3 Seizures in other models of cerebral ischemia

In the focal cerebral ischemia model with embolus, a clot is introduced into an artery in which it traverses along and blocks the blood flow (Kudo et al., 1982). Early seizures are observed within 2 hours in about 20% of the rats with the embolization of the CCA (Kudo et al., 1982; Wang et al., 2001; Shuaib et al. 2002). Two days after the embolization in the rats, the occurrence of early seizures declines to 10% (Shuaib et al., 2002). The behavioral manifestations of seizures range from non-convulsive seizures to rearing and falling (Kudo et al., 1982; Wang et al., 2001; Shuaib et al., 2002).

In addition to that seen with focal brain ischemia, global ischemia can also induce early seizures. In Levine's model, hypoxia-ischemia is induced by temporary unilateral carotid artery ligation in combination with hypoxic ventilation (Krugers et al., 2000). Seizures occur in about 40% of the animals during the 24 hours after the operation (Krugers et al., 2000). These seizures have durations of less than 30 seconds (Krugers et al., 2000). The seizures are characterized by spinning around the body axis, jerking movements, and clonic contractions of the paws (Krugers et al., 2000). In the 2-vessel model of global brain ischemia, the occlusion of both common carotid arteries is combined with hypotension (Uchino et al., 1996). Although no clinical signs of seizures are observed after 10 minutes of 2-vessel occlusion, electrographic seizures can be observed one hour after the recirculation in 86% of animals, after 3 hours in 57% of the animals and still after 16-18 hours in some animals (Uchino et al., 1996). In the 4-vessel model of global brain ischemia, electrocoagulation of vertebral arteries is combined with clamping of the carotid arteries 24 hours later (Moldovan et al., 2004). Following 3 or 10 minutes of ischemia, no clinical signs of seizure activity are observed (Moldovan et al., 2004). In contrast, after 30 minutes period of ischemia with the 4-vessel occlusion, seizures can be detected in 20% of the rats at 24 hours and in 40% at 72 hours (Ginsberg and Busto, 1998).

Global cerebral ischemia can be also produced with models that have been developed to study cardiac arrest. In these models, however, the seizures occur after various stimulations, usually not spontaneously (Siemkowicz and Hansen, 1978; Truong et al., 1994; Reid et al., 1996). For example, in a chest compression model, about 3% of the rats develop seizures elicited by human presence, cage movement or other minor stimuli and 58% exhibit sound-induced seizures (Reid et al., 1996). Sound-induced seizures, however, seem to continue for at least up to 5 months after the chest compression (Reid et al., 1996).

In addition to adult animals, the occurrence of seizures and epilepsy has been described in immature rats subjected to either focal or global cerebral ischemia. Recently, it was reported that injection of ET into the hippocampus results in the appearance of early seizures in 75-100% of the pups that are 12- to 25-days old at the time of ischemia induction (Mateffyova et al., 2006). Three months after the operation, non-convulsive seizures are observed in 63-100% of the rats (Mateffyova et al., 2006). After Levine's model of hypoxia-ischemia, 40% of the 7-day old rats develop spontaneous motor seizures (Williams et al., 2004b). The mean latency time from the operation to spontaneous motor seizures is 194 days and the animals exhibit one seizure every 4 to 5 days (Williams et al., 2004b).

To summarize, various focal and global cerebral ischemia models induce early seizures within the first two days (Uchino et al., 1996; Krugers et al., 2000; Shuaib et al. 2002; Moldovan et al., 2004). The development of epilepsy, however, has been described in far fewer models. In neonate rats, either a focal lesion in the hippocampus or global cerebral ischemia can induce epilepsy in later life (Williams et al., 2004b; Mateffyova et al., 2006).

2.3.4 Behavioral recovery and seizures after brain insults

Rats possess a great capacity to recover from a brain injury as assessed with various different behavioral tests (Corbett and Nurse, 1998). It has been suggested that seizures after brain insult may serve as an adaptive mechanism by which the brain tries to counteract the neural depression and to increase brain plasticity (Schallert et al., 1986; Witte and Freund, 1999). Conversely, the hyperexcitability present after a brain insult may disturb the re-establishment of normal function (Witte and Freund, 1999).

Previously, the effect of seizures on the recovery after brain insult has been investigated with chemical convulsants and with electrical stimulation. When the gamma-aminobutyric acid (GABA) antagonist, pentylenetetrazol, is administered during the first 24 hours after

unilateral sensorimotor cortex lesions, rats recover significantly faster from the somatosensory and motor asymmetry than the saline-treated control group (Hernandez and Schallert, 1988). Similarly, rats receiving two electroconvulsive seizures within the first 24 hours after the cortical lesion exhibit an accelerated recovery on the beam walking test (Feeney et al., 1987). However, when seven electroconvulsive seizures are applied, the treated group does not differ from the control group (Feeney et al., 1987). In an electrical kindling model, mild seizures from 2 to 6 days after the brain injury do not have any effect on the normal somatosensory recovery when the amygdala stimulation is started at 2 days (Hernandez and Warner, 1995). In contrast, when a more severe seizure occurs from 2 to 6 days after the brain insult, a somatosensory deficit is still observable at 4 months (Hernandez and Warner, 1995). Importantly, recovery is unimpeded when severe seizures occur more than a week after the lesion induction (Hernandez and Warner, 1995). In summary, it seems that the recovery process may be vulnerable to seizures during a certain critical period after the initial insult in rats. Second, the severity and the number of the seizures during the critical period might influence the extent of the recovery.

In addition to the effect of seizures, it is important to study whether administration of AEDs has any effect on the behavioral recovery after cerebral insults. A recent report describes that gabapentin treatment started 20 minutes after permanent MCAo reduces the number of seizures during the first post-operative day and the treatment is associated with a better neurological deficit score at 24 hours (Williams et al., 2006). In contrast, AED treatment with diazepam or phenobarbital hinders the recovery from asymmetry in a rat model of electrolytic unilateral lesion when treatment is started at 10 to 12 hours (Schallert et al., 1986). The asymmetry remains as severe as on the first post-operative day, even when the diazepam treatment is discontinued 22 days later and follow-up is carried out up to 3 months (Schallert et al., 1986). When diazepam treatment is postponed as long as 30 days after the lesion induction, a transient reinstatement of the asymmetry is observed in the majority of the animals during the first days of the treatment (Schallert et al., 1986). However, the asymmetry eventually disappears despite continuous drug treatment (Schallert et al., 1986). Taken together, there is evidence that AED treatment started hours after the brain insult might result in an impairment of recovery or a reinstatement of deficits.

2.4 Epileptogenesis-associated cerebral changes

An initial injury, such as a stroke, can trigger changes that alter the neural network and make it more prone to seizures. The process by which the normal brain is altered to a seizure-prone state is called “epileptogenesis”, and it can continue progressively from several weeks to even years after the initial insult (Mathern et al., 2002; Pitkänen et al., 2002). A variety of cerebral changes occur during epileptogenesis such as neuronal damage (Cavazos et al., 1994; Tuunanen et al., 1999; Mathern et al., 2002; Roch et al., 2002; Fabene et al., 2003), gliosis (Dawodu and Thom, 2005), axonal and dendritic sprouting (Salin et al., 1995; Buckmaster and Dudek, 1997; Ribak et al., 2000; Kato et al., 2001), alterations in the extracellular matrix (Perosa et al., 2002) and the induction of inflammation (Gahring et al., 1997; Vezzani et al., 1999; Turrin and Rivest, 2004).

Seizure activity has been associated with damage in several brain structures such as the amygdala (Fujikawa, 1996; Tuunanen et al., 1999; Roch et al., 2002; Fabene et al., 2003), the entorhinal and piriform cortices (Fujikawa et al., 1996; Roch et al., 2002; Fabene et al., 2003), the striatum (Fujikawa et al., 1996; Dreifuss et al., 2001; Fabene et al., 2003), and the thalamus (Roch et al., 2002; Dreifuss et al., 2001; Fabene et al., 2003). Further, one typical finding both in human and experimental epilepsy is cell loss and gliosis in the hippocampus, which is termed “hippocampal sclerosis” (Cavazos et al., 1994; Buckmaster and Dudek, 1997; Mathern et al., 1997; Proper et al., 2000; Mathern et al., 2002). In hippocampal sclerosis, neurons in the hilus and CA3 region are especially vulnerable to damage, whereas CA2 and the dentate gyrus are better preserved (Mathern et al., 2002). In the hilus, there may be loss of both glutamatergic mossy cells and GABAergic interneurons (Buckmaster and Jongen-Relo, 1999; Houser and Esclapez, 1996).

In addition to neuronal damage, neurogenesis has been associated with the network plasticity following seizures in adult rodents (Parent et al., 1997; Parent et al., 1998; Gray and Sundstrom, 1998). In the dentate gyrus, the majority of the newly born cells matures to neurons and resides at the base of the granule cell layer (Parent et al., 1997; Parent et al., 1998; Gray and Sundstrom, 1998). Newly born cells can also appear in ectopic locations within the hilus or in the superficial parts of the granule cell layer and extend axonal processes to abnormal locations within the inner molecular layer (Parent et al., 1997). However, it was suggested recently that the connectivity of new neurons can develop in order to mitigate the dysfunction in the epileptic brain (Jakubs et al., 2006).

Both axonal (Buckmaster and Dudek, 1997; El Bahh et al., 1999) and dendritic sprouting (Ribak et al., 2000; Kato et al., 2001) are also observed in association with seizures. Although the cortex and CA1 of the hippocampus can express axonal sprouting following seizures (Salin et al., 1995; Perez et al., 1996; Smith and Dudek, 2001), probably the most extensively studied form is mossy fiber sprouting in the dentate gyrus of the hippocampus. Normally, granule cell axons, i.e. mossy fibers, extend to hilar interneurons and the CA3 pyramidal cells (Amaral and Witter, 1989; Amaral, 1993). Seizure activity and the subsequent cell death in the hilus, however, are associated with mossy fiber sprouting to the inner molecular layer of the dentate gyrus, where abnormally sprouted mossy fibers make synaptic contacts with dendrites of the neighboring granule cells and form recurrent excitatory circuits (Wuarin and Dudek, 1996; Buckmaster and Dudek, 1997; Proper et al., 2000).

However, the role of mossy fiber sprouting in seizure occurrence still needs to be clarified. Williams and co-workers (2002) have shown that the development of spontaneous seizures in the pilocarpine model can occur without detectable mossy fiber sprouting or hippocampal neuron loss. Further, mossy fiber sprouting can be observed without the presence of spontaneous seizures in a status epilepticus model where there is electrical stimulation of the amygdala (Nissinen et al., 2001). Aging can also result as in aberrant mossy fiber sprouting as demonstrated with Timm's staining that locates the sprouting to the supragranular layer of the dentate gyrus (Cassell and Brown, 1984). Long-term potentiation induced with high-frequency electrical impulses to the perforant path in 10 consecutive days can also result as mossy fiber sprouting 7 days later (Adams et al., 1997). Lastly, although newly born neurons in the dentate gyrus can extend axonal processes to abnormal locations within the inner molecular layer, neurogenesis is not necessary for the development of aberrant mossy fiber sprouting after seizures (Parent et al., 1997; Parent et al., 1999).

2.5 Cerebral changes and excitability after focal ischemia

Focal cerebral ischemia results usually in dying neurons within an ischemic core that is surrounded by a penumbral zone in which neurons have the potential to survive. In addition, glial cells accumulate at the lesion border, prominent inflammation is present and neuronal reorganization and dendritic sprouting occur (Watson et al., 1985; Stroemer et al., 1995; Nudo et al., 1996; Bidmon et al., 1997; Barone and Feuerstein, 1999; Biernaskie and Corbett, 2001).

Around the lesion core, neither the average number of parvalbumin-labeled inhibitory interneurons nor the average number or the length of the dendritic processes arising from the labeled cells differ between control rats and ischemic rats with transient MCAo (Luhmann et al., 1995). Further, the expression of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors is decreased in the ischemic core and the penumbra at 24 hours and at 28 days (Jolkkonen et al., 2003; Sommer et al., 2003). In electrophysiological recordings, $GABA_A$ receptor-mediated inhibition appears to be decreased in the peri-infarct zone at day 7, but not at day 28, following the transient MCAo (Neumann-Haefelin and Witte, 2000). A reduction in overall excitability is also observed in the peri-infarct region for at least 28 days (Neumann-Haefelin and Witte, 2000).

In contrast to a large artery occlusion, after the cortical photothrombosis the number of parvalbumin-positive inhibitory interneurons becomes decreased within the immediate vicinity of the lesion at 7 days (Neumann-Haefelin et al., 1998). In addition, the interneurons appear shrunken and they have a reduced number of dendrites as an indication of degeneration for up to 1 mm from the necrotic lesion border (Neumann-Haefelin et al., 1998). In the core of the photothrombotic lesion, the $GABA_A$ receptors are up-regulated (Que et al., 1999b), but in the rim of the lesion they are down-regulated (Schiene et al., 1996; Que et al., 1999b). Cortical photothrombosis has also been associated with changes in the GABA receptor subunit composition and the messenger-ribonucleic acid expression (Neumann-Haefelin et al., 1998; Liu et al., 2002). NMDA-receptor expression, on the other hand, is increased in both hemispheres for at least 30 days after the photothrombosis, although the binding density of AMPA and kainate receptors does not seem to be altered (Que et al., 1999a). Electrophysiological recordings show a markedly decreased inhibition in the neurons from cortical layers IV and V at a distance of 2 to 3 mm from the photothrombotic lesion border (Witte and Freund, 1999). Further, the resting potential is less negative in the cortical neurons 1.5 to 2.5 mm lateral from the lesion than in the control neurons (Witte and Freund, 1999).

In addition to the primary lesion site, after focal cerebral ischemia damage can be observed in the thalamus (Ruballa et al., 1998; Dihne et al., 2002) and the substantia nigra (Dihne and Block, 2001). In the hippocampus, the damage seems to be related to the method which is used to induce focal cerebral ischemia. Permanent MCAo with or without clamping both common carotid arteries or transient MCAo with systemic hypotension seems to provoke

neuronal damage in the hippocampus (Zhu et al., 1995; States et al., 1996). However, recent results seem to imply that following cortical photothrombosis, the hippocampus is spared from injury as shown by Nissl and Fluoro-Jade-B stainings (Kharlamov et al., 2007). Further, the number of hippocampal interneurons does not change at 7 and 30 days after photothrombosis, when cells expressing glutamic acid decarboxylase-65/67 mRNA are labeled (Frahm et al., 2004). However, NeuN immunoreactivity becomes reduced in the ipsilateral hippocampus at 1, 3, 7 and 180 days when compared to the contralateral side (Kharlamov et al., 2007). A cortical infarct can also alter the voltage dependence of the calcium current inactivation in both CA1 and CA3 regions and this may lead to a stronger excitability of the hippocampal network (Diehm et al., 2003). In addition to cell damage, neurogenesis can be observed in the subgranular zone of the dentate gyrus of the hippocampus and in the subventricular zone after both large artery occlusion and cortical photothrombosis (Arvidsson et al., 2002; Kluska et al., 2005).

3. AIMS OF THE STUDY

The present study aimed to investigate the occurrence of post-stroke epilepsy in rats. For this purpose, three established focal cerebral ischemia models were evaluated: the transient intraluminal filament model of middle cerebral artery occlusion (I, IV), the endothelin-1 induced middle cerebral artery occlusion (II, IV) and cortical photothrombosis with Rose Bengal (III, IV). The aim was to answer the following questions:

(1) Is epileptogenesis associated with the type, size or location of the ischemic lesion?

(2) What are the characteristics of the late seizures occurring after focal cerebral ischemia?

(3) Does epileptogenesis associate with cell damage and mossy fiber sprouting in the hippocampus in the rats with focal cerebral ischemia?

(4) Does the occurrence of early seizures, increased spiking activity or epileptogenesis associate with behavioral recovery as measured by sensorimotor testing, the spatial learning and memory test, and the emotional learning and memory test after focal cerebral ischemia?

4. MATERIAL AND METHODS

4.1 Animals and housing conditions

Male Sprague-Dawley rats weighed 275-315 g (I, II) or 310-380 g (III) at the time of ischemia induction. Altogether, 22 rats were used for the filament model of MCA occlusion (I; 9 sham and 13 ischemic); 47 rats for the ET-induced MCA occlusion (II; 12 shams and 35 ischemic); and 51 rats for photothrombosis (III; 11 controls and 40 ischemic). In the photothrombosis model, an additional group of rats with lateral coordinates were prepared (n=6). The animals were single-housed in standard size cages in a humidity (50±10%) and temperature (21±1ºC) controlled environment with lights on from 7:00 to 19:00. Bottle water and pellet food were freely available for the rats. The procedures were approved by the Committee for the Welfare of Laboratory Animals of the University of Kuopio and the Provincial Government of Kuopio. All procedures were conducted in accordance with the European Community Council directives 86/609/EEC.

4.2 Study designs

As summarized in the study designs (Figure 1), video-EEG was recorded to detect spontaneous seizures after experimental stroke with the filament model of MCAo (I), after ET-induced MCAo (II; 6 and 12 months follow-up groups) or photothrombosis (III; 2 separate experiments from which Experiment 1 included only ischemic rats, and an additional group of rats with lateral coordinates). An additional aim was to investigate whether the severity of impairment in sensorimotor or learning and memory functions would differ between these rats with and without epilepsy. At the end of the studies, rats were perfused for histology to investigate the association between the brain damage severity and epileptogenesis.

4.3. Anesthesia and peri- and postoperative health care

Anesthesia with halothane (I, II). For the induction of MCAo with the ET and filament models, the rats were anesthetized in a plastic chamber containing a mixture of 5% halothane (Halothane, Rhodia Ltd, UK) in 30% oxygen and 70% nitrous oxide (Vaporizator Ohmer, BOC Ohmeda, Instrumentarium Oyj, Finland). When the animal was deeply anesthetized, it was removed from the chamber and connected to a nose mask. A surgical depth of anesthesia was maintained with 0.5-1% halothane. Air parameters of halothane, O_2 and N_2O were

monitored during the surgery (Capnomatic Ultima, Datex-Ohmeda, Instrumentarium Oyj, Finland).

Anesthesia with an anesthesia cocktail (I, II, III). In the photothrombosis model and for electrode implantations, the rats were anesthetized with an *intraperitoneal* injection of a mixture (6 ml/kg) containing sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulphate (127.2 mg/kg), propylene glycol (42.8%), and absolute ethanol (11.6%).

Peri- and postoperative health care (I, II, III). During the filament model of MCAo (I) and ET-induced MCAo (II), the body temperature of the rat was monitored and maintained automatically at 37ºC using a temperature controller connected to a heating pad and a thermometer (Harvard Homeothermic Blanket Control Unit, 50-7061, Harvard Apparatus Ltd, UK). Local anesthetic cream (2 % Lidocaine hydrochloride, Orion, Finland) and local antibiotics (Bacibact powder, Orion, Finland) were spread on the edges of the surgical incisions (I, II, III). To prevent surgery-related weight loss and dehydration, rats were *intraperitoneally* injected with saline (5 ml, 0.9% NaCl) during the initial postoperative days (I, II).

4.4. Experimental stroke models

4.4.1 Intraluminal filament model of middle cerebral artery occlusion (I)

To induce MCAo with the intraluminal filament model (Longa et al., 1989), a halothane-anesthetized rat was placed in a supine position under a light microscope and an incision was made on the right side of the neck. The external carotid artery (ECA) was cut with microscissors and the intraluminal filament with a rounded tip (0.25 mm in diameter, soaked in heparin) was introduced into the lumen of the ECA stump and advanced into the ICA until the rounded tip blocked the bifurcation of the MCA. This was ensured by advancing the intraluminal filament until the marking on the filament was reached (1.9-2.2 mm from the rounded tip of filament) and/or a slight resistance was felt. The animal remained deeply anesthetized during the 120 min of MCAo. At the end of the occlusion time, the intraluminal filament was removed, the stump of the ECA was electrocoagulated and the blood flow was restored. In the sham-operation, the CCA was exposed and the ECA was cut and electrocoagulated, but the intraluminal filament was not introduced into the lumen of the artery.

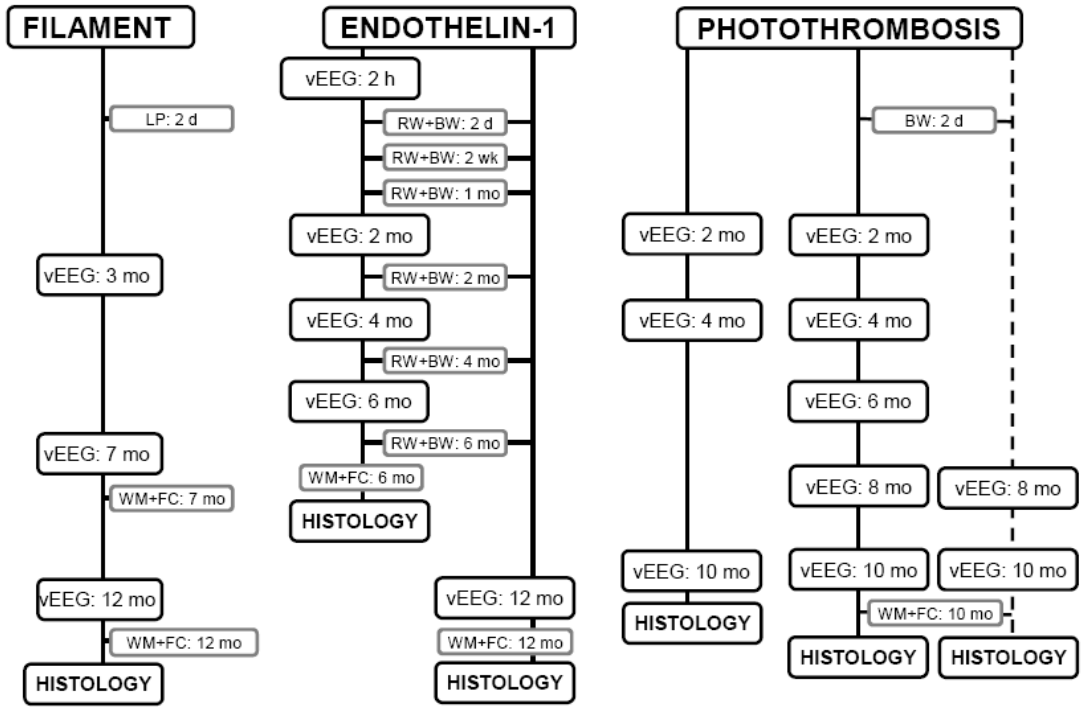

Figure 1. Flow charts represent study designs for video-EEG recordings and behavioral tests for the studies with the intraluminal filament model of middle cerebral artery occlusion (Filament), the ET-induced middle cerebral artery occlusion (Endothelin-1) and cortical photothrombosis (Photothrombosis). The dashed line indicates the additional group of photothrombotic rats with a lateral lesion and the grey line refers to behavioral tests. Abbreviations: BW, beam walking test; FC, fear conditioning test; LP, limb placing test; RW, running wheel test; vEEG, video-EEG; WM, water maze test.

4.4.2 Endothelin-1 induced middle cerebral artery occlusion (II)

For the ET-induced MCAo, a halothane-anesthetized rat was placed into a stereotaxic frame in the flat skull position (Kopf, USA; incisor bar 3.3 mm below zero according to the brain atlas of the rat by Paxinos and Watson, 1997; Biernaskie and Corbett, 2001). An incision was made in the midline of the head and the place of the bregma was identified with a light microscope. A hole was drilled into the skull overlying the site of ET-injection (0.9 mm anterior and 5.2 mm lateral to bregma; 8.7 mm below dura) and a small opening was made into the dura with a needle. Thereafter, 6 µl (120 pmol) of ET solution (20 pmol/µl in 0.9% NaCl; endothelin-1, Sigma-Aldrich Chemie GmbH, Germany) was injected stereotactically with a 10 µl syringe (Microliter Syringes, Hamilton Bonaduz AG, UK) to the proximal portion of the MCA over a period of 2 minutes. To minimize the backflow, the

needle was left in place for 5 minutes before it was slowly withdrawn. The sham operation included all of these procedures but injection of 0.9% NaCl was made instead of ET.

4.4.3 Cortical photothrombosis with Rose Bengal (III)

For the induction of the cortical photothrombosis, a pentobarbital-anesthetized rat was placed into a stereotaxic frame in the flat skull position (Watson et al., 1985; Zhao et al., 2005). An incision was made in the midline of the head and the place of the bregma was identified with a light microscope. Rose Bengal (20 mg/kg; 20 mg/ml; Sigma Aldrich Chemie, Germany) was infused into the femoral (Experiment 1) or saphenous vein (Experiment 2) at a speed of 150 µl/min. The center of the light beam (4 mm in diameter; Olympus, Denmark) was focused 1.8 mm posterior and 2.2 mm lateral to the bregma. A more lateral lesion was utilized in an additional group of animals with coordinates 0.5 mm posterior and 3.7 mm lateral. Photoactivation was performed during 10 min simultaneously cooling the skull surface with air. Two control groups were prepared, but their results were combined for the analysis. One group of controls was treated similarly with photothrombosis except that the light was not turned on and thus, the photoactivation leading to ischemia was not performed. The second control group underwent only the electrode implantation.

4.5. EEG recordings

4.5.1 Electrode implantation and EEG monitoring schedule

Following filament model of MCAo (I). Ten weeks after MCAo with the filament, two stainless steel screw electrodes (Plastics One Inc., USA) were inserted into the skull above the somatosensory cortex (3 mm posterior and 2 mm lateral from the bregma). Two screws positioned over the cerebellum served as indifferent and ground electrodes (10.3 mm posterior and 2 mm lateral). The electrode wires were connected to the plastic pedestal (Plastics One Inc., USA), and the headset was fixed with dental acrylic (Selectaplus CN, Dentsply DeTrey GmbH, Germany). The video-EEG was recorded continuously for 1 week at time points 3, 7, and 12 months after the MCAo or the sham-operation (Nervus EEG Recording System, Iceland or Stellate EEG Monitor System, Canada).

Following ET-induced MCAo (II). In the 6-month follow-up group, electrodes were implanted in the same session with the sham-operation or the ischemia induction with ET. In the 12-month follow-up group, electrodes were implanted 1 month before the video-EEG

monitoring (11 months after experimental stroke). In addition to two recording electrodes above the primary somatosensory cortex (3 mm posterior and 2 mm lateral) and an indifferent and a ground electrode above the cerebellum (10.3 mm posterior and 2 mm lateral), a hand made bipolar electrode was lowered into the ipsilateral hippocampus (two nylon insulated wires, 0.127 mm in diameter, soldered to a gold pin, Franco Corradi, Italy; 6.0 mm posterior and 4.6 mm lateral and 7.0 mm below the skull). The 6-month follow-up group was continuously monitored for the first 2 hours after the operation, and at 2, 4, and 6 months (2 weeks at a time, 24 hours/day). In the 12-month follow-up group, cortical and hippocampal electrodes were implanted at 11 months after operation and the video-EEG was recorded for 2 weeks at 12 months.

Following cortical photothrombosis (III). Electrodes were implanted immediately after the photothrombosis. In the additional group of rats with a lateral lesion, electrodes were attached one week before the first video-recording session (on post-ischemic week 27). The recording electrodes were implanted into the ipsilateral cortex (3 mm posterior and 2 mm lateral) and hippocampus (6.0 mm posterior and 4.6 mm lateral and 7.0 mm below the skull). Indifferent and ground electrodes were positioned over the cerebellum (10.3 mm posterior and 2 mm lateral). The video-EEG was recorded continuously for 7 or 10 days at 2, 4 and 10 months in Experiment 1. In Experiment 2, the photothrombotic and control group were recorded continuously with video-EEG for 7 or 14 days at 2, 4, 6, 8 and 10 months after the operation. The additional group of photothrombotic rats with a lateral lesion location was recorded for 14 days at 8 and 10 months after ischemia induction.

4.5.2 EEG equipment

EEG was collected using the recording system with an amplifier as described elsewhere (Nissinen et al., 2000; Nervus magnus 32/8 Amplifier; Nervus 2.4 or 3.0 software, Taugagreining, Iceland or Stellate EEG Monitor System, Canada). The behavior of the animals was recorded using a video camera (WV-BP330/GE, Panasonic) that was positioned above the cages and connected to a time lapse VCR (SVT-N72P, Sony). An infrared light (WFL-II/LED15W, Videor Technical GmbH, Germany) was used at night to allow visibility continuously for 24 hours/day.

4.5.3 Analysis of EEG

Seizures (I, II, III). The occurrence of spontaneous seizures was based on the visual analysis of digitized EEG on a computer screen. A paroxysmal discharge with rhythmic, repetitive waveforms that lasted at least 5 seconds, had a clear onset and offset and temporal evolution in amplitude and frequency was considered as an electrographic seizure. The number of seizures and their duration was assessed and the occurrence of spontaneous seizures per recording day was calculated.

When electrographic seizure activity was observed, then the severity of the behavioral seizure was classified from time-locked video recordings according to a modified Racine's scoring scale (Racine, 1972): 0, electrographic seizure without any detectable motor manifestation; 1, mouth and face clonus, head nodding; 2, clonic jerk of one forelimb; 3, bilateral forelimb clonus; 4, forelimb clonus and rearing; and 5, forelimb clonus with rearing and falling.

The term "early seizure" was used for electrographic seizures that were detected within 2 weeks after the ischemia induction. Electrographic seizures that occurred after the 2 week time point were called "late seizures". Late seizures were considered as spontaneous seizures representing the outcome of epileptogenesis, i.e. epilepsy.

Spikes (II). Spike analysis was performed at 2, 4, 6 (6-months follow-up group), and 12 (12-months follow-up group) months after ET-induced MCAo. At each time-point, a 24-hour artifact-free EEG from the beginning of each 2-week monitoring period was selected for the analysis. The entire 24-hour hippocampal EEG was transformed to the one channel European data format and processed with the Clampfit-program (version 9.0.1.07, Axon Instruments Inc., USA). The EEG was visually inspected on the computer screen to detect movement-related and other artifacts and an artifact-free portion of hippocampal EEG was used for the analysis. Spiking was considered pathologic when it was >2SDs (standard deviations) above the mean of sham animals.

4.6. Behavioral tests

4.6.1 Sensorimotor tests

The limb-placing test (I). To confirm the successful MCAo with filament, the limb-placing test was performed at 2 days. The limb placing test consisted of seven tasks, which were scored from 0 to 2 points according to the performance of the rat: 0 point, the rat did not

perform normally; 1 point, the rat performed with a delay of more than 2 seconds and/or incompletely; and 2 points, the rat performed normally (Jolkkonen et al., 2000). Both sides of the body were tested. The maximum possible score achieved by the sham-operated rats was 14. Only ischemic rats with a score of 10 or lower were included in this study.

The running wheel test (I, II). The running wheel test was used to detect sensorimotor impairment in the forelimb function. The running wheel test was performed at 7 and 12 months after the filament model of MCAo and at 2 days, 2 weeks, 4 weeks, 2 months, 4 months and 6 months after ET-induced MCAo. The running wheel (29 cm in diameter; steps 2.5 cm apart; transparent, plastic sides) was rotated by a motor at a speed of 4 rpm for 150 seconds. Rats were trained in the wheel on three consecutive days before ischemia induction. The performance of the rats in the wheel was video-recorded for later slow-motion analysis of the slips and the steps taken with the forelimbs (Jolkkonen et al., 2000). The slip ratio (number of slips/number of steps taken) was calculated and used for statistical analysis of sensorimotor performance.

The tapered beam-walking test (II, III). The tapered beam walking test was used to detect sensorimotor impairment of the hindlimb function and it was performed 2 days, 2 weeks, 4 weeks, 2 months, 4 months and 6 months after ET-induced MCAo and 2 days after the cortical photothrombosis. The beam (length 140 cm) was 40 cm above the floor and it was placed along the wall equipped with a mirror to help in the detection of the limb movements. The horizontal surface of the beam narrowed from 6.0 cm at the start to 1.5 cm at the end. A 1.5-cm wide horizontal lower level ran on both sides of the beam. A bright light (100 W; 30 cm above the beam) illuminated the beginning of the beam and motivated the rat to escape from the beam into a black box (20.5 cm x 25 cm x 25 cm), which was located at the narrow end of the beam. Rats were trained on three consecutive days (three trials each day) before ischemia induction. The performance of the rat on the beam was video-recorded for slow-motion analysis. The numbers of steps on the upper level, slips to the vertical surface of the beam, and slips to the lower level were counted for the hindlimbs. The score for each trial was presented as follows (Schallert and Woodlee, 2005): [(vertical slips $*$ 0.5 + lower level slips)/(upper level steps + vertical slips + lower level slips)] $*$ 100%.

4.6.2 Learning and memory tests

The water maze (I, II, III). The water maze test was used to investigate impairment in spatial learning and memory of the animals 7 and 12 months after the filament model of MCAo, 6 or 12 months after ET-induced MCAo and 10 months after cortical photothrombosis as described before (Puurunen et al., 2001). The testing system was composed of a black pool (150 cm in diameter) that was filled with water (temperature 20±2°C) and surrounded with visual cues (posters and lights). A black square platform (10 x 10 cm) was located 25 cm from the pool rim and 1.5 cm below the water surface. The swimming pattern of a rat was recorded using a video camera that was positioned directly above the pool and connected to a computerized image analysis system (HVS image, Imaging Research Inc., UK). The test was carried out on 3 consecutive days (5 trials per day). The starting position was changed after each trial. The rat was allowed to swim for 70 seconds to find the platform. When the rat failed to find the hidden platform within 70 seconds, it was guided to the platform. After each trial, the rat was allowed to remain on the platform for 10 seconds and thereafter, it rested in a cage for 30 seconds or 1 minute. On the third testing day, the sixth trial was done without the platform (the probe trial).

The fear conditioning test (II, III). To investigate emotional learning and memory of the rats, the fear conditioning test was carried out 7 months or 13 months after ET-induced MCAo and 11 months after cortical photothrombosis as described before (Narkilahti et al., 2003). On the first day, a rat was habituated to the fear-conditioning box without a tone or foot shock for 20 minutes. On the second day, a tone (20 seconds, 10 kHz and 75 dB) was combined with an electric foot shock that was delivered at the end of the tone (0.5 seconds, 0.5 mA; San Diego Instruments Incorporated, SD Instruments). The association of a tone and foot shock was repeated twice. The time between stimuli varied randomly from 1 to 5 minutes. On the third day, the protocol from day 2 was repeated. On the fourth day, the test was performed in a novel environment (a novel box in a novel room, and a novel odor was used for cleaning). The rat was placed into the box for 2 minutes and then a tone was introduced for 3 minutes. Freezing time was defined as the time the rat spent immobile (only respiratory-related movements observed). The duration of freezing was assessed by viewing videotapes. Freezing during the 20-second period immediately preceding the onset of the tone was used as a measure of contextual fear conditioning. Freezing during the 20-second delivery of the tone was used as a measure of cued fear conditioning.

4.7. Histology

4.7.1. Tissue processing

After the filament model of MCAo, the ventral part of the right hippocampus was frozen on dry ice and stored at −80 ºC for histological procedures at the end of the 12-month follow-up (I).

Six or 12 months after ET-induced MCAo (II) and 10 months after photothrombosis (III), rats were deeply anesthetized with an *intraperitoneal* injection of the anesthesia cocktail and transcardially perfused using Timm's sulphide method (Sloviter, 1982). Briefly, rats were perfused with buffered sulphide solution (10 min, 30 ml/min) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; 10 min, 30 ml/min). The brains were removed from the skull and postfixed in buffered 4% paraformaldehyde solution (4 h in ice bath). Then the brains were cryoprotected with 20% glycerol in 0.02 M potassium phosphate buffer (24 h at -4ºC), frozen in dry ice and stored at -70ºC until further processing. Coronal sections (30 µm thick) were cut from the frozen brains with a sliding microtome (1-in-5 series). The first series of sections was stored in buffered 10% formalin solution at room temperature. The rest of the sections were stored in tissue collection solution at -20ºC (30% ethylene glycol and 25% glycerine in 0.05 M phosphate buffer).

4.7.2. Thionin staining

The first series of sections was stained with thionin to determine the infarct size and depth (II, III), to assess the severity of neuronal damage in the hippocampus (II, III), and to confirm the placement of the hippocampal electrode (II, III).

Lesion volume (II). For lesion volume analysis after ET-induced MCAo, digitized images of sections spaced 600 µm apart were captured and depicted on a computer screen (Swanson et al., 1990). The hemispheres were manually outlined in each section and surviving tissue with optical densities above the threshold value was recognized and measured automatically by the image analysis program (MCID, Imaging Research Inc., Canada). To calculate the area of lesion in each section, the area of the surviving tissue of the ipsilateral hemisphere was subtracted from that of the contralateral hemisphere. The mean infarct area was calculated and multiplied by 0.6 (section interval), and the volumes were summed together to obtain a total infarct volume.

Severity of neuronal damage in the hippocampus (II). Thionin-stained sections were also used for semiquantitative analysis of the severity of neuronal damage in the granule cell layer, hilus, CA3 (c, b and a subfields) and CA1 subregions of the ipsilateral and contralateral hippocampus after ET-induced MCAo. The analysis was started in the section in which the suprapyramidal and infrapyramidal blades of the septal dentate gyrus fused together and continued through the entire septotemporal extent of the hippocampus. The severity of neuronal damage was scored as follows: 0, no damage; 1, damage involves less than 20% of neurons; 2, damage involves 20-50% of neurons; and 3, damage involves more than 50% of neurons in the area of interest (Pitkänen et al., 1996).

Hilar cell countings (III). After photothrombosis, damage to the hippocampal hilar neurons was assessed using a stereological approach with the optical fractionator method using Stereo Investigator software (MicroBrightField Inc., USA) as described before (Pitkänen et al., 2002; West et al., 1991). Briefly, a color video camera (Hitachi HV-C20) interfaced with the microscope (Olympus BX50) was used to view digitized sections on the monitor. Neuroanatomical borders of the hilus were outlined on the screen and subsequent counting of the cells was performed within these borders. The motorized stage of the microscope was under the control of the computer. The hilar fields in every section were surveyed at evenly-spaced 70 x 70 µm intervals using a counting frame of 20 x 20 µm. Counting was performed in a 1-in-15 series of systematically sampled sections throughout the hippocampus as described previously (West et al., 1991).

4.7.3. Timm's staining

One series of sections was stained with Timm's staining for native tissue (I; Jolkkonen et al., 1997) or Timm's staining for sulphide-perfused tissue (II, III; Sloviter, 1982; Narkilahti et al., 2003) to investigate the sprouting of mossy fibers in the dentate gyrus of the hippocampus. The analysis was performed throughout the hippocampus including septal and temporal-dorsal and temporal-ventral portions (Narkilahti et al., 2003). The rating was based on the density of Timm's granules in the supragranular region and in the inner molecular layer of the dentate gyrus as follows: 0, no granules; 1, sparse granules; 2, granules evenly distributed; 3, almost a continuous band of granules; 4, a continuous band of granules; and 5, a confluent and dense laminar band of granules (Cavazos et al., 1991).

4.8 Statistical analysis

All statistical analyses were performed with SPSS software for Windows (SPSS Inc., USA). Non-parametric tests were used because data were not normally distributed and the number of cases was low. Analysis of variance for repeated measures was carried out when a behavioral test was repeated. A *p*-value of less than 0.05 was considered to be statistically significant.

5. RESULTS

5.1 Mortality and exclusions

Filament model of MCAo (I). Altogether, two ischemic rats from 13 were excluded after filament-induced MCAo. One rat was excluded because it did not exhibit a sufficiently severe sensorimotor deficit in the limb-placing test and the other was omitted because of infection. Six rats died during the 12-month follow-up period. Thus, five ischemic rats with a 12-month follow-up were available.

ET-induced MCAo (II). Of 47 operated rats, one sham-operated rat died due to hemorrhage during the first EEG-recording; 1 ET-injected animal died during the replacement of a headset for EEG recordings and 5 rats had to be killed during the follow-up (4 shams, 1 ET-injected). Histologic analysis revealed no measurable infarct in 7 of the 35 ET-injected rats, and these animals were excluded from the analysis. Thus, 11 rats (3 shams and 8 ET-injected) were included in the 6-mo follow-up group and 22 (4 shams and 18 ET-injected) in the 12-mo follow-up group.

Cortical photothrombosis (III). Cortical photothrombosis was induced in 40 rats of which one rat died during the ischemia induction and two during the experiment. Two rats were excluded because of a poor EEG and one rat because no confirmable lesion could be detected histologically. The headset fell off from one rat before the 2-month recording, from one rat before the 4-month and from 5 rats before the 10-month video-EEG recording sessions. The headset was reimplanted in 2 rats before the 6-month video-EEG recording period. Thus, altogether 36 ischemic rats were available for the EEG analysis (16 ischemic rats in Experiment 1 and 20 ischemic rats in Experiment 2; 11 controls). In addition, one ischemic rat was excluded from the mossy fiber analysis and hilar cell counting because its hippocampus was deformed.

5.2. EEG recordings

5.2.1 Early seizures

Early seizure occurrence was not investigated after the filament model of MCAo (I) or after cortical photothrombosis (III). After ET-induced MCAo (II), non-convulsive, electrographic seizures were observed in 7 out of 8 ET-injected rats (88%) within 2 h after surgery. The seizures appeared, on average, 50 minutes after ET injection. The mean seizure frequency was 0.79 seizures per hour and the mean seizure duration was 76 seconds. The

sham-operated rats (3 out of 8, 38%) also exhibited early seizures and one of them died during the recording. The mean seizure frequency for the two surviving shams was 0.75 seizures per hour with a mean duration of 95 seconds. The rat which died had 2.6 seizures per hour, spent a total of 25 minutes in seizure activity, and eventually died while still in seizures at 3 hours 55 minutes after the saline-injection.

5.2.2 Late seizures

Late seizures were considered as spontaneous seizures representing the outcome of epileptogenesis, i.e. epilepsy. No spontaneous late seizures were detected during the intermittent video-EEG recordings, which were arranged up to 12 months after the filament model of MCA occlusion (I; Table 3).

Following ET-induced MCAo, one rat developed epilepsy 6 months after the ischemia induction (6 months follow-up group; II; Table 3). The seizure frequency for that rat was 0.21 seizures per recording day with a mean seizure duration of 113 seconds (range from 78 to 174 seconds). Behaviorally, the seizures ranged from bilateral forelimb clonus to rearing and falling (scores 3 and 5).

Following cortical photothrombosis, 18% of the rats exhibited electrographic seizures (III; Table 3). The mean seizure frequency was 0.39 seizures per recording day and mean seizure duration was 117 seconds (range from 36 to 358 seconds). Electrographic seizures were accompanied by bilateral forelimb clonus, rearing, or rearing and falling (scores 3, 4 or 5). The latency time from photothrombosis to the first seizure varied from 71 to 297 days. No electrographic seizures were detected in the rats with the control treatment. Further, none of the rats in the additional photothrombotic group with a lateral lesion location developed seizures.

5.2.3 Spikes

After ET-induced MCAo, the mean hippocampal spike frequency did not differ between sham and ischemic group at 2, 4, or 6 mo, but at the 12-mo recording, the ET group had a higher mean spike frequency than the sham group ($p<0.05$; II). The infarct volume correlated with the spiking frequency at 12 mo ($p<0.01$, $r=0.540$), i.e. those rats with larger lesions exhibited a higher spiking frequency at 12 mo. No increase in spiking frequency was observed in sham-operated rats.

Table 3. Summary of results from the studies using the filament model of MCAo, ET-induced MCAo and cortical photothrombosis.

	Filament model of MCAo	ET model of MCAo	Cortical photothrombosis
Seizure characteristics			
Number of rats with epilepsy	0/5 (0%)	1/26 (4%)	7/36 (18%)
Latency time from ischemia to seizures (d)	-	192	71-297
Seizure score	-	3 or 5	3-5
Mean seizure frequency (seizures/recording day)	-	0.21	0.39
Mean seizure duration (s)	-	113	117
Histology in rats with seizures			
Hippocampal cell damage	-	no¤	no
Aberrant mossy fiber sprouting	no	no¤	yes
Behavioral impairment after focal ischemia			
Morris' water maze	yes	No	yes
Fear conditioning	-	No	no
Sensorimotor tests	acute: yes chronic: yes	acute: yes chronic: no	acute: yes chronic: -

Abbreviations: -, data not obtained; ¤, statistical analysis could not be carried out; Seizure score, severity of behavioral seizures scored from 0 to 5 according to Racine (Racine, 1972).

5.3 Behavioral deficits

5.3.1 After ischemia

The rats subjected to the filament model of MCAo slipped more than shams in the running wheel after 7 and 12 months ($p<0.05$; I; Table 3). Further, MCA occluded animals had a longer escape latency (at 7 months $p<0.01$ and at 12 months $p<0.001$) and path length (at 7 months $p<0.001$ and at 12 months $p<0.001$) in the water maze than their sham-operated counterparts.

The rats subjected to ET-induced MCAo slipped more than the shams in the running wheel after 2 days ($p<0.01$), but no difference was found between ischemic and sham rats in the beam walking test (II; Table 3). In addition, ET-injected rats performed as well as the sham-operated in the water maze and fear conditioning tests.

Rats with photothrombosis slipped more on the beam than the control group after 2 days ($p<0.05$) and spent more time trying to find the platform in the water maze test when this was

assessed at 10 months ($p<0.05$) compared to the control group (III; Table 3). However, photothrombotic rats and control rats performed similarly in the fear conditioning test.

5.3.2 After seizures and spiking

The behavioral data could not be compared between rats with and without seizures after the filament model of MCAo, because none of the rats developed seizures during the follow-up. After ET-induced MCAo, the existence of early seizures or increased spiking did not influence the performance in the running wheel or on the beam (II). Unfortunately, no comparison between rats with and without late seizures was possible after ET-induced MCAo and photothrombosis, due to the low number of rats in the groups (II, III).

5.4. Histology

5.4.1 Lesion volume and depth

Following the ET-induced MCAo (II), lesions were detected in both parietal and temporal cortical regions and striatum. The mean lesion volume in ET-injected rats was 32 ± 22 mm^3 (range from 3 to 76 mm^3). The ET-injected rat with late seizures had a lesion volume of 10 mm^3. After photothrombosis (III), the lesion depth varied from layer I to VI both in epileptic and non-epileptic rats.

5.4.2 Neuronal damage in the hippocampus

Following the ET-induced MCAo, semiquantitative analysis of neuronal densities in the hippocampus did not reveal any neuronal loss outside the electrode tract (II; Table 3). Similarly, the estimation of hilar cell number did not reveal any massive loss of hilar neurons following cortical lesioning with photothrombosis (III; Table 3).

5.4.3 Effect of ischemia and age on mossy fiber sprouting

Following the filament model of MCAo, Timm's staining did not reveal any aberrant mossy fiber sprouting in the hippocampus (I). Similarly, the mean Timm's score did not differ between sham rats and rats with ET-induced MCAo (II). At 12 mo, however, rats with ET-induced MCAo had a slightly higher total mean Timm's score than ischemic rats at 6 mo ($p<0.05$; II). Furthermore, the mean Timm's score did not differ between non-epileptic photothrombotic rats and control rats (III).

5.4.4 Effect of seizures on mossy fiber sprouting

None of the rats with filament induced MCAo developed epilepsy (I). Interestingly, after ET-induced MCAo, sprouting of mossy fibers was not detected in the epileptic rat (II; Table 3). In contrast, after photothrombosis, the epileptic rats had slightly denser ipsi- and contralateral mossy fiber sprouting when compared to the situation in the control rats or photothrombotic non-epileptic rats ($p<0.05$; III; Table 3).

6 DISCUSSION

6.1 Methodological considerations

Experimental groups. Focal cerebral ischemia was induced in rats using three different methods to study the occurrence of post-stroke epilepsy in the animals. Two of the techniques, the filament model and the ET-model resulted in occlusion of MCA. The results suggested that the percentage of young rats developing seizures is low after the large MCA territory infarct, which is consistent with the results of a previous study (Kelly et al., 2006). In contrast, a small cortical lesion induced epileptic seizures in 18% of the rats. Previously, it has been shown that up to 50% of the rats can develop epilepsy after cortical photothrombosis (Kharlamov et al., 2003). The difference might relate to different lesion size due to the dose of Rose Bengal dye, the diameter of the light beam, and the duration of illumination. Further, in the present study, electrical discharges shorter than 5 s were not counted as seizures. Detection of seizures was also based on the ipsilaterally placed hippocampal depth electrode and the contralateral cortical electrode and thus, short lasting and local discharges around the lesion may have gone unnoticed.

Stroke models. Focal cerebral ischemia models were selected for the present study, because they are believed to be relevant for modeling human stroke (Ginsberg and Busto, 1998). In particular, transient MCAo models are of special interest, because in humans the MCA is often affected by occlusion and is usually accompanied by recanalization (Saito et al., 1987). The intraluminal filament model represents probably one of the most extensively used stroke model that has relevance to the clinical condition of the MCAo (Ginsberg and Busto, 1998). The ET-induced MCAo produces an artery spasm achieved by stereotactic injection of a vasoconstricting peptide (Biernaskie and Corbett, 2001) whereas the cortical photothrombosis with Rose Bengal produces thrombotic occlusion of small cortical vessels (Watson et al., 1985).

Although stroke can occur in any age group, it is most often observed after 60 years of age (Giroud et al., 1998). Most of the experimental studies, however, use young adult rats aged 2 to 3 months at the time of ischemia induction. Aged rats are expensive to purchase and they have increased mortality after surgical procedures meaning that a relatively large number of animals need to be operated to obtain a relevant study groups.

Video-EEG recordings. Long-term video-EEG recordings were performed to detect seizures after focal cerebral ischemia. EEG monitoring is important, especially during the

acute phase after the experimental stroke, because seizures are typically non-convulsive (Williams et al., 2004a; Williams et al., 2006). Further, the latency time between ischemic insult and the appearance of the first late seizure can vary from days to months (Kharlamov et al., 2003) and thus, intensive long-term monitoring is necessary.

Previously it was suggested that chronic implantation of an electrode into the amygdala could be sufficient to evoke epileptiform changes in hippocampal electrical activity (Niespodziany et al., 1999). In the present experiment, both skull electrodes and intrahippocampal electrodes were used in the rats with ET-induced MCAo or with the cortical photothrombosis. Importantly, no late seizures were detected in control treated animals that underwent also the implantation of the depth and cortical electrodes.

6.2 Comparison between human and experimental post-stroke epilepsy

The present experiment was aimed to study the development of epilepsy after stroke using three established techniques to induce focal cerebral ischemia. No seizures were observed after the intraluminal filament model of MCAo. After the ET-induced MCAo, 1 rat of 26 exhibited late seizures. Further, the cortical photothrombosis induced seizures in 7 rats of 36. Seizure frequency varied from 0.21 to 0.39 seizures per recording day in those rats developing seizures in the ET-model and in the cortical photothrombosis, respectively. In humans, stroke can be highly variable in size and location and from 3% to 14% of the patients experience late seizures (Gupta et al., 1988; Ryglewicz et al., 1990; Kotila and Waltimo, 1992; So et al., 1996; Bladin et al., 2000; Dhanuka et al., 2001). Seizure frequency can range from one to two seizures per month (Ryglewicz et al., 1990).

6.2.1 Electrophysiological findings

At acute time points after human stroke, the EEG can detect four times more seizures than can be seen clinically (Vespa et al., 2003). In agreement, non-convulsive seizures were observed in 7 out of 8 rats with ET-induced MCAo within 2 h after the ischemia induction. The occurrence of early seizures was not monitored after the filament model of MCAo and cortical photothrombosis. It remains to be investigated whether the physiological variables during and after the induction of focal cerebral ischemia can contribute to the occurrence of early seizures after the focal cerebral ischemia.

Seizures after stroke are thought to be partial with or without generalization (Silverman et al., 2002). Recently, it was also shown that in humans the median duration of secondarily generalized tonic clonic seizures is 130 seconds (Jenssen et al., 2006). In the present study, all observed late seizures were secondarily generalized, ranging from bilateral forelimb clonus to rearing and falling (behavioral seizure scores from 3 to 5) and the seizure duration varied from 36 to 358 seconds.

It has been suggested that in humans, seizures arise from the perilesional cortex (Witte and Freund, 1999; Cosgrove, 2001). Previously, it was shown in experimental models that during the early phase after MCAo with the filament, early seizures were initiated in the ischemic hemisphere and then spread bilaterally to all cortical regions as demonstrated with 10 electrodes covering bilaterally frontal, parietal, occipital and temporal areas (Williams and Tortella, 2002; Hartings et al., 2003). Similar kinds of findings have been obtained from the photothrombosis model at later time points (Kelly et al., 2001).

In humans, ictal EEG recordings are rarely obtained during routine epilepsy assessment. However, interictal epileptiform discharges, i.e. spikes can be used to support a diagnosis of an epileptic disorder and to localize the epileptogenic foci. In the present study, increased spiking activity was observed at 12 months after ET-induced MCAo, but not in the age-matched sham-treated animals. These results suggest that although late seizures occur rarely, excitability might be increased during the long term follow-up of the ischemic animals with ET-induced MCAo.

6.2.2 Lesion size, location and depth

In humans, a large lesion size on computed tomography correlates with the occurrence of seizures and epilepsy (Gupta et al., 1988; Ryglewicz et al., 1990; Bladin et al., 2000). Furthermore, an anterior location in the MCA territory seems to be associated with development of seizures and epilepsy (Burn et al., 1997; Lamy et al., 2003). In contrast to the human data, the results from the present and previous experimental studies imply that the large infarcts in the MCA territory rarely induce seizures in rats, whereas the development of epilepsy is observed more often after small cortical photothrombotic lesions (Kelly et al., 2001; Kharlamov et al. 2003; Kelly et al., 2006). Furthermore, in the present study, those epileptic rats, both after ET-induced MCAo and photothrombosis, did not exhibit the largest lesions in their study groups.

In humans, certain brain regions are often associated with seizures after stroke, e.g., the cerebral cortex (Bladin et al., 2000; Dhanuka et al., 2001; Lossius et al., 2002). The location of the lesion seems to play an important role also in epileptogenesis after experimental stroke. Previously, it was reported that the occurrence of seizures is most frequent in rats with photothrombotic lesions either in the midfrontal (1.2 mm rostral and 1.8 mm lateral to the bregma) or the frontoparietal cortex (1.8 mm caudal and 2.2 mm lateral; Kelly et al., 2001). In the present study, photothrombosis induced epilepsy in 7 rats of 36 when the lesion center was focused at a site located above the hippocampus (1.8 mm posterior and 2.2 mm lateral). The effect of lesion location on epileptogenesis after the photothrombosis was investigated by preparing one group of rats with lateral coordinates (0.5 mm anterior and 3.7 mm lateral). No electrographic seizures were observed in the six rats with lateral coordinates during 14 days of continuous EEG-recordings at 8 and 10 months after photothrombosis. These present and previous findings suggest that the location of the lesion might play a role in the development of epilepsy after the focal cerebral ischemia (Kelly et al., 2001).

The depth of the cortical photothrombotic lesion has been linked to the extent and grade of reduced inhibition. Electrophysiological recordings suggest that if the lesion affects cortical layers that extend to the subcortical white matter, then ipsi- and contralateral depression may be observed, instead of a slight ipsilateral change followed by a shallower lesion (Buchkremer-Ratzmann and Witte, 1997). Consistently, shallower and smaller lesions were less effective in inducing epilepsy after cortical photothrombosis in aged rats (Kelly et al., 2001). In the present study, however, the lesion depth in epileptic animals varied from a shallow layer I lesion to a deep layer VI lesion.

6.2.3 Behavioral recovery

In humans, early seizure occurrence may be related to a better outcome of the stroke survivors (Reith et al., 1997), whereas late seizures may be followed by transient or permanent worsening of the neurological deficit (Bogousslavsky et al., 1992; Lamy et al., 2003; Vespa et al., 2003; De Reuck et al., 2006a). Similarly, faster recovery is associated with the occurrence of rare stimulated seizures after the brain lesion in animals (Feeney et al., 1987; Hernandez and Schallert, 1988), whereas recovery might be hindered following the appearance of seizures from 2 to 6 days after the brain insult (Hernandez and Warner, 1995). In the present study, no association was found between early seizures or spiking activity and

the behavioral results following ET-induced MCA occlusion. Unfortunately, the behavioral data could not be compared between rats with and without late seizures due to the low number of rats developing seizures. Because the number of rats in the groups was low, more studies will be needed to investigate the association between behavioral recovery and seizures after focal cerebral ischemia.

6.3 Comparison between photothrombosis and other models of spontaneous seizures

In the present study, the highest number, i.e. 18% of the rats, had seizures after photothrombosis. In contrast, the number of rats developing seizures is significantly higher in other models of spontaneous seizures: 43-50% after the lateral fluid percussion (Kharatishvili et al., 2006), 45-59% after kainate injection (Stafstrom et al., 1992; Mascott et al., 1994), 88-91% after electrically stimulated status epilepticus (Bertram and Cornett, 1993; Nissinen et al., 2000), and 90-100% after pilocarpine injections (Priel et al., 1996).

6.3.1 Electrophysiological findings

In the present study, the first seizure was observed at 71 d after the cortical photothrombosis. It is important to state that video-EEG monitoring was started 2 months after experimental stroke. Continuous video-EEG study is, however, needed to assess the duration of post-stroke epileptogenesis accurately. In the ET model, the epileptogenesis phase seems to be more prolonged than in the photothrombosis model, because the first late seizure was recorded 6 months after the ischemia induction. In the individual animals, the latency period varied from 10 weeks to over 10 months. This is consistent with the model of traumatic brain injury, where the latency period can vary from 7 weeks to 1 year (Kharatishvili et al., 2006). In contrast, the first spontaneous seizures occur consistently about at 1 month following chemical or electrical status epilepticus induction (Cavalheiro et al., 1991; Bertram and Cornett, 1993; Nissinen et al., 2000; Stafstrom et al., 1992). In these models, the longest latency period has ranged from 30 to more than 80 days (Stafstrom et al., 1992; Bertram and Cornett, 1993; Cavalheiro et al., 1991; Nissinen et al., 2000), which is clearly shorter than the 10 months found in the present study.

In this study, the seizure frequency was 0.21 seizures per recording day for the single epileptic rat that exhibited seizures after ET-induced MCAo. Following cortical photothrombosis, the mean seizure frequency was 0.39 seizures per recording day. In

chemical and electrical stimulation models of spontaneous seizures, the seizure frequency can be significantly higher (Cavalheiro et al., 1991; Mello et al., 1993). For example, one rat was reported to experience over 120 spontaneous seizures during one day after the status epilepticus with electrical stimulation of the amygdala (Nissinen et al., 2000).

6.3.2 Learning and memory impairments after seizures

Previous experimental studies have indicated that both brief and prolonged seizures can alter the learning and memory functions of the animals. The stimulation of even a few seizures in the dorsal hippocampus can disrupt the performance in the Morris water maze as assessed by direct swims to the hidden platform (Hanneson et al., 2004). When rats are kindled twice daily in the dorsal hippocampus until three fully generalized seizures are evoked, the animals require more time to escape to the platform and use less direct routes (Hannesson et al., 2001). It has been suggested that this is a reflection of disruption of spatial learning and/or short-term memory (Hannesson et al., 2001). Seizures have also been reported to disrupt learning and memorizing the place of the platform in different status epilepticus models (Halonen et al., 2001a; Halonen et al., 2001b; Mohajeri et al., 2003). In addition, emotional learning and memory, as assessed by the fear conditioning test, is claimed to be disrupted by seizures (Szyndler et al., 2002; Kemppainen et al., 2006). Unfortunately, the effect of seizures on the spatial and emotional learning and memory after focal cerebral ischemia will need to be investigated in future studies, because the number of rats in the groups was too low in the present study to draw any meaningful conclusions.

6.4 Histological findings

6.4.1 Hippocampal cell damage

In addition to the lesion, cell loss and gliosis may be observed in the hippocampus of stroke patients (Leverenz et al., 2002). Previously, it was shown that global cerebral ischemia can induce loss of GABAergic interneurons and terminals with a concomitant increase in the glutamatergic terminals in the CA3 region of the hippocampus after 2 months (Epsztein et al., 2006). These changes were associated with spontaneous interictal epileptiform discharges, a reduction in the frequency of inhibitory postsynaptic potentials and an increase in the frequency of glutamatergic postsynaptic currents in the CA3 region (Epsztein et al., 2006). Further, a positive correlation exists between seizure activity and the severity of the

hippocampal lesion 3 months after the intrahippocampal injection of ET in 12 or 25-day-old rat pups (Mateffyova et al., 2006). In contrast, following cortical photothrombosis, the hippocampus seems to be spared from injury as shown by Nissl and Fluoro-Jade-B stainings and glutamic acid decarboxylase-65/67 mRNA labeling (Frahm et al., 2004; Kharlamov et al., 2007). In the present experiment, no estimation of the cell loss was performed after the intraluminal filament model of MCAo. Following ET-induced MCA occlusion, the severity of neuronal damage was estimated in the granule cell layer, hilus, CA3 and CA1 regions. The results indicated that ET-induced MCAo had not induced any major cell loss in the hippocampus. The semiquantitative method used, however, may not be sensitive enough to detect subtle differences between ischemic and control rats. Following photothrombosis, stereological techniques were utilized to count the number of the hilar neurons. No difference was found in the hilar cell number between the rats with photothrombosis and control treatment.

This study also aimed to explore whether the occurrence of late seizures was associated with neuronal damage in the hippocampus. Epilepsy-associated hippocampal sclerosis has been reported to be characterized by selective damage to target cells of the mossy fibers in the hilus and CA3, where as CA2 and the dentate gyrus are better preserved (Mathern et al., 2002). Hippocampal sclerosis is observed in association with seizures both in humans and animals (Cavazos et al., 1994; Buckmaster and Dudek, 1997; Mathern et al., 1997; Proper et al., 2000; Mathern et al., 2002). However, there is variation in the epilepsy-associated hippocampal damage, because there are patients with lesion-associated epilepsy who exhibit no significant neuropathological changes in the hippocampus (Blumcke et al., 2000). In the present experiment, the severity of the neuronal damage in the hippocampus could not be compared between epileptic and non-epileptic rats after ET-induced MCAo because only one rat developed epilepsy. Following photothrombosis, the number of neurons in the hilus did not differ between those rats with and those without seizures.

6.4.2 Aberrant mossy fiber sprouting

In the present study, the three focal cerebral ischemia models did not induce observable increase in the mossy fiber sprouting in rats without epileptic seizures when scoring was performed from Timm's-stained sections. In contrast to focal cerebral ischemia models, global cerebral ischemia does induce 30-50% loss of CA3 pyramidal cells and 67% of the rats

demonstrate supragranular mossy fiber sprouting in the dentate gyrus after 100 d (Onodera et al., 1990). Age can also have an effect on mossy fiber sprouting since Timm's staining can be found in the supragranular layer of older people (Cassell and Brown, 1984). In the present experiments, the study design in the ET model allowed observations from two groups of different aged rats. In agreement with human data, the mossy fiber sprouting was slightly denser in the older 12-months old rats when compared to younger 6-months old rats after ET-induced MCAo.

One aim was to investigate whether aberrant sprouting of granule cell axons would associate with the occurrence of late seizures after focal cerebral ischemia. As shown previously, both dendrites and axons can undergo changes during epileptogenesis (Buckmaster and Dudek, 1997; El Bahh et al., 1999; Ribak et al., 2000; Kato et al., 2001). In the global cerebral ischemia model, structural alterations in the dendrites of CA3 pyramidal cells have been associated with the occurrence of convulsions (Semchenko et al., 2001). In the dentate gyrus, the reorganization of granule cell axons, i.e. mossy fiber sprouting, has been detected in both epileptic humans and animals (Cavazos et al., 1991; Buckmaster and Dudek, 1997; El Bahh et al., 1999; Nissinen et al., 2001; Kharatishvili et al., 2006). Previously, it has been shown that the severity of the cell loss in the hilus associates with the aberrant sprouting of mossy fibers (Buckmaster and Dudek, 1997; El Bahh et al., 1999) and that the frequency of spontaneous seizures can correlate positively with mossy fiber sprouting (Mathern et al., 1997). Since there was no difference in the hippocampal cell damage between epileptic and non-epileptic animals and the seizure frequency was low, it was anticipated that there would be only a mild increase in the mossy fiber scores of epileptic animals. After ET-induced MCAo, no aberrant mossy fiber sprouting was detected in the rat with late seizures. In contrast, after cortical photothrombosis seizures occurrence was associated with a slightly denser bilateral sprouting of mossy fibers. In summary, the results of the mossy fiber sprouting assessment after the cortical photothrombosis indicate that the epileptogenesis, in addition to the evoking lesion might also evoke changes in distant brain structures.

6.5 Cerebral changes affecting excitability after focal cerebral ischemia

Previous studies indicate that the inhibitory interneuron survival and receptor expressions around the lesion site may be different between the large artery occlusion models and cortical photothrombosis (Luhmann et al., 1995; Schiene et al., 1996; Neumann-Haefelin

et al., 1998; Que et al., 1999b; Jolkkonen et al., 2003; Sommer et al., 2003). This may lead to only a moderate and transient decrease in the inhibition adjacent to the lesion following large artery occlusions, whereas after the cortical photothrombosis, a markedly decreased level of inhibition is observed (Neumann-Haefelin and Witte, 2000; Witte and Freund, 1999). In addition, excitability might be affected by the loss of control from the damaged structures to the area of interest (von Giesen et al., 1994; Classen et al., 1997). Experimental results also indicate that contralateral frontoparietal infarct can have an inhibitory influence on the seizures originating from the amygdala (Schwartz et al., 1983).

Several changes have been related to the enhanced excitability in the cortex following the photothrombosis. First, the neurons may be more likely to generate action potentials due to a less negative membrane potential (Witte and Freund, 1999). Second, the decreased GABA-mediated inhibition may result from a decreased number of interneurons, alterations in the GABA receptor expression or changes in the GABA receptor subunit composition (Schiene et al., 1996; Neumann-Haefelin et al., 1998; Neumann-Haefelin et al., 1999; Que et al., 1999b; Liu et al., 2002; Frahm et al., 2004). Third, changes in NMDA receptor expressions may contribute to the hyperexcitability after cortical photothrombosis (Buchkremer-Ratzmann et al., 1998; Mittmann et al., 1998; Kim and Todd, 1999; Que et al., 1999a). Fourth, glial cells accumulate at the lesion border after focal cerebral ischemia and can affect brain excitability, for example, by modulating the extracellular ion balances or by secreting signaling molecules (Watson et al., 1985; White et al., 1986; Bidmon et al., 1997; Fedele et al., 2005). In addition, although hippocampal neurons seem to survive after the photothrombosis, their function and activities might be altered (Diehm et al., 2003; Frahm et al., 2004; Kharlamov et al., 2007). Enhanced neurogenesis is also observed in the subgranular zone of the dentate gyrus after photothrombosis and the newly born cells might play a role in epileptogenesis after focal cerebral ischemia (Parent et al., 1997; Kluska et al., 2005). Although both structural and functional modifications have been associated with excitability changes after the cortical photothrombosis, their role in epileptogenesis after focal cerebral ischemia still needs to be elucidated.

6.6 Future perspectives

The results from previous and these present studies suggest that the photothrombosis model produces a higher percentage of the rats developing epilepsy compared to that obtained with the large artery occlusion models (Kelly et al., 2001; Kharlamov et al., 2003; Kelly et al., 2006). The cortical photothrombosis model also incorporates features of secondary epileptogenesis (Dudek and Spitz, 1997). A latency time of 10 weeks to over 10 months was observed between the induction of cortical photothrombosis and the occurrence of spontaneous seizures (Kharlamov et al., 2003). In addition, cortical photothrombosis was associated with changes not only in the lesion site but also in distant areas. In the present study, the axonal sprouting, i.e. mossy fiber sprouting, was slightly increased in the dentate gyrus of the hippocampus of those epileptic rats obtained with the photothrombosis model. Previous studies also imply that the function of neurons in the ipsilateral hippocampus might be altered after photothrombosis (Diehm et al., 2003; Kharlamov et al., 2007).

However, further studies are needed to investigate the validity of the photothrombosis model as a tool to study epileptogenesis after stroke. The possible toxicity of the Rose Bengal dye and its effects on the seizure occurrence after cortical photothrombosis needs to be investigated. The reproducibility of epileptogenesis after cortical photothrombosis needs to be tested. It also remains to be investigated whether seizures respond to AED treatment. In addition, future studies will need to strive to elucidate the causes and consequences of epilepsy after focal cerebral ischemia and how they correspond to human post-stroke epilepsy.

Compared to other models of spontaneous seizures, focal cerebral ischemia models induce a lower number of epileptic rats with a low seizure frequency and with a variable latency time between the onset of ischemia and the occurrence of late seizures. All of these variations will limit the usefulness of focal cerebral ischemia models in the development of new AEDs. Focal cerebral ischemia models, especially photothrombosis, however, may be applied in basic research when investigating the underlying mechanisms of epileptogenesis during the time course from the initial injury to the process of epileptogenesis, and to the occurrence of spontaneous, recurrent seizures.

7 SUMMARY AND CONCLUSIONS

The aim was to investigate the occurrence of post-stroke epilepsy in rats using three models: the transient intraluminal filament model of MCAo (I, IV), ET-induced MCAo (II, IV), and the cortical photothrombosis with Rose Bengal (III, IV). The main findings are summarized as follows:

(1) Differences in the development of epilepsy between models. No electrographic seizures were observed in the rats with the filament model of MCAo. After ET-induced MCAo, however, one rat out of 26, and after the photothrombosis 7 rats out of 36, did develop seizures. Thus, the percentage of rats with epilepsy seems to vary between the stroke models. Taken together, previous and these present studies indicate that the location of the lesion, rather than the size, seems to play a role in determining whether epileptogenesis will occur after experimental focal cerebral ischemia.

(2) Seizure characteristics. The latency time from focal cerebral ischemia to the first late seizure varied from 10 weeks to over 10 months and the mean seizure frequency from 0.21 to 0.39 per recording days in the ET- and photothrombosis model, respectively. The mean seizure duration was about 2 minutes. Behaviorally, these seizures manifested as secondarily generalized.

(3) Histology. After ET-induced MCAo, the single epileptic rat did not exhibit aberrant mossy fiber sprouting in the dentate gyrus. After photothrombosis, the hippocampal hilar cell number did not differ between the rats with and without seizures, but the occurrence of seizures was associated with a slightly denser bilateral mossy fiber sprouting.

(4) Behavior. The occurrence of early seizures or increased spiking activity after ET-induced MCAo did not associate with poor performance in the behavioral tests.

In conclusion, post-stroke epilepsy seems to be difficult to reproduce in rat focal cerebral ischemia models. Of the three investigated stroke models, cortical photothrombosis with Rose Bengal dye in adult Sprague-Dawley rats might be suitable for studies of epileptogenesis after small cortical thrombotic vessel occlusion.

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APPENDIX:

ORIGINAL PUBLICATIONS

I–IV

I

LONG-TERM FUNCTIONAL CONSEQUENCES OF TRANSIENT OCCLUSION OF THE MIDDLE CEREBRAL ARTERY IN RATS - A 1-Y FOLLOW-UP OF THE DEVELOPMENT OF EPILEPTOGENESIS AND MEMORY IMPAIRMENT.

Karhunen H, Pitkänen A, Virtanen T, Gureviciene I, Pussinen R, Ylinen A, Sivenius J, Nissinen J, Jolkkonen J.

Epilepsy Research 2003;54(1):1–10.

II

A LONG-TERM VIDEO-EEG AND BEHAVIORAL FOLLOW-UP AFTER ENDOTHELIN-1 INDUCED MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS.

Karhunen H, Nissinen J, Sivenius J, Jolkkonen J, Pitkänen A.

Epilepsy Research 2006;72(1):25–38.

III

EPILEPTOGENESIS AFTER CORTICAL PHOTOTHROMBOTIC BRAIN LESION IN RATS.

Karhunen H, Bezvenyuk Z, Nissinen J, Sivenius J, Jolkkonen J, Pitkänen A.

Neuroscience, in press.

IV

EPILEPTOGENESIS AFTER EXPERIMENTAL FOCAL CEREBRAL ISCHEMIA.

Karhunen H, Sivenius J, Jolkkonen J, and Pitkänen A.

Neurochemical Research 2005;30(12):1529−1542.