Charles University
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Consequences of early ischemic hippocampal lesion in rats

Ph.D. Thesis

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CONTENTS

List of abbreviations

AP-1.....activator protein 1
ATP.....adenosine 5'-triphosphate
ICAM-1.....intracellular adhesion molecule-1
AMPA .....α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
FGF.....basic and acidic fibroblastic growth factor
BDNF.....brain derived neutrophic factor
Ca2+.....calcium
CRE.....cAMP response element
CNS.....central nervous system
CBF.....cerebral blood flow
Cu^{2+}.....copper
cAMP.....cyclic-adenosine monophosphate
cGMP.....cyclic-guanosine monophosphate
coxs.....cyclooxygenases
DNA.....deoxyribonucleic acid
ERGs.....early response genes
EEG.....electroencephalogram
ECE.....endothelin-converting enzyme
ETs..... endothelins
fVL.....factor V Leiden
Fe^{2+}.....ferrum
FFAs.....free fatty acids
GDNF.....glial derived growth factor
HSP.....heat shock proteins
h.....hour
H_2O_2.....hydrogen peroxide
Cl^-.....chloride
INF-γ.....interferon-γ
IL-1.....interleukin-1
IL-2.....interleukin-2
Mg^{2+}.....magnesium
Mn.....manganese
min.....minute
MCAO.....middle cerebral artery occlusion
MCP-1.....monocyte chemoattractant peptide-1
NGF.....nerve growth factor
NO.....nitric oxid
NOS.....nitric oxide synthase
NMDA.....N-methyl-D-aspartate
NOS.....NO syntase
Osteo.....osteopontin

PLs..... phosholipases

PLA_2..... phospholipase A2

PLC.....phospholipase C

PARP.....polyADP-ribose polymerase

K^+.....potassium

ROS.....reactive oxygen species

s.....second

SE.....status epilepticus

Na^+.....sodium

SHR... .....spontaneously hypertensive rat

SOD.....superoxide dismutase (SOD)

TGF.....transforming growth factor super family

TNF..... .....tumor necrosis factor

H_2O.....water

Zn^{2+}.....zinc

I. INTRODUCTION

1. Stroke

Stroke is an important cause of neurological morbidity in infants and children, with an incidence of approximately 8 cases per 100 000 per year *(Giroud et al. 1995)*. In children, stroke is often under-recognized/unrecognized because of variation in the presentation, evaluation, and diagnosis. In many cases, asymmetry of reaching and grasping, failure to reach developmental milestones, or post-neonatal seizures that appear some months after the acute event are the only mark of previous stroke.

1.1. Incidence and prognosis

The outcome after neonatal stroke varies among studies due to differences in functional measures, stroke type, length of follow-up, and clinical sample studies. Hemiplegia, language deficits (prose and comprehension), visual impairments, seizures, and learning disorders are long-term sequelae of stroke (*Lynch et al. 2002)*. In the USA, on the basis of a review of epidemiological studies of perinatal stroke over the last 30 years, 40% of infants with perinatal stroke were later neurologically normal, 57% were neurologically or cognitively abnormal, and 3% died *(Lynch and Nelson 2001)*.

The death rate from ischemic stroke is much higher in the first year after birth than during childhood and the first half-century of life (Figure 1) *(Murphy SL. Deaths: final data for 1998: National Vital Statistics Report, vol 48, number 11. Hyattsville, MD: National Center for Health Statistics, 2002)*. Despite the decrease in childhood deaths from intracerebral or subarachnoid haemorrhage, deaths from ischemic stroke have not declined over recent decades.

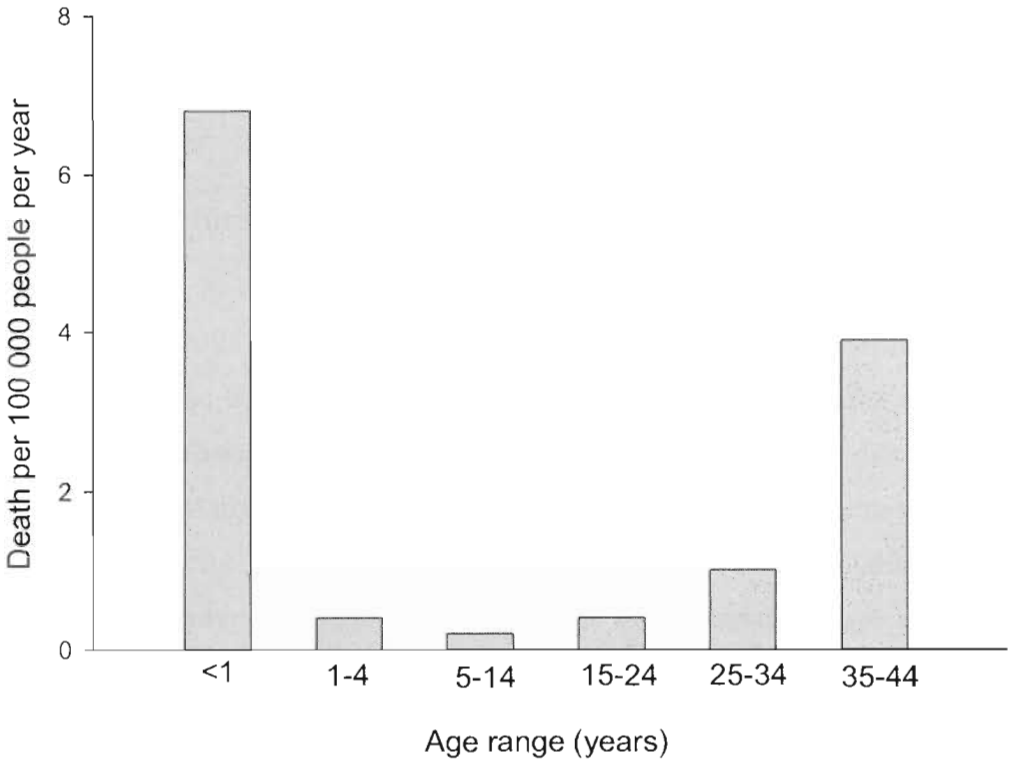

Figure 1. Mortality rates for ischemic stroke in the USA

USA data show that stroke mortality is maximal in the first year of life. Despite this, Czech statistic concentrates only on elder people and no statistic data showing the incidence of stroke in Czech newborns is available.

1.2. Etiology

It is common to recognize 3 types of strokes; hemorrhagic stroke, sinovenous thrombosis stroke, and arterial ischemic stroke. The incidence of arterial strokes is 93 out of 100 000 births. Arterial ischemic stroke usually presents with lethargy or seizures, causing 12% to 14% of neonatal seizures *(Andrew et al. 2001).* This stroke usually involves large vessels, most commonly the middle cerebral artery. Arterial ischemic stroke in neonates is usually caused by a thromboembolism from intracranial

or extracranial vessel, the heart, or the placenta. There is a preponderance of left-hemisphere lesions.

1.3. Risk factors

Perinatal stroke is not the same fundamental entity as adult stroke, because the causes for it can be substantially different in the childhood relation to the age. The neonatal coagulation system is immature and more susceptible to clot formation than the adult one. Many factors associated with arterial thrombotic events in the newborn, such as the factor V Leiden (fVL) and prothrombin events mutations, are chiefly associated mainly with venous disease rather than arterial disease as in adults. In children, coexisting infections seem to predispose to arterial stroke, whereas in adults venous thromboembolic disease is affected much less by inflammation.

1.4. Stroke and seizures

Seizures are the most common and specific presentations of neurological disease in the neonatal period, with a reported incidence of 1.5-14/1000 live births. The wide variation in the reported incidence is due to the wide variation among the population studied. Commonly seizures occur in childhood stroke and that a later onset of initial seizure and/or the presence of cortical involvement are risk factors for recurrent seizures *(Yang et al. 1995)*. Many seizures are focal and may occur in the absence of other signs of neonatal encephalopathy-such as abnormalities of tone or feeding, or depressed level of alertness. Clinical observations suggest that prolonged seizures can deteriorate ischemia induced brain injury.

1.5. Cerebral ischemia

In cerebral ischemia, blood flow within the brain is reduced severely or totally blocked *(Hossmann 1999)*. There are two major forms: global (forebrain) ischemia and focal (stroke) ischemia *(Siesjö et. al 1990; Siesjö et al. 1995; Siesjö 1992a; Siesjö 1992b)*. In global ischemia, the reduction of cerebral blood flow (CBF) involves the whole brain or forebrain. In focal cerebral ischemia, the disturbance in CBF occurs in a localized area.

1.6. Focal ischemia

The neonatal brain is vulnerable selectively to focal ischemia due to enhanced post-synaptic development of the NMDA receptor *(Yager and Thornhill 1997)*.

We can distinguish between a core of tissue (the focus) with relatively dense ischemia and perifocal tissues (penumbra), which are less densely ischemic because they receive a collateral blood supply from leptomeningeal branches of the major arteries. The core and penumbra are dynamic in space and time. The CBF is reduced to <15% in the core and <40% in the penumbra. Both core and penumbra zone become infarcted *(Zhao et al. 1997)*, whereas the extra penumbral zone only shows death of isolated neurons.

There are major differences in physiology and biochemistry between the core and penumbra and, indeed, these represent another way in which the regions are differentiated (Figure 2) *(Dirnagl et al. 1999)*.

Events in the penumbra are less drastic, although they still can lead to infarct owing to ongoing excitotoxicity or to secondary deleterious phenomena, such as a spreading depression (a depolarization wave that propagates at a rate of 2-6 mm/min triggered electrically, mechanically or by various toxic factors such as high extracellular potassium (K^+) concentration), free radical production, post-ischemic inflammation and apoptosis. Although there is ample evidence that the penumbra exists in human stroke patients *(Furlan et al. 1996; Read et al. 1998)*, the extent and

temporal dynamics of this area are less well defined: it might be smaller and exist for a shorter time period in humans *(Kaufmann et al. 1999).*

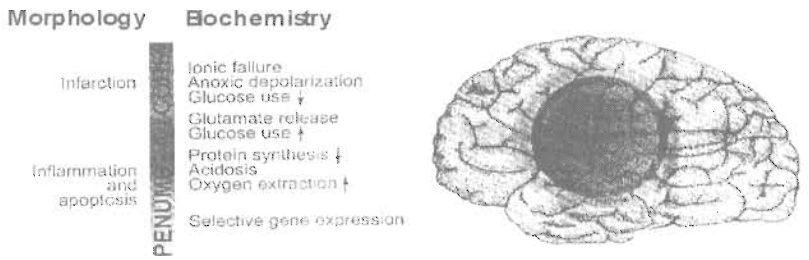

Figure 2. The ischemic core and penumbra *(Dirnagl et al. 1999).*

2. Pathophysiology of cerebral ischemia

Focal impairment of CBF restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients *(Martin et al. 1994).* Because of energy depletion, membrane potential is lost and neurons and glia depolarize *(Katsura et al. 1994).* Thus, somatodendritic as well as presynaptic voltage-dependent calcium (Ca^{2+}) channels are activated and excitatory amino acids are released into the extracellular space. At the same time, the energy-dependent processes, such as presynaptic reuptake of excitatory amino acids, are impeded, therefore there is a further increase of the glutamate accumulation in the extracellular space (Figure 3) *(Dirnagl et al. 1999).*

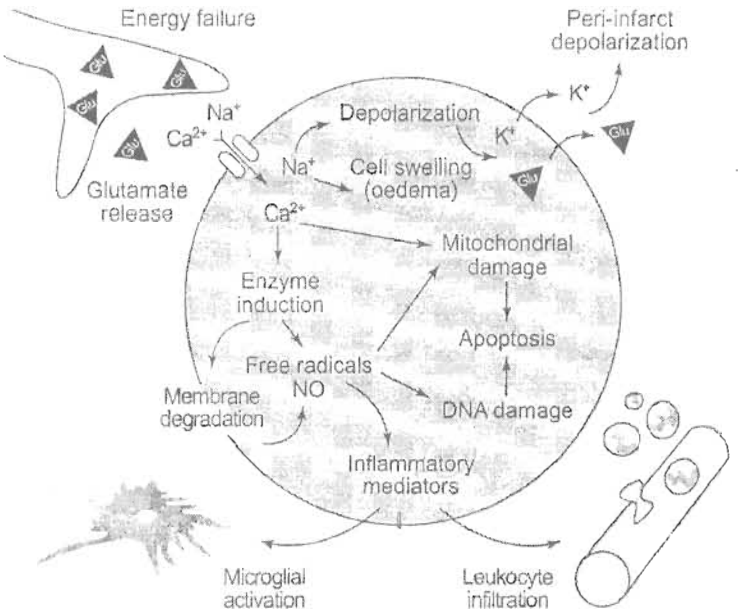

Figure 3. Pathophysiological mechanisms in the focally ischemic brain *(Dirnagl et al. 1999).*

Ischemic brain injury results from a complex sequence of pathophysiological events that evolve over time and space. The major pathogenic mechanisms of this cascade include excitotoxicity, peri-infarct depolarizations, free radical production, inflammation and cell death (Figure 4) *(Dirnagl et al. 1999).*

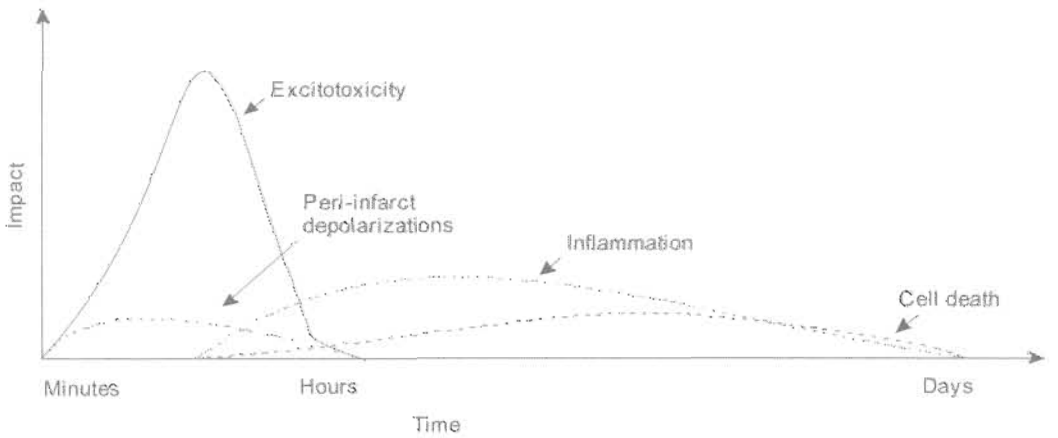

Figure 4. Putative cascade of damaging events in focal cerebral ischemia *(Dirnagl et al. 1999).*

2.1. Excitotoxicity

Glutamate is the major excitatory neurotransmitter working at a variety of excitatory synapses in the nervous system. Pharmacological studies in rodents and recent clinical studies in humans have shown that extracellular concentration of glutamate increases to toxic levels in ischemic neurons *(Benveniste et al. 1984; Bullock et al. 1995; Davalos et al. 1997; Hagberg et al. 1985).* Furthermore, several studies indicate that glutamate receptor-mediated excitotoxicity plays a key role in neuronal cell death and that it is more critically involved in the developing brain than in the adult brain *(Barks and Silverstein 1992; Choi and Rothman 1990; Johnston 2001; Martin et al. 1997; McDonald et al. 1988).*

Cell death due to excitotoxicity occurs in many types of cells in the newborn brain, and the initial trigger seems to be the impairment of glutamate uptake by glia, resulting in overactivation of glutamate receptors *(McDonald and Johnston 1990, Johnston et al. 2001).* It is due to the fact that many excitatory circuits are enhanced during development to promote activity-dependent neuronal plasticity *(Cohen-Cory 2002, Sheng and Kim 2002).*

Glutamate as a di-carboxylic acid molecule with considerable structural flexibility, mediates excitatory synaptic transmission through activation of ionotropic glutamate receptors sensitive to NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) or kainate receptors and G-protein linked metabotropic glutamate receptors. The pathophysiological role of ionotropic glutamate receptor activation has been well established *(Choi 1998; Gill and Lodge 1997; Lipton and Rosenberg 1994).*

2.1.1. NMDA receptors

NMDA receptors mediate normal brain development and function by promoting proliferation and migration of neuronal precursors, and synaptic development and plasticity *(Komuro and Rakic 1993; McDonald and Johnston 1990).* However, they can be especially devastating under certain conditions in the immature brain.

NMDA receptors are fully activated under depolarization of plasma membrane that abolishes the inhibition of NMDA receptors by magnesium (Mg^{2+}). Since a brief (>3 min) activation of NMDA receptors is sufficient to trigger neuronal death, activation of NMDA receptors has been proposed as primary cause of neuronal death after focal cerebral ischemia.

NMDA receptors consist of NR1 subunits (a-h), NR2 (A-D) and NR3 (A, B) subunits *(Wenthold et al. 2003)*. The heteromeric NMDA receptors are highly permeable to calcium (Ca^{2+}) as well as sodium (Na^{+}) and potassium (K^{+}). Ca^{2+} influx through NMDA receptors mediates the early NMDA excitotoxicity, while Na^{+} influx contributes to the swelling of neuronal cell body.

The pharmacological and biophysical properties of the receptor are determined mainly by the NR2 (or NR3) subunits. On the other hand, the NR1 subunit is essential for the formation of functional ligand-gated ion channel *(Cull-Candy et al. 2001; Dingledine et al. 1988; Sucher et al. 1996)*. During development, the expression of the NR2 subunits changes from a relatively high level of subtype 2B during the first 2 postnatal weeks in the rat, to a predominance of the 2A subunit in the adult *(Gurd et al. 2002; Sheng et al. 1994; Zhong et al. 1995)*. This developmentally regulated alteration in the ratio of NR2A:NR2B is reflected in altered receptor properties, including increase Ca^{2+} flux on glutamate activation, which may contribute to the increased sensitivity of the neonatal brain to hypoxic-ischemic injury *(Johnston 1995)*.

2.1.2. AMPA receptors

AMPA receptors consist of a combination of GluR1-GluR4 subunits *(Hollmann and Heinemann 1994; Pellegrini-Giampietro et al. 1997)*. The key subunit in determining the ion channel properties is GluR2, which is responsible for Ca^{2+}-impermeability of these receptors. The receptor channel is mainly permeable to Na^{+} and K^{+} and likely subserves primarily rapid excitatory neurotransmission in the CNS. However, Ca^{2+}/Zn^{2+}-permeable form of AMPA receptors lacking GluR2 subunit was discovered recently. Most of them are formed in early neonatal life. They could

contribute to synaptogenesis and formation of neuronal circuitry during early development *(Pellegrini-Giampietro et al. 1997, Sanchez et al. 2001)*. However, later in life, this form of receptor is associated with neurological disorders and enhances the toxicity of endogenous glutamate *(Tanaka et al. 2005, Weiss and Sensi 2000)*.

In contrast to NMDA receptors, a prolonged (>60 min) activation of AMPA receptors (containing all subunits) is required to trigger neuronal death *(Koh et al. 1990)*.

2.1.3. Kainate receptors

There are five subunits that can constitute kainate receptors- KA1, KA2, GluR5, GluR6, and GluR7 *(Hollmann and Heinemann 1994)* which are permeable to Na^+ and K^+. While GluR5-7 homomers are functional kainate gated ion channels *(Herb et al. 1992; Schiffer et al. 1997)*, KA1 and KA2 only form functional channel as heteromers with subunits from the low affinity group *(Herb et al. 1992; Werner et al. 1991)*. Like AMPA-mediated slow excitotoxicity, a prolonged (>60 min) exposure to kainate is needed to trigger neuronal death.

2.2. Peri-infarct depolarizations

Adult brain tissue has a relatively high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production. However, the glucose metabolism in the immature brain differs from the adult in ways that confer both resistance and vulnerability to energy failure during ischemia. Rates of cerebral energy metabolism are low in the immature brain and utilize substrates other than glucose, i.e. lactate and ketone bodies β-hydroxybutyrate and acetoacetate, to satisfy its cerebral energy requirements *(Edmond et al. 1985; Nehling and Pereira de Vasconcelos 1993)*.

Ischemic insults both in mature and immature brain induce changes in the balance of ion gradients, e.g. Na^+, K^+, Ca^{2+}, and chloride (Cl^-) gradients across cellular

membranes, which are basically caused by reduced energy production *(Hansen et al. 1982; Jiang et al. 1992).* Failure to maintain the ion gradients results in depolarization of cell membranes. The depolarization results in activation of a variety of voltage-sensitive and receptor-operated ion channels *(Fujiwara et al. 1987; Silver and Erecinska 1990).* Under ischemic circumstances the activation of Ca^{2+} channels in neurons causes enhanced release of glutamate. The activation of Na^{+} channels also induces the release of glutamate via reverse transport of glutamate by the glutamate transporters *(Taylor et al. 1995).* Moreover, there is evidence that the release of glutamate is also enhanced by Cl^{-} channel activation induced by cell swelling under hypertonic conditions during ischemia *(Philips et al. 1997).*

The same cells can depolarize again in response to increasing glutamate or K^{+} levels, or both, which accumulate in the extracellular space. This phenomenon is called 'peri-infarct depolarization' *(Hossmann 1996)* and it can be observed for at least 6 to 8 h after the infarct onset.

There is a difference in occurrence of depolarization in the core and in the penumbra of the ischemic brain tissue. In the core region, cells can udergo an anoxic depolarization and never repolarize. This core anoxic depolarization lasts 1-3 min. Anoxic depolarizations do not depend on NMDA-type of glutamate receptors *(Grigg and Anderson 1990; Kral et al. 1993; Lauritzen and Hansen 1992; Rader and Lanthorn 1989; Xie et al. 1995)* and depend on non-NMDA glutamate receptors only very slightly *(Xie et al. 1995)* or not at all *(Lauritzen and Hansen 1992).* There is a concomitant extracellular K^{+} increase to ~ 70 mM, sometimes with transient periods of baseline values *(Gido et al. 1997; Nedergaard and Hansen 1993).* In the same time, there is a large decrease in extracellular Ca^{2+} due to the intracellular accumulation *(Harris and Symon 1984; Harris et al. 1981).* During the reperfusion period, 2 h after temporary focal ischemia, extracellular K^{+} level returns to control levels. Then it increases slightly (to 5 mM) in the following 24 h recirculation period. Despite this return to near normalcy, the insult produces severe damage with a large infarct including all of core *(Gido et al. 1997).* Level of adenosine 5'-triphosphate (ATP) falls down to ~ 25% of the basal values during the first 5 min after the ischemic insult in the core and remains at this level for 4 h *(Folbergova et al. 1992; Folbergova et al. 1995; Sun et al. 1995; Welsh et al. 1991).*

On the other hand, in penumbra zone, cells can repolarize, but the repolarization requires further energy consumption. The cells in penumbra are threatened by irregularly occurring depolarization waves (intraischemic depolarizations) accompanied by K^+ efflux and Ca^{2+} influx, by the damage of the microcirculation, and by chemical mediators of secondary ischemic damage (free radicals and other mediators of inflammatory reactions) that are released from the densely ischemic tissue *(Siesjö 1992a; Siesjö 1992b)*. These events can cause progressive neuronal damage, and an extension of the infarct into the penumbral zone *(Gingsberg and Pulsinelli 1994; Hossmann 1994)*. Since ionic gradient alterations in the penumbra are not irreversible, there is a time window for drug therapy *(Astrup et al. 1981; Strong et al. 1983; Symon 1980)*. On the contrary to anoxic depolarization, the frequency of intraischemic depolarizations is markedly dependent on both classes of ionotropic glutamate receptors *(Back et al. 1996; Chen et al. 1993; Iijima et al. 1992; Mies et al. 1994)*. Levels of ATP in the penumbra average ~ 50-70% of normal values during ischemia.

2.3. Free radical production

Activation of voltage-dependent Ca^{2+} channels and Ca^{2+}-permeable glutamate receptors due to energy failure results in production of free radicals through activation of prooxidant pathways including phosholipases, nitric oxide synthase (NOS), xanthine oxidase, and loss of mitochondrial potential *(Au et al. 1985; Dawson et al. 1992; McCord et al. 1985; Radi et al. 1994; Sevanian et al. 1983; Zhang et al. 1990)*. Others than Ca^{2+}, transition metals such as ferrum (Fe^{2+}), copper (Cu^{2+}), and zinc (Zn^{2+}) could also contribute to generation of free radicals in ischemia.

Endogenous defences that detoxify reactive oxygen species (ROS) in the brain include superoxide dismutase (SOD), which exists as Cu, Zn-SOD (SOD1) in the cytoplasm and manganese (Mn)-SOD (SOD2) in the mitochondria. Both of these enzymes actively scavenge oxygen free radicals by converting them to hydrogen peroxide (H_2O_2), which can then be effectively detoxified by the action of catalase or glutathione peroxidase and eliminated as water (H_2O). Both, catalase and glutathione

peroxidase are dependent on the cellular energy state. Clearly, when these systems fail (oxidative stress) as in hypoxia-ischemia (due to accumulation of Ca^{2+} in mitochondria), the brain suffers the consequences of oxidative damage to cellular macromolecules and death.

In contrast to ion and metabolite changes, there are few amounts of free radicals in the core with respect to penumbra *(Solenski et al. 1997)*. In the penumbra, free radicals level increases early during ischemia, remains elevated throughout 3 h, and then becomes further elevated with the onset of reperfusion. On the contrary, there is no increase in the core during the first 3 h of ischemia, however after perfusion the number of free radicals increases.

The immature brain differs from the adult in its sensibility to production of free radicals. In fact, the immature brain has poor antioxidant capabilities (especially glutathione peroxidase) and a high concentration of free Fe^{2+} *(Fullerton et al. 1998, Vannucci and Hagberg 2004)*.

2.3.1. Xanthine oxidase

Accumulation of Ca^{2+} in mitochondria induces activation of the neutral protease calpain. This results in conversion of xanthine dehydrogenase into xanthine oxidase. Xathine oxidase catalyzes oxidation of xanthine and hypoxanthine into uric acid, producing superoxide as a by-product *(McCord et al. 1985)*.

2.3.2. Transition metals

There is an increase in the brain level of the transition metals (Fe^{2+}, Cu^{2+}, and Zn^{2+}) in the brain during ischemia. These metals mediate formation of hydroxyl radical through the iron-catalyzed or copper-catalyzed Haber-Weiss reactions *(Haber and Weiss 1934)*.

2.3.3. Reactive nitrogen radicals

Nitric oxide (NO) plays a physiological role in blood vessels as vasodilator (endothelial-derived relaxing factor), but it also plays an important role in the pathogenesis of neuronal injury during cerebral ischemia *(Beckman and Koppenol 1996; Dirnagl et al. 1999).*

NO promotes oxidative damage by reacting with superoxide anion to form peroxynitrite (a strong oxidant), and by altering iron metabolism *(Beckman et al. 1990; Reif and Simmons 1990).* NO, either directly or through its derived species, causes energy failure, damages of DNA (deoxyribonucleic acid), inhibits DNA synthesis and triggers programmed cell death by activating poly (ADP-ribose) synthetase *(Bonfoco et al. 1995).* Furthermore, NO might exacerbate the damage by enhancing the post-ischemic release of excitatory neurotransmitters *(Montague et al. 1994).*

NO is synthesized by the enzyme NO syntase (NOS) after oxidation of L-arginine. NO may exert neurotoxicity or neuroprotenction depending upon the isoforms and localization of activated NOS *(Dalkara et al. 1994; Dirnagl et al. 1999).*Three isoforms of NOS have been characterized and cloned: neuronal NOS (nNOS or type I), inducible macrophage or 'immunological' NOS (iNOS or type II), and constitutive endothelial NOS (eNOS or type III) *(Griffith and Stuehr 1995).*

NO production is enhanced in the all steps of cerebral ischemia. Immediately after the onset of ischemia, parenchymal and microvascular NO production are driven by upregulation of eNOS. In this step, this NO is protective, mainly by promoting vasodilation. However, after development of ischemia, NO produced by overactivation of nNOS and, later, NO release by de novo expression of iNOS contribute to the brain damage *(Moro et al. 2004).*

2.3.4. Phospholipase activation

Free fatty acids (FFAs), and in particular free arachidonic acid, are released during cerebral ischemia as a consequence of the activity of both phospholipase C (PLC) (activated by depolarization) and phospholipase A_2 (PLA_2) (activated by increased

intracellular Ca^{2+}) *(Drenth et al. 1976; Moskowitz et al. 1984, Muralikrishna Adibhaltla R and Hatcher JF 2006).* Elevated concentrations of these FFAs persist into the early reperfusion phase *(Abe et al. 1987; Bazan 1970; Katsuki and Okuda 1995; Rehncrona et al. 1983; Umemura 1990; Yasuda et al. 1985; Yoshida et al. 1980).* During reperfusion arachidonic acid is converted into eicosanoids with concomitant production of superoxide by cyclooxygenases (coxs) and 5-lipooxygenase *(Kontos 1987; Krause et al. 1988).*

2.4. Expression of early responses genes and inflammation

After the interruption of CBF, tissue injury begins with inflammatory reaction, which is a common response of the cerebral parenchyma to various form of injury. This requires the infiltration of leukocytes, both polymorphonuclear leukocytes and monocytes/macrophages (but not lymphocytes), which are the cellular mediators of subsequent microvessel obstruction, edema formation, cellular necrosis, and tissue infarction *(Huang et al. 2006).* The early stage of inflammation, which starts a few hours after the onset of ischemia, is characterized by the expression of adhesion molecules on the vascular endothelium as well as by circulating leukocytes. However, the expression of a broad range of up-regulated transcription factors and heat-shock proteins occurs before inflammatory reaction alone (Figure 5) *(Arvin et al. 1996).*

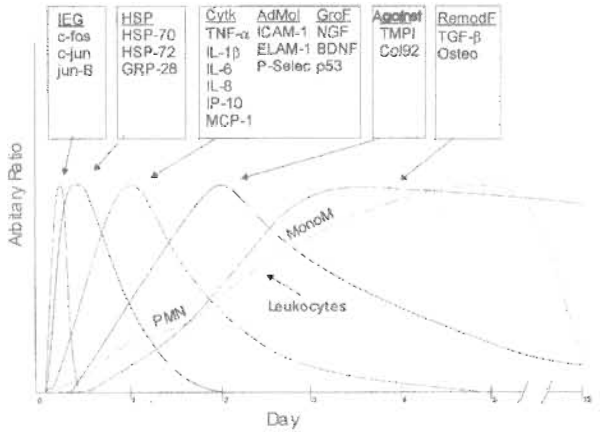

Figure 5. Time course of gene expression following focal ischemia in rat cortex induced by middle cerebral artery occlusion *(Arvin et al. 1996).*

According the Figure 5 in the first phase, increased intracellular concentration of Ca^{2+} leads to the transcriptional activation of so-called 'cell-death genes'. Within minutes of ischemia onset, early response genes (ERGs), such as c-fos, c-jun and genes that encode zinc-finger proteins such as zif268, are expressed. However, they disappear very quickly. ERGs triggers the expression of a wide variety of target genes by the activation of activator protein 1 (AP-1) and cAMP response element (CRE) promoter elements *(Akins et al. 1996)*.

Consequently, in the second phase, the expression of heat shock proteins (HSP70, HSP72) within 1-2 hours is initiated. Their expression is downregulated within 1-2 days *(Nowak and Jacewicz 1994; Sharp and Sagar 1994)*. HSP90 is constitutively present in cells and is bound to heat shock elements. As a result of injury, the complex between HSP90 and shock elements dissolves and heat shock elements bind to denatured proteins in an effort to preserve their functional ability. Then HSP90 can then enter the nucleus where it binds to specific DNA sites and promotes the synthesis of HSP70, HSP72 and HSP27 among others. These inducible HSPs are protective against neuronal damage mainly by preventing protein denaturation. Gene expression of HSPs is limited to regions in which CBF decreases below 50% of normal, and it seems to occur only in injured cells that are vital after ischemic insult.

A third phase starts after about 1 to 3 h and comes up to a peak at about 12 h. By 2 to 3 days, it is usually completely downregulated, however for some genes, it lingers on for several days. In this phase, the expression includes genes encoding for inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, monocyte chemoattractant peptide-1 (MCP-1)) is taking place. They are produced by a variety of activated cell types, including endothelial cells, platelets, leukocytes and fibroblasts, to serve as intercellular messengers mediating inflammatory and immunological responses. After ischemia, their synthesis is increased also in microglia, astrocytes, and neurons *(Huang et al. 2006)*. Microglial cells as the primary immunodefector cells of the CNS, are especially activated in the penumbra *(Tarozzo at al. 2002)*. The overall role of microglia is not clear at present. It is very likely that microglia at different time points plays different roles, with protective or regenerative activities occurring days or even weeks after the onset of ischemia *(Mergenthaler et al. 2004)*.

Cytokines regulate the interaction of immune cells such as T cells, B cells and monocytes/macrophages and thereby orchestrate immune responses *(Arai et al. 1990b)*. They can have beneficial effects by promoting cell growth, but on the other hand, they can be toxic and facilitate expansion of brain lesions *(Merrill and Benveniste 1996)*. Cytokines induce expression of adhesion molecules in the cerebral vasculature (intracellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule (ELAM-1), P-selectins and E-selectins) and initiate the inflammatory reaction that occurs in the ischemic brain. Adhesion molecules interact with complementary surface receptors on neutrophils. The neutrophils, in turn, adhere to the endothelium, cross the vascular wall and enter the brain parenchyma. Macrophages and monocytes follow neutrophils, they migrate into the ischemic brain and become the predominant cells 5 to 7 days after ischemia *(Iadecola 1997)*. In addition, cytokines might activate expression of inflammation-related genes, such as iNOS and cox-2 *(Nowaga et al. 1998)*. Cox-2 mostly expressed in the penumbra, is likely to have a damaging effect on the penumbral tissue *(delZoppo et al. 2000, Emsley and Tyrrell 2002)*.

Moreover, in this phase, the expression of growth factors (nerve growth factor (NGF), brain derived neutrophic factor (BDNF), glial derived growth factor (GDNF), basic and acidic fibroblastic growth factor (FGF), and members of the transforming growth factor super family (TGF)) and the tumor suppressor gene (p53)) occurs *(Feuerstein et al.1996, Li et al. 1994)*. Growth factors are thought to confer protection mainly by interfering with apoptotic death.

The fourth phase of new gene expression was identified which may well be associated with the acute inflammatory reaction to brain ischemia. This phase includes proteolytic enzymes (metalloproteases, Col92) implicated in damage to extracellular matrix, and their endogenous protease inhibitors TIMP1, following focal stroke *(Romanic et al. 1998; Rosenberg et al. 1996; Wang 1998a)*. The expression of these genes in stroke appears to be related to the influx of inflammatory cells, and it is associated with secondary brain injury and repair processes following stroke.

Finally, the fifth phase of new gene expression includes anti-inflammatory cytokines (TGF-β, IL-10) and osteopontin (Osteo), which downregulate inflammation and have a protective effect in the cerebral ischemia *(Ellison et al. 1998; Wang et al. 1995; Wang et al. 1998b, Strle et al. 2001)*. This phase appears to be important for

tissue remodeling, including the later glial changes and scarring that follow the inflammatory brain reaction in the brain injury *(Clark et al. 1993; Clark et al. 1994).*

2.5. Cell death

Brain cells that are compromised by excessive glutamate-receptor activation, Ca^{2+} overload, oxygen radicals or by mitochondrial and DNA damage can die by necrosis or apoptosis (programmed cell death), which depends on the nature and intensity of the stimulus, the type of cell and the stage it has reached in its life-cycle or development *(Leist and Nicotera 1998).*

Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion. It was shown to occur in the core of the ischemic lesion, a zone in which the degree of injury was severe. It represents passive degeneration of cells. It is characterized by membrane dysfunction and cell swelling, and usually causes an inflammatory response *(Choi 1992).*

On the other hand, in milder injury, cell suicide becomes unmasked and resembles apoptosis, particularly within the ischemic penumbra. In contrast to necrosis, apoptosis requires an active commitment of the cell to degrade its own DNA, according to an internal program of self-destruction. This process is energy dependent *(Manjo and Joris 1995; Kerr et al. 1972).* Apoptosis is morphologically characterized by primary nuclear changes, for example, chromatin condensation, marginalization and segregation followed by fragmentation, while the cell membrane is relatively preserved. There are several lines of evidence that the apoptotic processes are involved in cerebral ischemia in immature brain *(Benjelloun et al. 1999; Derugin et al. 2000; Johnston 2001).*

2.6. Endothelins

Cerebral microvessels are the target of ischemia. Microvessels responses to ischemia depend in part on their structure. Cerebral capillaries consist of endothelial

cells (endothelium), bounded by the basal lamina, and encompassed by end-feet of astrocytes *(delZoppo 1994)*. Astrocytes, important glial component of the blood brain barrier, provide a link between endothelial cells and neurons. Pericytes and histiocytes also contribute to this complex *(Ookawara at al. 1996)*.

Under normal circumstances the endothelium presents to the blood an actively antithrombic and antinfllammatory surface. However, under ischemia, it can udergo a dramatic transformation and become actively prothrombotic and proinflammatory. Mechanisms involved in this conversion include expression of adhesion receptors for leukocytes, production and release of IL-1, platelet activating factor, factor VIII/von Willebrand factor, TXA_2, the rapid inhibitor of t-PA, superoxide anion, and endothelins *(delZoppo and Hallenbeck 2000)*.

Endothelins (ETs), the 21 amino acid peptides, are important in imparting tissue damage due to their vasoconstricting effect *(Bian et al. 1994; Faraci and Heistad 1998; Nikolov et al. 1993; Willette et al. 1993)*.

There are at least three isoforms of ET named: ET-1, ET-2 and ET-3 *(Inoue et al. 1989; Yanagisawa et al. 1988)* Figure 6 *(Masaki 2004)*.

In the CNS, both ET-1 and ET-3 are produced by the vascular, neural and/or non-neural elements. There is no evidence of ET-2 in the brain.

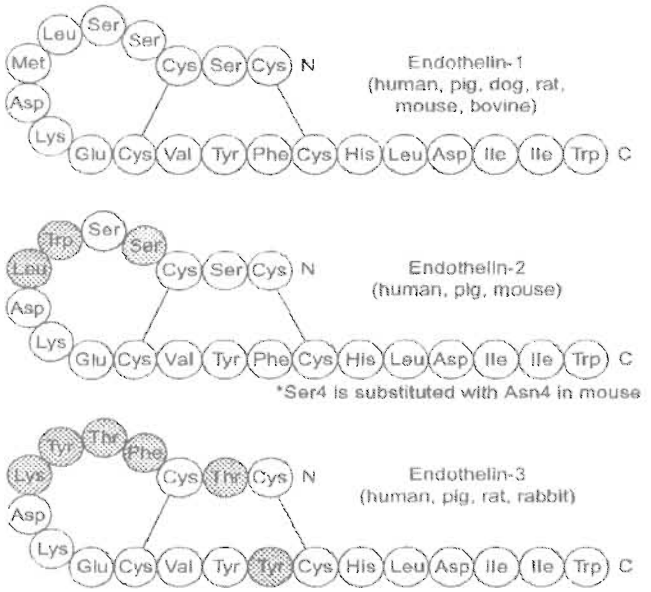

Figure 6. The structure of the three isoforms of endothelin (ET) *(Masaki 2004)*.

2.6.1. Biosynthesis of ET

Similar to other hormones and neurotransmitters, ET is synthetized from the precursor polypeptide, prepro-endothelin, through proteolytic processing. Prepro-endothelin is proteolytically cleaved to form inactive intermediate called big-endothelin. Conversion of the big-endothelin is the final key step in endothelin biosynthesis. This conversion has been postulated to be catalyzed by an endothelin-converting enzyme (ECE) *(Emoto and Yanagisawa 1995)* Figure 7 *(Stjernquist 1998).*

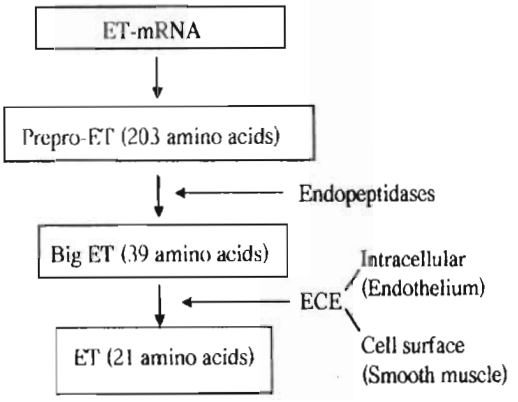

Figure 7. Schematic presentation of the biosynthesis of endothelin (ET) *(Stjernquist 1998).*

2.6.2. ET receptors

The activities of ET peptides are mediated by two types of heterotrimeric G protein-coupled receptors (ET_A and ET_B) with different affinities *(Arai et al. 1990a; Goto et al. 1996; Sakurai et al. 1990).* Signal transduction mechanisms that are possibly affected by activation of ET receptors include: 1. stimulation of several types of G proteins; 2. activation of PLC and phosphatidylinositol hydrolysis; 3. increase of cytosolic Ca^{2+} and/or mobilization of intracellular Ca^{2+}; 4. activation of PLA_2 and arachidonic acid metabolism; 5. activation of Na^+ /H^+ exchange; 6. production of

cyclic-adenosine monophosphate (cAMP) and/or cyclic-guanosine monophosphate (cGMP); 7. signaling into the nucleus that causes long-term effects.

The ET_A receptor has high specificity to ET-1 *(Arai et al. 1990a)*, whereas ET_B receptor is non-selective and accepts the three subtypes of ET almost equally *(Sakurai et al. 1990)*. In the cerebral circulation, ET_A receptors mediate a potent and long-lasting vasoconstriction *(Robinson and McCulloch 1990)*; however, the function of ET_B is more controversial. The selective activation of ET_B receptors induces vasodilation mediated by the generation of the endothelin-derived relaxing factor (NO and/or PGI_2) *(Kobari et al. 1994; Schilling et al. 1995; Touzani et al. 1995)*. Under some conditions ET_B can also induce vasoconstriction *(Touzani et al. 1995; Zuccarello et al. 1998; White et al. 1998)*. This dual role of ET_B receptor, has led to a proposed subdivision of this receptor into ET_{B1} receptors (on endothelial cells) that mediate vasodilatation and ET_{B2} receptors (on smooth muscle cells) that mediate vasoconstriction *(Masaki et al. 1994; Webb 1997; Zuccarello et al. 1999)*.

Moreover, ET_A and ET_B receptors are differently distributed among cell type in the brain. In the rat brain, ET_A receptors are mainly expressed in vessels of cerebral cortex and they are responsible for vasospasms after brain injuries *(Clozel and Watanabe 1993; Hori et al. 1992; Itoh et al. 1994)*, while ET_B receptors are present in the human cerebral cortex *(Fernandez-Durango et al. 1994)*, hippocampus *(Williams et al. 1991)*, and in the rat striatum *(Tayag et al. 1996)*. Moreover, ET_B receptors are abundant in resting and reactive astrocytes, activated microglia *(Furuya et al. 2001; Hori et al. 1992; Rogers et al. 2003)* and are expressed in some populations of neuronal cells *(Webber et al. 1998)*. Several studies have shown that ET-1 affects numerous cell functions of cultured astrocytes such as control of ion channel activity *(Bychkov et al. 2001)*, glutamate efflux *(Sasaki et al. 1997)*, and uptake *(Leonova et al. 2001)*, glucose utilization *(Tabernero et al. 1996)*, permeability of gap junction communications *(Blomstrand et al. 1999; Giaume et al. 1992)*, and calcium signaling *(Blomstrand et al. 1999; Venance et al. 1997)*. In turn, astrocytes are a major source of ET-1 *(Ehrenreich et al. 1991; Ehrenreich et al. 1993)* and seem to play an important role in the regulation of ET-1 levels within CNS *(Ehrenreich et al. 1999; Hasselblatt et al. 1998; Hasselblatt et al. 2001)*.

2.6.3. Endothelin-1 (ET-1)

Three lines of evidence point to a major role of ET-1 in the pathophysiology of cerebral ischemia:

1) the ability of exogenous ET-1 to reduce CBF to levels that induce infarction *(Macrae et al. 1993)*;
2) the increase in plasma, cerebrospinal fluid, and cerebral tissue ET-1 levels after cerebral ischemia *(Lampl et al. 1997; Viossat et al. 1993)* ; and
3) the efficacy of selective ET_A/ET_B receptor antagonists in some, but not all, studies to reduce brain damage after experimental stroke *(Barone et al. 1995; Patel 1996; Touzani and McCulloh 2001).*

3. Animal models of focal cerebral ischemia

Human ischemic stroke is very heterogenous in its manifestations, causes and anatomical sites. A wide variety of animal models for stroke have been developed in order to find different approaches to study ischemic brain injury *(Gingsberg and Busto 1989; Hossmann 1999).* There are two different approaches to study ischemia: *in vitro* and in *vivo* models. *In vitro* models are based on using the brain slices and cell cultures. The main advantage of these models is that they facilitate analysis of mechanisms of early changes, although the conclusions from these models cannot be simply extended to *in vivo* situations. The absence of blood flow as a variable, and the absence of blood vessels themselves in cultures, facilitate interpretations but eliminate what may be important components of the damage processes. Moreover, these models show different morphologies from *in vivo* tissue because the preparation of the slices itself induces transcription of some stress-related mRNA and immediate early genes. *In vivo* models are used many animal species. Although larger animal species (notably cats, dogs, rabbits, and subhuman primates) have been used to study cerebral ischemia, rodents are equally suitable and more desirable *(Garcia 1984; Molinari and Laurent 1976).* The most studies use rats. This reflects cost, ease of experimentation and suggested similarity in cranial circulation of rat and human,

particularly when compared to gerbil, cat and dog *(Macrae IM. Br J Clin Pharmacol 34: 302-308, 1992; Yamori et al. 1976).* However, rat is not a good model in terms of collateral blood supply *(Hunter et al. 1995).* The validity of these cerebral ischemia models is highly dependent on the strict control of physiological variables, such as body temperature, blood pressure, blood gases and glucose levels, which may fluctuate and lead to variability in the results *(Gingsberg and Busto 1998).*

There are several experimental models available for focal cerebral ischemia studies in adult rats *(Ginsberg and Busto 1989; Hunter et al. 1995; McAuley 1995).*

Model	**Advantages (+) / disadvantages (-)**
Middle cerebral artery occlusion (MCAO): permanent	+ selection of the occlusion site allows to some extent the choice of the affected brain area - penetration of drugs to the infarcted area is limited - no recanalization involved - variation in size of infarcted area - some models need craniectomy
Middle cerebral artery occlusion: transient	+ probably the most widely used experimental stroke model + possible to assess reperfusion damage following recanalization + possible drug penetration to the occluded area

	- variation in size of infarcted area
Stroke in the spontaneously hypertensive rat (SHR)	+ MCA occlusion develops large cortical hypertensive rat (SHR) damage- useful in controlled experimental studies - cerebral vasculature differs from that of normotensive strains - influence of dietary factors on the development of strokes in stroke prone SHR
Photochemically induced focal cerebral thrombosis	+ possibility for precise localization and size of infarcted cortical area + possibility to study antiplatelet and thrombolytic therapy - end-arterial occlusion, which is resistant to therapies based on enhacement of collateral perfusion - differs in some respects from human stroke, e.g. no penumbra
Miscellaneous models of focal cerebral embolism and thrombosis	- random and unpredictable location and size of the lesion

(Ginsberg and Busto 1989; Hunter et al. 1995; McAuley 1995)

There are also several models using an endogenous vasospasm-inducing peptide ET-1. It can be applied near the cerebral arteries *(Biernaskie et al. 2001; Robinson et al. 1990; Sharkey et al. 1994; Ward et al. 1998)*, microinjected into selected brain regions *(Fuxe et al. 1992; Gilmour et al. 2004; Hughes et al. 2003)* or applied directly onto the cortical surface *(Fuxe et al. 1997)* to produce ischemic damage.

Sharkey et al. demonstrated that microinjections of ET-1 adjacent to the rat MCA result in a reproducible pattern of focal cerebral infarction *(Sharkey et al. 1994)*. Application of ET-1 (60 pmol in 3 µl saline) near MCA reduced CBF to 30-50% in the cortex and striatum, and significant reduction persisted for 16-22 h in the cortex and 7-10 h in the striatum *(Biernaski et al. 2001)*. Fuxe et al. examined the possibility that lesions induced by intrastriatal injections of ET-1 into the rat brain (ET-1, 0.43 nmol/ 0.5 µl) are ischemic *(Fuxe et al. 1992)*. Their study showed that ET-1 injected in the neostriatum may produce lesions by causing ischemia, related to its vasoconstrictor activity and possibly also to an activation of ET-1 receptors in the astroglial-endothelial complex. In the following work Fuxe et al. developed graded lesions by application of ET-1 on the dorsal frontoparietal cortex of the rat *(Fuxe et al. 1997)*. This study established the usefulness of ET-1 to induce localized, reproducible focal cortical ischemic lesions. A few years later Hughes et al. demonstrated that microinjection of ET-1 into specific brain areas can be used to induce precise and reproducible focal ischemic lesions in grey and white matter, without disruption of the blood brain barrier *(Hughes et al. 2003)*.

At present there are no effective therapeutical strategies for the treatment of injury to developing brain, and perinatal brain injury remains a leading cause of death and disability in children. Therefore, at present, there is an urgent need to develop appropriate models that provide insights into the experimental treatments for potential application during the perinatal period. Using experimental models in which ischemic injury is induced in healthy immature rat brain is a direct approach to addressing the question of whether ischemia without underlying pathology leads to seizure development and psychomotoric impairment. The number of models of ischemic brain damage suitable for immature animals is, however, sparse.

To my knowledge, there are only four publications regarding focal cerebral ischemia in immature rodents using MCA occlusion. Ashwal et al. reported a reproducible model of a transient MCAO at spontaneously hypertensive rats in the

postnatal day-14-18 (P14-P18) *(Ashwal et al. 1995)*. In 1998, Derugin et al. and Renolleau et al. reported two different models of transient MCAO focal cerebral ischemia in the P7 rat pups with or without craniotomy, respectively *(Derugin et al. 1998; Renolleau et al. 1998)*. In 2004, Wen et al. used permanent MCAO models at P7 rats *(Wen et al. 2004)*. In addition, in experimental animals (immature rats, rabbits, guinea pigs, sheep and monkeys), structural brain damage from hypoxia-ischemia has been produced. Out of the several available animal models, the fetal and newborn rhesus monkey and immature rat have been studied most extensively because of their similarities to humans in respect to the physiology of reproduction and their neuroanatomy at or shortly following birth. The most used experimental model was proposed by Rice et al., based on the Levine procedure *(Levine 1960; Rice et al. 1981; Vannucci and Vannucci 1997),* and consists of unilateral hypoxic-ischemic brain damage in 7-day-old rats obtained by the association of unilateral carotid occlusion with subsequent exposure to a hypoxic atmosphere lasting for hours. These conditions restrain possibilities to study seizure development.

However, technical difficulties associated with the small size of cerebral vessels in neonatal rats precluded investigators from studying the pathophysiology of neonatal focal ischemia. Moreover, due to the fact that reperfusion has been reported to a deleterious event in young and adult rats, there is necessity to develop models based on reperfusion.

In comparison to previously mentioned models of MCAO in neonatal rats, the advantage of ischemia which is developed after application of ET-1 into the different brain areas is that it represents less invasive model than MCAO based on reperfusion. Unfortunately, there are not available any publications using this model in neonatal rats. The main goal of our work was to develop this model.

4. Evaluation of experimental ischemia consequences

4.1. Behavior after focal ischemia

Survivors of stroke manifest abrupt development of neurological deficit that can range from nausea, headache, blurred vision, convulsions and loss of consciousness. A wide range of motor and sensory motor deficits including tremor, lack of coordination and partial paralysis is also seen *(Rogers et al. 1997)*. Evidence suggests that apart from motor impairment, cognitive deficit along with motor deficits are also seen in survivors of stroke *(Tatemichi et al. 1994)*. Therefore, the assessment of cognitive and motor deficits is considered. A variety of simple tests have been performed to assess general sensory-motor and cognitive function following focal ischemic injury in the rat (see below) *(Corbett and Nurse 1998)*.

Behavior test	Behavior task	Behavior assessment
Beam-walking test	Walking on a beam	Ability to maintain balance and hindlimb slips during walking
Cylinder test	Forelimb usage in vertical movements	Asymmetry in forelimb usage
Foot-fault test	Walking on a grid	Limb misplacement asymmetry while moving around a grid
Ladder rung walking test	Walking on a ladder	Forelimb and hindlimb placing, stepping and inter-limb coordination
Limb-placing test	Forelimb and hidlimb placement	Response to proprioceptive and tactile stimuli
Montoya's staircase test	Reaching and grasping of food pellet	Fine movements of forepaws

Open field	Behavior in open field	Locomotion and exploration, spatial habituation learning
Passive avoidance test	Avoidance of aversive stimulus	Associative memory
Plus-maze test	Spatial learning	Reference and working memory
Prehensile traction test	Prehensile traction	Time of traction
Rota-rod test	Walking on a rotating rod	Motor coordination and balance
Radial arm maze	Spatial learning	Reference and working memory
Running wheel test	Running in a wheel	Forelimb slips during running
Sensory inattention test	Orientation to sensory (visual, olfactory or tactile) stimuli	Tendency to orientate and investigate impinging stimuli
Step-down inhibitory avoidance test	Learning of inhibitory avoidance	Working memory
Tape test	Removing of sticky tapes from forepaws	Extinction, preference for removing of adhesive stimuli
T-maze	Spatial learning	Reference and working memory
Vertical screen test	Ability to stay on a vertical screen	Forelimb and hindlimb muscular strenght
Water-maze test	Memorizing the location of a hidden platform	Spatial learning and memory

There is little agreement about which tests should be used, and what the minimum number of tests should be. Choices often are guided by individual investigator preferences. First of all, the tests should be reliably sensitive to the location and extent of the injury and the degree to which the intervention affects plasticity beneficially. Because restoration of function in humans is graded and usually incomplete, the tests

should assess the degree of damage to the intended structure as well as residual impairment *(Corbett and Nurse 1998).*

4.1.1. Somatosensory tests

An examination of the behavioral outcomes from focal ischemia studies reveals that most of the sensory/motor tests only detect deficits within days or a week or two after injury. This is problematic since it suggests that there is either a much greater capacity for recovery of function in the rodent than in humans and/or that behavior tests are insensitive/inappropriate. In the human there is often a significant recovery of movement of the limbs, however, it is the fine control of the fingers that often does not show as much improvement *(Gowland 1987).* Therefore, the staircase test provides a good approximation of one of the most pronounced long-term deficits in humans, finger dexterity. This test appears especially useful since it is very sensitive to focal ischemic injury of the forelimb region of cortex as well as the dorsolateral striatum which also participates in forelimb use *(Whishaw et al. 1986).* On the other hand, there are some disadvantages. This test requires an extensive training over a number of sessions before ischemic insult and a recovery time of several days after postischemia before functional assessment can be carried out. In contrast, many other sensory/motor tests are relatively rapid, simple, and objective, and, mainly, require minimal pretraining before the onset of ischemia. However, as mentioned above, they only detect deficits within days or a week or two after injury.

4.1.2. Cognitive tests

There is some evidence to suggest that cognitive impairments may persist longer than somatosensory deficits *(Markgraf et al. 1992; Smith et al. 1997).* There are some tests of working and reference memory that have been used to test rats after focal ischemia, including the open field, the passive avoidance, the plus-maze, the radial

arm test, the T-maze and the water-maze test. Cognitive deficits following focal ischemia have been found in tests measuring associative memory such as passive avoidance *(Hirakawa et al. 1994; Wahl et al. 1992; Yamamoto et al. 1988)* or spatial memory such as water maze *(Jolkkonen et al. 2003; Puurunen et al. 2001a; Puurunen et al. 2001b; Yonemori et al. 1996; Yonemori et al. 1999)* and radial arm maze *(Okada et al. 1995; Sakai et al. 1996)*. These results suggested that neuronal damage also affected the main centers responsible for learning and memory- the hippocampus and the cholinergic system. However, transient MCAO did not lead to any impairment of memory and learning in some experiments. The reason can be the time window of ischemia in which the hippocampus was not affected *(Gupta et al. 2002)*.

4.2. Electrophysiology

In most behavioral tests there is often considerable recovery following ischemia. In many cases, deficits in behavioral tasks are only observed within the first few weeks after the insult *(Andersen et al. 1991; Corbett et al. 1992; Markgraf et al. 1992)*. Some studies have provided evidence that neuronal death can continue for months after an ischemic episode *(Dietrich et al. 1993; Colbourne and Corbett 1994)*. Thus, there are many cases where a functional assessment of outcome will need to be made in months after ischemia. While there are few behavioral tests which could still be effective indicators of neuronal damage and/or dysfunction at later time points, there is always the difficulty of interpretation when a behavioral test is used. Brain damage is also distributed among many brain areas in focal ischemic models, thereby complicating the interpretation of results from behavioral tasks.

It is often desirable to be able to compare histological results with a functional assessment of the same brain area. This approach requires a behavioral task which is a sensitive indicator of damage of the cell group being studied, however no behavioral tasks have absolute specificity. Therefore, an alternative approach is to examine the area of interest electrophysiologically. This strategy has been used to assess cortical neurons after photochemically induced focal ischemia *(Buchkremer-Ratzmann et al. 1996)*. Results showed that a neocortical infarction leads to hyperexcitability not only

in its direct vicinity but also in the contralateral hemisphere. Such hyperexcitability may contribute to increased activation of contralateral brain areas and to functional reorganization after stroke. There is an imbalance between excitatory and inhibitory system, NMDA receptors are up-regulated whereas the $GABA_A$ receptors are down-regulated in the ipsi- and contralateral neocortex after photothrombotic focal cerebral ischemia *(Qu et al. 1998)*. Moreover, double or multiple epileptiform discharges occurred in more than 30% of the recordings *(Buchkremer-Ratzmann et al. 1996)*. It is known, than in 5 to 15% of stroke patients a brain infarct can provoke epileptic seizures *(Bladin and Norris 1998; Kotila and Waltimo 1992)*. Distinct electroencephalographic (EEG) abnormalities, such as increased slow delta activity, voltage depression and epileptiform discharge, have been identified in stroke patients. There are several reports of epileptic seizures in vivo following experimental stroke in adult rats. In 2001, Lu et al. reported a new rat model of EEG topographic mapping during permanent and transient MCAO (*Lu et al. 2001)*. This study showed that there are the striking similarities in EEG profiles seen in stroke patients and in MCAO rats what was already observed in another studies with primates *(Auer et al. 1996)* and rodents *(Cohen et al. 1994; Moyanova et al. 1998)*. Moreover, a few pharmacological studies have used improvement of EEG as a measure of drug efficacy in animal models of stroke *(Cohen et al. 1994; Phillips et al. 2000; Williams et al. 2000)*.

Using EEG monitoring, Hartings et al. demonstrated spontaneous nonconvulsive seizures in animals with both permanent and transient MCAO with perfusion. Periodic lateralized epileptiform discharges appeared over penumbral regions in the injured regions in the injured hemisphere while intermittent rhythmic delta activity recurred in the contralateral hemisphere with frontoparietal dominance *(Hartings et al. 2003)*.

Due to the fact that the changes in CBF are closely connected to changes in EEG activities some authors used vasoconstrictor peptide ET-1 to examine the effect of it on EEG. Moyanova et al. showed that application of ET-1 to the MCA in conscious rats caused a significant shift to a lower EEG frequency, i.e., augmentation of slow waves and a reduction of alpha-like and faster EEG waves *(Moyanova et al. 1998)*. Later, Yager et al. mentioned the occurrence of convulsive seizures in animals with focal ischemia induced by the injection of ET-1 to the MCA in both immature (P10) and adult (3 and 6 month) rats *(Yager et al. 2005)*. They however did not provide

detailed description of seizures. In immature CD1 mice, ligation of unilateral carotid artery without general hypoxia induced behavioral seizures in 75% of animals suggesting that immature brain may be prone to ischemia-induced seizures *(Comi et al. 2005)*. In immature rats with ligation of the common carotid artery development of immediate convulsions was never described, but hypoxia-ischemia damage led to dynamic changes of susceptibility to bicuculline-induced seizures *(Cataltepe et al. 1995)*. Unfortunately, till now, there is not available any work showing the changes in EEG after application ET-1 into the some specific areas of the brain.

II. AIMS OF THE STUDY

The purpose of this thesis was to estimate both the short- and long-term functional and morphological sequelae of focal ischemia induced by the intrahippocampal injection of endothelin-1 (ET-1) in 12- (P12) and 25-day-old (P25) immature rats. Experiments were designed to answer four major questions:

1. What is the risk of acute seizure development after ET-1 injection at different ages?
2. What is the risk of epileptogenesis after ET-1 injection at different ages?
3. What is the extension and pattern of ET-1-induced lesion at different ages?
4. What is the risk of developmental behavioral changes and cognitive decline as a result of ET-1-induced lesion at different ages?

Results of these experiments are published in two articles:

Mateffyova A, Otahal J, Tsenov G, Mares P, Kubova H. Intrahippocampal injection of endothelin-1 in immature rats results in neuronal death, development of epilepsy and behavioral abnormalities later in life. Eur J Neurosci 24(2): 351-360, 2006

Tsenov G, Mateffyova A, Mares P, Otahal J, Kubova H. Intrahippocampal injection of endothelin-1,a new model of ischemia-induced seizures in immature rats. Epilepsia suppl., in press

III. METHOD

3.1. Animals

Experiments were performed in male Wistar albino rats on postnatal day 12 (P12), or 25 (P25). The day of birth was defined as day 0 and the animals were weaned on P28. Rats were housed in a controlled environment (temperature 22 ± 1°C, humidity 50-60%, lights on 06:00 –18:00 h) with free access to food and water. Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directive 86/609/EEC.

3.2. Acute experiment

3.2.1. ET-1 injection

Surgery was performed under ether anesthesia. Cannula for endothelin-1 (ET-1) infusion (PlasticOne, Bilaney Consultants GmbH, Germany; outer diameter 0.2 mm) was implanted into dorsal hippocampus at coordinates AP=3.0; V=3.0; L=3.0 in P12 and AP=3.5; V=3.5; L=3.5 relative to bregma in P25 animals.

To register EEG in freely moving animals, flat silver electrodes were placed bilaterally over the sensorimotor cortex (AP=0, L=2 mm) and twisted steel electrode was implanted in the dorsal hippocampus at the same coordinates as cannula in the contralateral hemisphere. An indifferent electrode was inserted into the nasal bone, ground electrode into the occipital bone. All electrodes and cannula were fixed to the skull by means of dental acrylic, the animals were allowed to recover for at least 1 h.

Endothelin-1 (# E 7764, Sigma, USA) was dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4) and infused using an infusion pump (KDS210, KD Scientific,

New Hope, USA; 1μl/5 min) to freely moving animals. Controls received 0.01 M PBS in corresponding volume instead of ET-1 solution. Three doses of ET-1 were used in both age groups: 10 pmol, 20 pmol, and 40 pmol (40pmol/1μl). During the entire period of separation from their mothers, the body temperature of P12 animals was maintained at 32±2°C with an electric heating pad connected to a digital thermometer to compensate the immature thermoregulation at this age *(Conklin and Heggeness 1971)*.

3.2.2. Video/EEG registration and evaluation

Before ET-1 infusion, baseline EEG was registered for 20 min. After that, ET-1 was infused and EEG was further recorded for the next 100 min. Animals were then returned to their homecages. When technically possible, animals of both P12 and P25 groups were registered 22 h after ET-1 injection for the second time again for 100 min. Latency to the 1st seizure was measured. The number and duration of electrographic seizures (i.e. at least 5-s sections of rhythmic sharp elements) was estimated during both registration periods. Total seizure duration was expressed as time spent in seizures per registration period. Behavior was videomonitored during whole period of EEG registration. Presence of stereotypic behavior (face washing, scratching), clonic movements (unilateral or bilateral clonus, crawling or pedalling movements, head bobbing), other specific convulsions (circling, barrel rolling), and generalized tonic-clonic seizures was registered. If an electrographic seizure was detected, its behavioral correlate in the corresponding video recording was checked and behavioral correlates of seizures were registered. The severity of convulsions was scored according to following scale:

0 – normal behavior,

1 – stereotypic behavior (face washing, scratching), isolated myoclonic jerks,

2 – clones (unilateral or bilateral clones, crawling, pedalling, head bobbing),

3 – intense circling,

4 – barrel rolling with clonus,

5 – generalized clonic seizures with a loss of righting reflexes.

Each animal was assigned a score for the most severe behavioral characteristics and average score was then calculated for each dose-and-age group.
Mortality was registered till the end of the entire experiment.

3.2.3. Histology

All rats surviving for 24 h were anesthetized with urethane (2 g/kg i.p.) and transcardially perfused with 4% paraformaldehyde. Brains were postfixed in fixative, cryoprotected in gradual sucrose and frozen in dry ice. Tissue was then cut with a cryocut Leica CM 1900 in the coronal plane (50 μm, 1-in-5 series) and sections were stored in a tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until processed. To detect and localize degenerating neurons, adjacent series of 1-in-5 sections were processed for cresyl violet and Fluoro-Jade B. Fluoro-Jade B histochemistry was performed according to *Schmued et al. (1997)*. Fluoro-Jade stained sections were examined using Olympus AX 70 fluorescent microscope (filter cube for fluorescein, excitation band 450-490 nm and emission band above 515 nm).

The extent of hippocampal damage was assessed semiquantitatively using the modified rating scale developed for common artery ligation model by *Wirrell et al. (2001)*. For regional determination of the severity of injury, areas CA1 and CA3, the dentate granule cell layer, and the hilus were evaluated separately. Severity of the damage was scored from 0 to 4 according to extension of damaged area (0: 0-5% of the area is damaged; 1: 6-25%; 2: 26-50%; 3: 51-75%; 4: >75%). Average score was calculated for each hippocampal area and than summarized for each animal. Total score therefore ranged from 0 to 16.

3.2.4. Statistics

In the text, data are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses for comparison among groups were performed using a one-way ANOVA with all pairwise multiple comparison procedures (Bonferroni t-test). Differences between two groups were compared with t-test. Incidences were compared using Fisher's exact test. Correlation was performed using Spearman's rank test. All parameters were analyzed using SigmaStat® (SPSS Inc., Chicago, IL) software. A p value less than 0.05 was considered as significant.

3.3. Long-term experiment

3.3.1. ET-1 injection

Intrahippocampal injection of ET-1 was performed under halothane anaesthesia (1.5%; Narcotan, Zentiva, Czech Republic). Rat pups were placed into stereotaxic apparatus and cannula (Plastics One, Bilaney, UK; outer diameter 0.2 mm) for intrahippocampal injection was inserted into the left dorsal hippocampus. ET-1 in doses of 20 and/or 40 pmol was injected as mentioned above. After the injection, the cannula was kept in place for two more minutes and then removed. The scalp was closed by means of tissue adhesive (Collodion; Penta, Czech Republic). Surgery lasted for approximately 15 min.

Animals were then placed into an observation box and their behavior was video-recorded and severity of behavioral correlates was evaluated according to scale described above. During the entire period of separation from their mothers, the body temperature of P12 animals was maintained at 32±2°C with an electric heating pad connected to a digital thermometer.

Body weight was checked daily for 1 week and thereafter 13, 26, and 56 day after surgery. These data were used to calculate relative body weight [(body weight at the time of measurement/body weight at P12) * 100%] during the course of the study to investigate the effects of early ET-1 injection on normal growth.

3.3.2. Effects on sensorimotor development

Behavioral testing started 24 h after ET-1 injection. Before testing, animals were allowed to adapt to the testing room for at least 2 h. All tests were performed between 08:00 and 15:00 h and the order of tests was always the same. Testing of motor abilities was repeated six times in both P12 and P25 groups (1, 3, 6, 13, 26 and 56 day after injection; detailed experimental schedule is in Figure 8).

P12

	Days	0	1	3	6	13	26	56	60-64	75-79
Test	ET1 application	x								
Negative geotaxis			x							
Wire mesh test			x	x	x					
Bar holding test			x	x	x	x	x	x		
Rotarod test						x	x	x		
Morris-water maze									x	
EEG/video monitoring										x

P25

	Days	0	1	3	6	13	26	56	60-64	75-79
Test	ET1 application	x								
Bar holding test			x	x	x	x	x	x		
Rotarod test			x	x	x	x	x	x		
Morris-water maze									x	
EEG/video monitoring										x

Figure 8. Experimental schedule.

The following motor tests were used to characterize maturation of motor abilities:

Bar holding. The rats were held so that their forelimbs touched a 25 cm long wooden bar extended between two poles 50 cm high. Latency to fall down was registered up to 120 s.

Rotarod. Motor coordination was measured using an automatic 4-lane rotarod unit 3376-4R (diameter of the rod 6.5 cm; Technical and Scientific Equipment GmbH, Germany). Animals were placed on the rotating rod and time spent on the rod was recorded up to 180 s. All animals were pre-trained using rotation speed 8 r.p.m. The first trial was realized with the same rotation speed (8 r.p.m.). In subsequent trials, motor abilities were tested in two 180-s sessions with higher rotation speeds (15 and 30 r.p.m.).

Because of different levels of maturation of the two age groups studied, specific age-related tests *(Altman and Sudarshan 1975)* were used for P12 animals:

Negative geotaxis. Pups were placed on an inclined (30^0) surface with their heads facing downward. The time necessary to turn to 90^0 and consequently to 180^0 was recorded. The animals were tested for a maximum of 90 s.

Wire-mesh ascending. A 10-mm wire mesh, 45 cm high and 15 cm wide, was placed at an angle of 70^0 in contact with a platform on the top and an edge of laboratory desk at the bottom. To motivate a pup to ascend from the bottom of the wire mesh, its littermates were placed on the top platform. The time necessary to rejoin the siblings was measured up to 120 s.

3.3.3. Effects on cognitive functions in adulthood

Morris water maze. Animals were tested in a Morris water maze 2 months after surgery. The water-maze consisted of a blue circular pool (diameter 195 cm, height 55 cm) filled with clear water (depth of 25 cm; temperature 20^0C). A round clear

Plexiglas platform (escape platform, diameter 11 cm) was placed 1 cm below the water surface and its position was kept the same throughout the experiment.

Each rat received one training session (8 swims) per day for 5 consecutive days. A trial began by placing the rat into the water at one of four randomly selected starting points (north, west, south, or east; each starting point was used twice). The rat was allowed to swim until it found the escape platform or had swum unsuccessfully for 60 s. In case of an unsuccessful trial, the rat was guided to the platform by hand and its latency in that exposure was recorded as 60 s. Animals were allowed to rest on the platform for 15 s. The escape latency (time to reach the hidden platform) was measured.

To characterize the best performance reached by each animal in this test, sum of all latencies during day 3, 4 and 5 of training (cumulative latency) was calculated. This period was chosen because animals of all groups already reached asymptotic performance by the 3rd day of training, i.e. the performance of animals did not further improve. The correlation between cumulative latency and severity of hippocampal damage as well as severity of seizures in chronic phase was calculated.

3.3.4. Video-EEG monitoring of spontaneous seizure activity in adulthood

Recording electrodes were implanted after finishing the behavioral testing (i.e., approximately 3 months after injection; Figure 8) in both P12 and P25 animals. Rats were anesthetized with halothane (1.5%). Silver ball cortical electrodes were placed epidurally over the left and right sensorimotor cortical areas (AP = 0; L = 2.5 mm). In addition, twin stainless steel electrodes (wire diameter 100 μm) isolated up to the tips were implanted into the left and right dorsal hippocampus (AP = 3.5; L = 3; H = 3.5 mm). An indifferent electrode was inserted into the nasal bone, a grounding electrode into the occipital bone. The animals were allowed to recover for at least 1 week. For video-EEG monitoring (BrainScope system, M&I, Czech Republic), rats were individually placed into plastic boxes and provided with water and food. Behavior of the rats was registered using a video camera (Panasonic WV BP 330; Matsushita Electric industrial Co., Ltd., Japan) mounted over the plastic boxes. Animals were

monitored continuously (24 h/day) for 5 days. Only animals with at least 20-h duration of recording each day were included into evaluation. Some rats (11 of 67 recorded animals) quickly learned how to destroy wires and therefore their recordings were only episodic and these animals were excluded from evaluation.

Computerized analysis of the video-EEG started immediately after the end of recording sessions. For spike analysis, the digitized EEG was partitioned into successive 10-s epochs. Occurrence of spikes was determined separately in each of the four channels using a custom-made program. A spike was defined based on its duration (<70 ms) and amplitude (≥ 3 times the amplitude of background activity). Sections of EEG with spikes were analyzed in detail. Isolated spikes were classified as interictal activity and scored as "present" or "not present". If the spike or sharp-wave activity continued for at least 5 s it was considered ictal activity (i.e. an electrographic seizure) and its duration was measured. If an electrographic seizure was detected, its behavioral correlate in the corresponding video recording was checked. The occurrence of motor seizures was scored as "yes" or "no".

3.3.5. Neuropathologic analysis

After video-EEG recording, rats were deeply anesthetized with urethane (2.5 g/ kg, i.p.) and perfused according to Timm-fixation protocol with 0.37% sulfide solution (1 ml/g) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (1 ml/g), +4^{0}C, for 10 min. The brains were collected and processed as described above and than cut in the coronal plane (30 μm, 1-in-5 series). Adjacent series of sections were used for Nissl and Timm staining.

Nissl staining. To identify the cytoarchitectonic boundaries and the distribution and severity of neuronal damage, the first series of 1-in-5 sections was stained with cresyl violet. The amount of cell damage in the hippocampus was assessed in the whole rostrocaudal extent of the lesion. Severity of hippocampal damage was assessed semiquantitatively using scoring system described above.

Timm histochemistry. To assess mossy fiber sprouting, all sections (1-in-5 series) in which the hippocampus was present were mounted on gelatin-coated slides, air-dried, and stained using the Timm sulfide-silver method *(Sloviter 1982).* Mossy fiber sprouting was analyzed in the suprapyramidal region and the inner molecular layer along the entire septotemporal axis of the hippocampus. For scoring, the hippocampus was divided into the septal and temporal ends. At the septal end, the analysis was started at the level at which the suprapyramidal and infrapyramidal blades of the dentate gyrus fused together, and continued caudally until the septal hippocampus fused with the more temporal hippocampus. That is, the analysis included sections between the levels -2.3 to -6.0 from bregma *(Paxinos and Watson 1986).* In each section, the tip, mid and crest portions of the supragranular region and inner molecular layer were analyzed, and the highest density of Timm-positive granules in any of these regions was assigned as score for that particular section (typically the highest score was found in the tip of the infrapyramidal blade). Then, the mean score was calculated from all sections. At the temporal end, the analysis of mossy fiber sprouting was done in sections, in which the septal and temporal ends of the granule cell layer become fused and form an "oval-shape" layer (corresponding to levels 6.1 to 6.7 posterior to the bregma according to *Paxinos and Watson 1986).* In each section, the septal and temporal halves were scored separately, and the mean of the two scores was assigned for that particular section. Finally, the mean score was calculated from all sections including the temporal hippocampus.

The density of sprouting was scored from 0 (no sprouting) to 5 (confluent dense band of sprouting covering the entire inner molecular layer) according to *Cavazos et al. 1991.*

3.3.6. Stereological assessment of hippocampal volume

To assess changes in the hippocampal volume three days after the ET-1 injection, additional groups of animals were prepared according to protocol for ET-1 injection used for long-term study. Animals were perfused according to above mentioned protocol for tissue preparation 3 days and 3 months after ET-1 injection and brains

were processed as described above. One series of 50 μm sections was Nissl stained and used to measure the hippocampal volume.

Stereological estimation of hippocampal volume (3 days and 3 months after injury) was performed using the CAST 2 software (Olympus, Denmark A/S) on the computer coupled to the light BX50 Olympus microscope with a three axis computer-controlled motorized stage and *CCD color video camera*. The areas were outlined using a 4x 0.16 objective. The volumes were estimated with the Cavalieri method *(Gundersen and Jensen 1987)*. This method requires randomly superimposing lattices of test points on sectional images generated by systematic random sampling and counting those points which hit specified tissue compartments. To estimate the volume of a given brain compartment, we used the equation

$$est\ V = \sum P \cdot a_P \cdot d,$$

where *est V* represents the estimated volume. $\sum P$ is the sum of test points hitting the compartment seen on section, a_P is the planar area associated with a lattice test point, and d is the distance between sampled section planes.

The hippocampal volume was estimated from 13-18 and 16-19 sections in P12 and P25 animals, respectively. The volume of a region in a single section was estimated by using the traditional lattice point probe. The distance between single points was 0.8 mm. Volume of the injected hippocampus was compared between controls and experimental animals. Because there was no difference between the effects of two tested doses, data were polled together for statistical evaluation.

3.3.7. Statistics

Except extension of the lesion, there were no significant differences between individual dose groups in functional parameters (motor and cognitive abilities and seizure parameters). Therefore, data from the two doses were collapsed together for each age group. If there were significant differences between two doses in some parameters they are mentioned in the text.

Data are presented as mean ± standard error of the mean in the text. Statistical analyses for comparison among groups were performed using a one-way ANOVA

with all pairwise multiple comparison procedures (Bonferroni t-test); comparison between two groups was performed using Mann-Whitney's Rank Sum Test. Behavioral parameters (latencies) were evaluated by two-way ANOVA with one grouping factor with two levels (ET-1 and control groups) and one repeated measures factor (repeated measurements). The Newman-Keuls test was used for post hoc multiple comparisons. Incidence was compared using Fisher's exact test. Correlation was performed using Spearman's rank test; all experimental animals from one age group were pulled together for correlation. Parameters were analyzed using SigmaStat® (SPSS Inc., Chicago, IL) software. A p value of less than 0.05 was considered to be significant.

IV. RESULTS

4.1. Acute effects of intrahippocampal injection of ET-1

Intrahippocampal injection of ET-1 induces both electrographic and behavioral seizures. As revealed by video/EEG monitoring, seizures never developed in control animals. Only isolated spikes were detected after PBS infusion in the hippocampus and then they slowly diminished. In both age groups of ET-1 injected rats, electrographic seizures were formed by fast rhythmic spikes or sharp waves with a frequency of 3-4 Hz; this frequency decreased towards the end of seizure. Amplitude of sharp elements was at least three times higher than amplitude of background EEG (Figure 9).

4.1.1. ET-1-induced seizures at P12

Seizure development was observed after all doses of ET-1. Electrographic seizures were detected in 6 of 7 animals with 10 pmol, 9 of 11 animals with 20 pmol and in all 12 animals with 40 pmol of ET-1. Latencies to the first seizure ranged around 30 min and there was no difference among groups. Seizure parameters varied considerably among individual animals in each dose group. Seizure severity tended to increase with a dose of ET-1, however, differences in seizure frequency (5.7±0.7, 4.3±1.3, and 7.1±2.1 seizures /100 min, respectively) or average seizure duration (18.4±2.5 s, 41.1±13.9 s, and 40.2±6.5 s, respectively) were not significant. Total seizure duration tended to increase with the dose of ET-1 (Figure 10). During the 2nd observation period 22 h after ET-1 injection, number of animals exhibiting seizures decreased significantly in 10 pmol group (to 1 of 6, $p=0.026$), but not in other groups. Total seizure duration tended to decrease in all dose groups (Figure 11), but differences

were not significant. Shorter duration of seizure activity compared to the 1st observation period occurred in 69% of animals.

In behavior, face washing or scratching occurred in both control and ET-1 animals. This behavior was never accompanied by epileptifom activity in the EEG. Electrographic seizures were usually accompanied by convulsions, non-convulsive seizures were observed occasionally after low doses of ET-1. Clonic movements were the most common phenomenon after all tested doses of ET-1. The incidence of more severe convulsions (i.e. circling, barrel rolling and generalized clonic seizures) tended to increase with the dose of ET-1, however the differences were not significant (Figure 12). Barrel rolling occurred in 14 to 58% and generalized clonic seizures in 14% to 42.7% of animals in individual groups. During the 2nd observation, electrographic seizures were not accompanied by convulsions. Only 1-2 s lasting abnormal movements (whole body flexion, hindlimb flexion, tail erection) were observed. In total, 7 animals died within 2 h after ET-1 injection. Six of them died during generalized clonic seizures.

4.1.2. ET-1-induced seizures at P25

Seizure developed in 5 of 6 animals with 10 pmol, and in all animals with both 20 (9 of 9) and 40 (10 of 10) pmol.

Latency to the 1st seizure ranged around 20 min in all groups. Even thought there was a clear tendency to increase seizure severity with a dose of ET-1, no significant differences were observed in either seizure frequency (2.7±0.9, 2.2±0.5, and 7.2±2.3 seizures /100 min, respectively) or average seizure duration (17.5±2.3 s, 26.0±10.2 s, and 39.1±12.7 s) among the three dose groups. Also, total seizure duration did not differ among groups (Figure 13).

There were no significant differences in seizure frequency or mean duration between the 1st and 2nd registration period. Total seizure duration however tended to decrease (Figure 14). Shorter duration of seizure activity compared to the 1st observation period occurred in 47% of animals.

Similarly to P12 animals, behavioral seizures were always accompanied by electrographic seizures. In addition to convulsive seizures, non-convulsive seizures were also detected and their proportion was higher in animals with lower doses of ET-1. Clonic movements were seen after all doses of ET-1. Barrel rolling was observed in 5 of 10 animals with 40 pmol, but not after lower doses. Behavioral score in both 10 pmol (1.9±0.3) and 20 pmol (1.8±0.4) was significantly lower than in 40 pmol (3.4±0.4) rats (Figure 15).

Generalized clonic seizures occurred only in two animals with 40 pmol. One of these animals died.

Comparison between age groups did not reveal any differences in characteristics of electrographic seizure except higher seizure frequency in P12 rats with 10 pmol compared to P25 animals. No convulsions were detected in the 2nd period.

4.1.3. Neuropathology

In controls, only sparse FJB-positive neurons were detected in close vicinity of electrode track. Pattern of the ET-1 induced hippocampal lesion was similar in both age groups. Size and distribution of the lesion varied considerably among animals. In both age groups, distribution of the hippocampal damage exhibited specific "patchy" pattern (Figure 16). In Nissl stained sections, affected parts of the hippocampus exhibited decrease of staining and vacuolization, which was more evident in principal cells in both the pyramidal and granular layer. Typically, there was accumulation of glial cell nuclei in affected hippocampal regions (Figure 16).

From each age group, three brains could not be evaluated because of tissue destruction.

4.1.3.1. Rats with ET-1 injection at P12

The hippocampal injury was evident in all animals with ET-1 injection (Figure 16). Damage was localized in dorsal part of the hippocampus, only in some animals (5 of 20) isolated FJB-positive neurons were found also in the dorsal thalamic nuclei. Neither severity nor number of damaged hippocampal areas differed among dose groups.

4.1.3.2. Rats with ET-1 injection at P25

All three doses induced damage in the dorsal hippocampus (Figure 16), there was however no difference in severity of the damage among individual groups. Also, number of damaged hippocampal structures did not differ among groups.

Comparison between age groups showed that ET-1 in doses 20 and 40 pmol induced more severe morphological damage in P12 (10.5±1.6 and 11.0±1.1) (Figure 17) than in P25 (3.1±0.9; $p=0.001$ and 5.3±1.3; $p=0.006$) (Figure 18).

Data from the 1st observation revealed positive correlation between total seizure duration and severity of morphological damage in both P12 ($r=0.428$; $p=0.029$) and P25 ($r=0.641$; $p=0.002$). There was also positive correlation between severity of convulsions and severity of morphological damage in both age groups ($r=0.684$; $p<0.001$ and $r=0.678$; $p<0.001$, respectively).

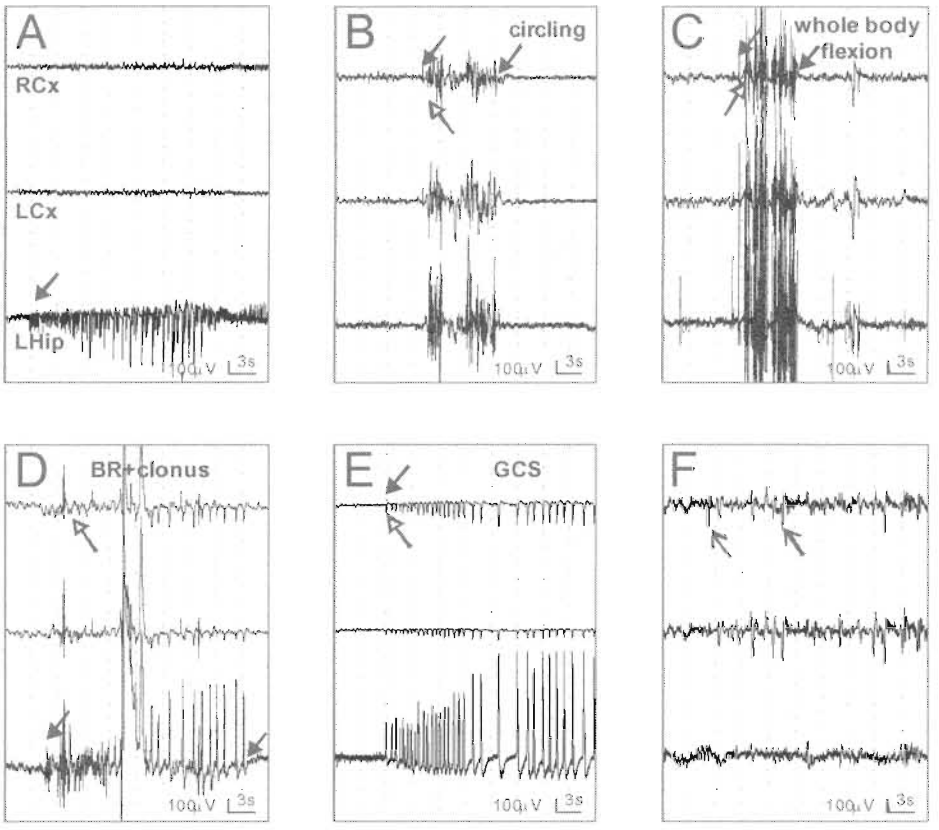

Figure 9. Examples of electroencephalographic recordings from animals with ET-1 injection. The beginning and the end of the electrographic seizures are indicated with black arrows, empty arrows indicate the beginning of behavioral seizures. **(A)** An electrographic seizure with onset in the hippocampus contralateral to the injection registered within 100 min after ET-1 injection (40 pmol) at P12. Electrographic seizure was associated with clonic movements. **(B)** A generalized seizure associated with circling in P12 registered 63 min after ET-1 (40 pmol). **(C)** Seizure starting with brief whole-body flexion registered from the same animal 22 h later. **(D)** An electrographic seizure associated with barrel rolling (BR) and clonus in a rat with ET-1 (40 pmol) at P25. **(E)** Gereralized clonic seizure (GSC) registered in the same animal 12 min later. **(F)** Interictal spikes (open arrows) registered in P25 animal with 20 pmol during the 2nd registered period.Time marks 3s, amplitude calibration 100µV. Individual leads: RCx – right sensorimotor cortex; LCx – left sensorimor cortex; Lhip – left hippocampus, all in reference connection.

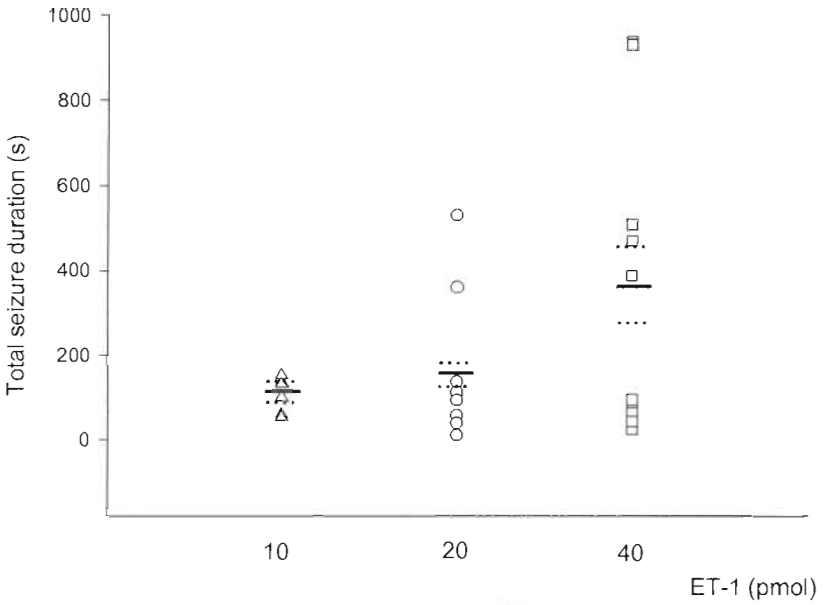

Figure 10. Total seizure duration (s) per 100 min after ET-1 (10, 20 and 40 pmol) injection on the PD 12. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=6), circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=12) (on the bottom).The black lines represent mean ± SEM (dotted lines). The differences between different doses of ET-1 were not significant (Kruskal-Wallis (ANOVA) test).

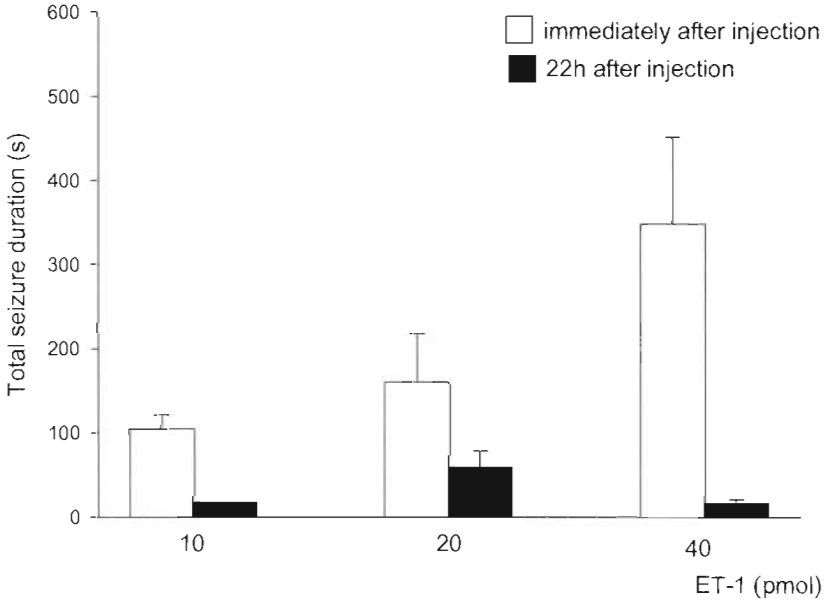

Figure 11. Comparison of total seizure duration (s) in EEG per 100 min (registered in the same animals) immediately after ET-1 (10, 20 and 40 pmol) injection (white columns) and 22 h after injection (black columns) on the PD 12. Symbols represent values from individual animals. Values are expressed as mean ± SEM. Note that there were no significant differences in total seizure duration between both individual dose groups and the time after injection in animals.

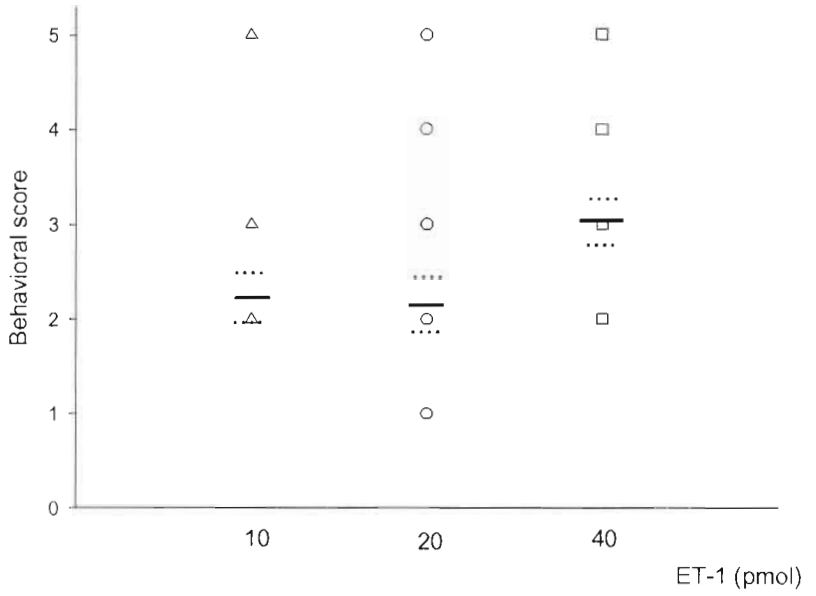

Figure 12. Severity of convulsions per 100 min expressed as behavior score after ET-1 (10, 20 and 40 pmol) injection on the PD 12. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=6), circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=12) (on the bottom). The black lines represent mean ± SEM (dotted lines). The differences between different doses of ET-1 were not significant (Kruskal-Wallis (ANOVA) test).

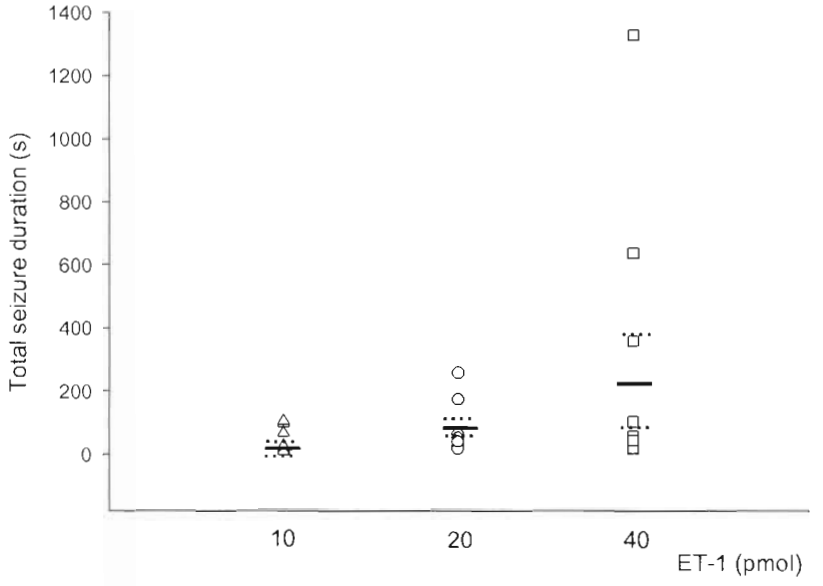

Figure 13. Total seizure duration (s) per 100 min after ET-1 (10, 20 and 40 pmol) injection on the PD 25. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=5) circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=10) (on the bottom).The black lines represent mean ± SEM (dotted lines). The differences between different doses of ET-1 were not significant (Kruskal-Wallis (ANOVA) test).

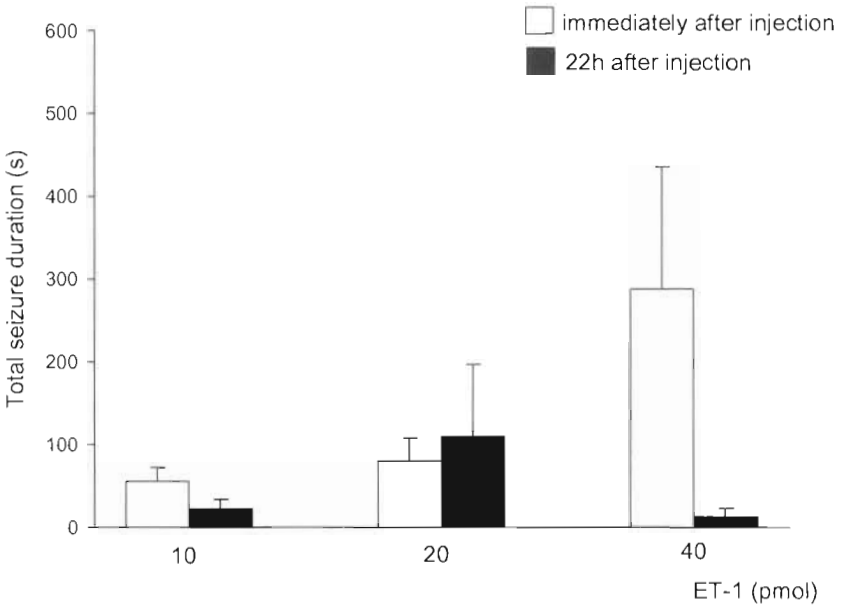

Figure 14. Comparison of total seizure duration (s) in EEG per 100 min (registered in the same animals) immediately after ET-1 (10, 20 and 40 pmol) injection (white columns) and 22 h after injection (black columns) on the PD 25. Symbols represent values from individual animals. Values are expressed as mean ± SEM. Note that there were no significant differences in total seizure duration between both individual dose groups and the time after injection in animals.

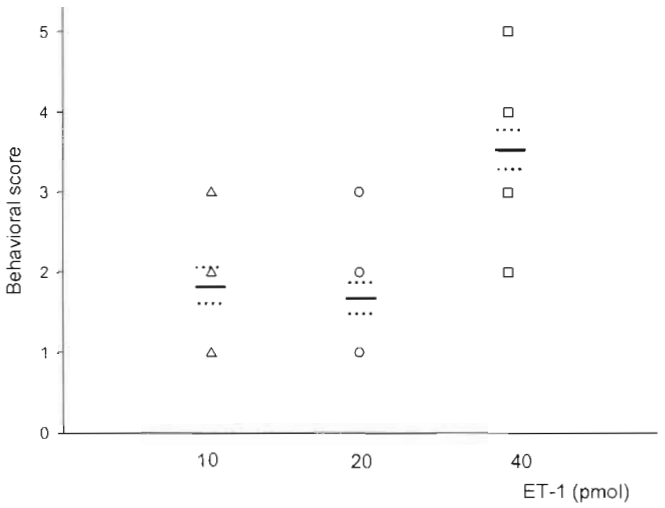

Figure 15. Severity of convulsions per 100 min expressed as behavior score after ET-1 (10, 20 and 40 pmol) injection on the PD 25. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=5), circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=10) (on the bottom). The black lines represent mean ± SEM (dotted lines). The difference between 10pmol vs 40 pmol and 20 pmol vs 40 pmol, were significant (p=0.011 and p=0.003) (Kruskal-Wallis (ANOVA) test).

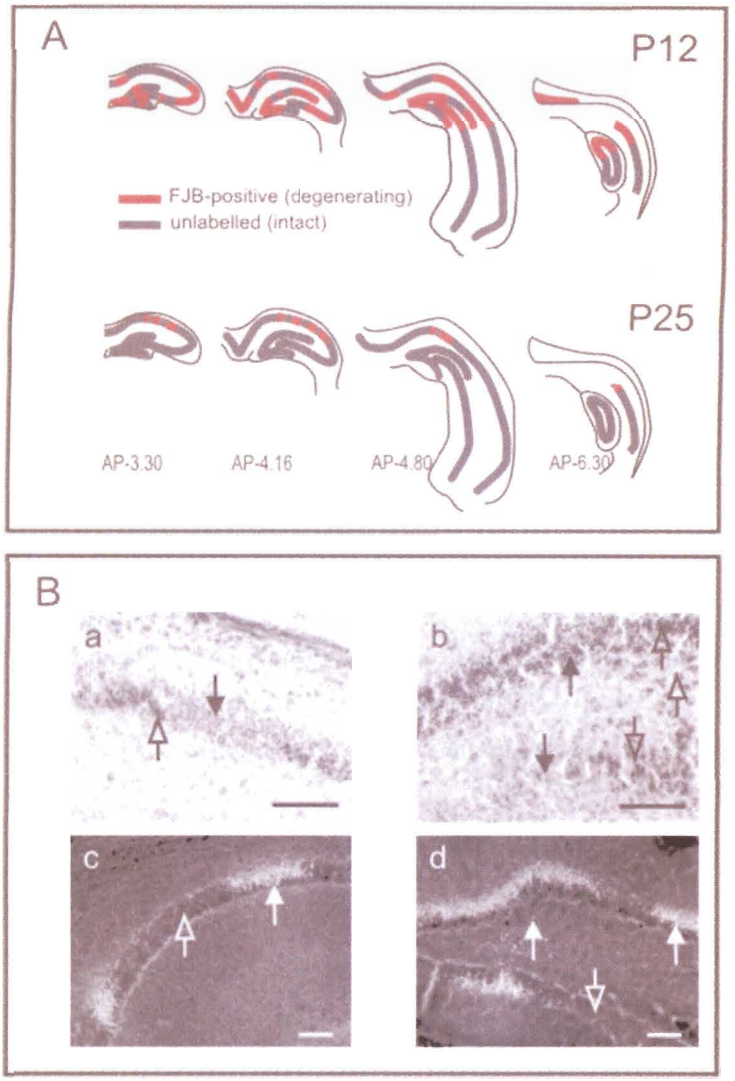

Figure 16. Distribution of neuronal damage in the hippocampus 24 h after infusion of ET-1 in a dose of 40 pmol. **Panel A:** Schematic drawing illustrating pattern and extension of damage in the hippocampus of P12 (upper row) and P25 (lower row). Note specific "patchy" pattern of the damage and difference in the extension of the damage between P12 and P25 rat. Red line symbolizes segments with Fluoro Jade B-positive cell bodies, gray line demonstrates preserved segments (for details see Panel B c and d). on the bottom: corresponding AP levels according to Paxinos and Watson (1986). **Panel B:** Brightfield photomicrographs of Nissl-stained sections demonstrating cell loss and accompanying gliosis (black arrows) in the CA1 region **(a)** and dentate gyrus **(b)** of P12 rat with 40pmol of ET-1. Empty arrows indicate normal neuronal cells. Distribution of Fluoro Jade B positive neurons (indicated with white arrows) in the same animal in the pyramidal cell layer of the CA1 **(c)** and dentate gyrus **(d).** Empty arrows indicate preserved segments of the pyramidal **(c)** or granular **(d)** layer. Scale bar=100 μm

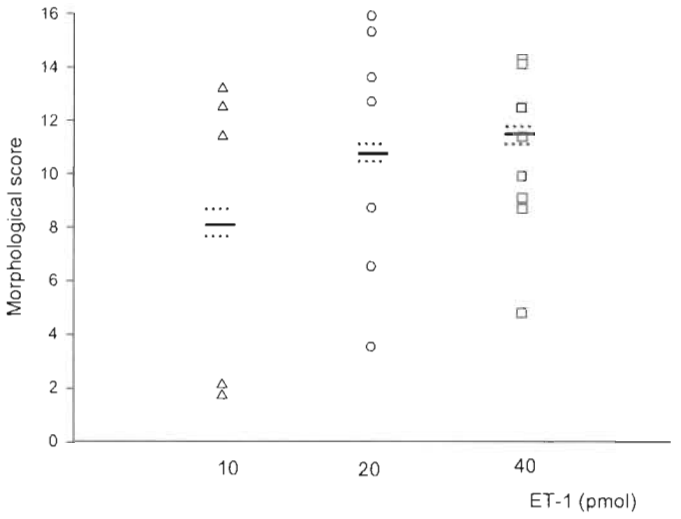

Figure 17. Severity of the hippocampal damage expressed as morphological score assessed 24h after ET-1 (10, 20 and 40 pmol) injection on the PD 12. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=6), circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=12) (on the bottom). The black lines represent mean ± SEM (dotted lines).

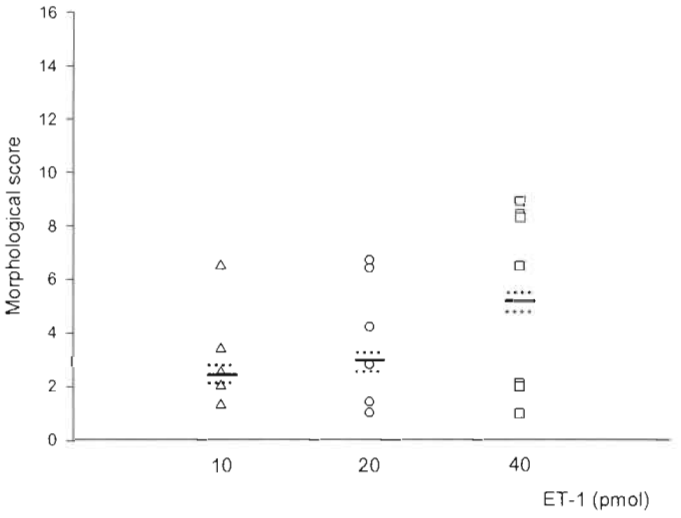

Figure 18. Severity of the hippocampal damage expressed as morphological score assessed 24h after ET-1 (10, 20 and 40 pmol) injection on the PD 25. Symbols represent values from individual animals. Triangles represent animals with 10 pmol of ET-1 (n=5), circles with 20 pmol of ET-1 (n=9) and squares with 40 pmol of ET-1 (n=10) (on the bottom). The black lines represent mean ± SEM (dotted lines).

4.2. Long-term effects of intrahippocampal injection of ET-1

4.2.1. Behavior and mortality following ET-1 injection

To characterize severity of acute seizures after ET-1 injection in individual animals used for long-term follow up, their behavior was evaluated according to behavioral scale described above.

4.2.1.1. Rats with ET-1 injection at P12

Behavioral score was lower in controls (1.3±0.2) compared to ET-1 animals (3.2±0.2; $p<0.001$). Two animals died in control group. Two animals died within 24 h after injection, both of them after the observation period. No animals died later.

4.2.1.2. Rats with ET-1 injection at P25

Injection of both doses of ET-1 significantly increased score compared to controls (2.5±0.3 vs. 1±0; $p=0.02$). Three animals died during the observation period after ET-1 injection (two of them during generalized clonic seizure, one without seizures). No animals died later.

There was no difference in seizure severity between age groups.

4.2.2. Body weight following ET-1 injection

Day-by-day comparison of relative body weight during the 1st week after ET-1 injection did not reveal any differences between control and experimental groups in either age group. No differences occurred in absolute body weights 1 week after injection between control and experimental animals in P12 (44.7±1.4 *vs.* 45.4±0.8g) or in P25 rats (96.8±2.4 *vs.* 96.7±1.5g). Differences between controls and ET-1 animals 2 months after injection did not reach level of significance in either age group (in P12 361±10 *vs.* 370.6±11.7g; in P25 383±14 vs. 406±12g).

4.2.3. Effects of ET-1 injection on sensorimotor development and cognitive function in adulthood

Animals from all age-and-treatment groups were able to pass behavioral tests within 24 h after the injection. The ET-1 groups did not differ from the corresponding controls in strategy they chose to solve tasks of individual behavioral tests.

4.2.3.1. Rats with ET-1 injection at P12

Motor development: Intrahippocampal injection of ET-1 did not change developmental profile in comparison to controls in any motor test used (Figure 19, 20, and 22). In bar holding test, controls spent significantly longer time on the bar than ET-1 animals 1 day (21.8±4.4 vs. 9.5±2.4; p=0.032), 3 day (55.8±12.3 vs. 17.7±4.9; p=0.025) as well as 56 day after injection (58.7±13 vs. 24.4±8.7; p=0.024) (Figure 23).

Morris water maze test: Latency to the hidden platform decreased from the first to the fifth testing day at the same rate ($p<0.0001$) in both experimental and control groups suggesting that ET-1 animals are able to learn this task. In contrast, escape

latencies were significantly longer in ET-1 animals than in controls ($p<0.001$) (Figure 24). Also, cumulative escape latencies were longer in animals with ET-1 compared to controls (Figure 25).

4.2.3.2. Rats with ET-1 injection at P25

Motor development: Injection of ET-1 in either dose did not affect motor performance in any test used (Figure 26, 27 and 28).

Morris water maze test: Latency to the hidden platform decreased from the first to the fifth testing day in control as well as ET-1 groups ($p<0.001$) (Figure 29).

There was no difference in cumulative escape latencies between control and ET-1 groups.

Comparison between age groups showed that cumulative escape latencies are shorter in P25 groups than in P12 groups with corresponding dose of ET-1 ($p<0.001$). There was no difference between P12 and P25 controls (Figure 25).

4.2.4. Epileptiform activity 3 months after ET-1 injection

4.2.4.1. Rats with ET-1 injection at P12

In controls, sporadic isolated spikes classified as interictal activity were detected in 1 of 15 animals. None of the control animals exhibited seizures.

Interictal activity was registered in 13 of 14 rats with ET-1. Clear-cut seizures occurred in 10 of 14 animals. The ictal activity was formed either by fast spikes with a frequency from 6 to 8 Hz (Figure 30Aa) or by huge sharp waves with a frequency between 1 and 2 Hz (Figure 30Ab). The ictal activity was usually generalized, only

two rats with the lower dose and one with the higher dose exhibited also partial seizures. EEG seizures were accompanied by freezing and/or automatisms like chewing, licking, and/or orienting reaction, never by convulsions. There was no difference between doses in parameters of electrographic seizures, however, seizure frequency as well as seizure duration varied considerably among individual animals (Figure 31 and 32). In 2 of 14 animals, seizure frequency was extremely high (97 and 75 seizures per 24 h). When they were excluded, the average seizure frequency was 2.3±0.6 per 24 h. Mean seizure duration was 9.1±1.2 s (Figure 33).

4.2.4.2. Rats with ET-1 injection at P25

Only one of 15 control animals exhibited interictal activity. Seizures were not registered in any control animal.

The interictal activity was registered in 11 of 12 animals with ET-1. Also, electrographic seizures, formed by series of spikes (6 to 8 Hz; (Figure 30B) occurred in 11 of 12 animals. Rats with the lower dose of ET-1 exhibited generalized as well as partial seizure activities, higher dose of ET-1 resulted always in generalized EEG seizures. Like in P12 rats, all seizures were nonconvulsive and there was high interindividual variability in parameters of electrographic seizures (Figure 31, 32, 33). One rat exhibited 50.4 seizures per 24 h on the average; if it was excluded, the mean number of seizures was 1.8±0.9 seizures per 24 h (Figure 31). Average seizure duration was 6.5±0.4 s (Figure 33), but seizures were significantly shorter in animals with 40 pmol of ET-1 than with 20 pmol (5.6±0.4 *vs* 7.0±04 s).

Mean duration of seizures (Figure 33) and total seizure activity per 24 h (Figure 32) were significantly higher in P12 than in P25 group suggesting higher severity of epilepsy developed after ET-1 injection in less mature brain ($p=0.038$ and $p=0.032$). There was no difference in number of seizures per 24 h between age groups (Figure 31).

4.2.5. Neuropathologic lesion 3 months after ET-1 injection

Lesion was present in all animals with ET-1 injection. Lesion was characterized by significant cell loss in affected hippocampal fields, accompanied by massive gliosis. Hippocampal lesion had segmental character and principal layers were more affected (Figure 34). As seen 24 h after ET-1 injection, size and distribution varied considerably among animals.

4.2.5.1. Rats with ET-1 injection at P12

Three brains (1 control and 2 ET-1 animals) were excluded from analysis for technical reasons (tissue destruction). Tissue damage was localized only in dorsal part of the hippocampus. Severity of the hippocampal injury expressed as score was 10.8±0.8. Difference between the two dose groups was significant ($p=0.037$, Figure 34J). Mean number of damaged hippocampal subfields was 3.7±0.1.

4.2.5.2. Rats with ET-1 injection at P25

Damage was observed in the dorsal part of the hippocampus in all animals with ET-1 injection. Extension of the hippocampal damage expressed as score was 4.0±0.6. Lower dose of ET-1 induced significantly milder damage than higher dose ($p=0.002$, Figure 34J). Mean number of damaged hippocampal subfields was 2.5±0.3 and difference between dose groups was significant (1.9±0.3 *vs.* 3.1±0.3; $p=0.033$). ET-1-induced lesion was more severe in P12 than P25 animals ($p<0.001$). Also, number of damaged hippocampal subfields was higher in P12 than in P25 ($p=0.001$) (Figure 34D, G, I and K). Differences between individual dose groups also reached level of significance (Figure 34J).

4.2.6. Timm score

4.2.6.1. Rats with ET-1 injection at P12

Intensity of Timm sprouting expressed as score was significantly higher in ET-1 compared to control rats in both injected (1.282±0.151 *vs.* 0.712±0.104; p=0.004) and contralateral (1.3±0.1 *vs.* 0.7±0.1) hippocampus. Intensity of sprouting increased with dose of ET-1 and difference was significant in both injected (0.9±0.1 *vs.* 0.4±0.1, p=0.022) and contralateral (0.8±0.1 *vs.* 0.5±0.1; p=0.021) (Figure 35 and 36).

4.2.6.2. Rats with ET-1 injection at P25

ET-1 injection in P25 rats had no effects on intensity of Timm sprouting three months later (1.0±0.1 in controls vs. 1.3±0.1 in ET-1 group at the injected site and 1.1±0.1 in controls vs. 1.1±0.1 in ET-1 animals at the opposite site) (Figure 37).

4.2.7. Changes in the hippocampal volume

4.2.7.1. Rats with ET-1 injection at P12

Compared to controls, volume of the injected hippocampus decreased 3 days after ET-1 injection by 17% (23.2± 0.8 vs. 16.4± 0.4 mm^3; p<0.001). Three months later volume of the injected hippocampus was by 30% lower in ET-1 animals compared to controls (47.1±1.2 vs. 33.3±3.6 mm^3; p=0.005) (Figure 38).

4.2.7.2. Rats with ET-1 injection at P25

Three days after injection, volume of the injected hippocampus of ET-1 animals was lower by 6% compared to controls (35.2±1.4 vs. 31.9±1.4 mm^3; p=0.113). Similarly to P12 rats, the difference between ET-1 animals and controls increased with time. Three months after the injection volume of the injected hippocampus was by 17% lower in ET-1 than in control animals (41.1±1.5 vs. 33.2±1.9 mm^3; p=0.004) (Figure 39).

4.2.8. Correlation between severity of the hippocampal damage and seizure severity and/or cognitive impairment in chronic phase

There was positive correlation between seizure activity (total seizure time/24 h) 3 months after ET-1 injection and severity of the hippocampal lesion in both P12 (r=0.640; p=0.001) and P25 (r=0.768; p<0.001) animals.

Severity of the lesion assessed 3 months after ET-1 injection correlated positively with cumulative latency in the Morris water maze in P12 (r=0.359; p=0.04) but not in P25 animals (r=-0.094; p=0.592).

In P12, there was positive correlation between intensity of sprouting and extension of the hippocampal lesion (both ipsilaterally to the ET-1 injection; r=0.573; p=0.004) but not total seizure time (r=0.280; p= 0.187).

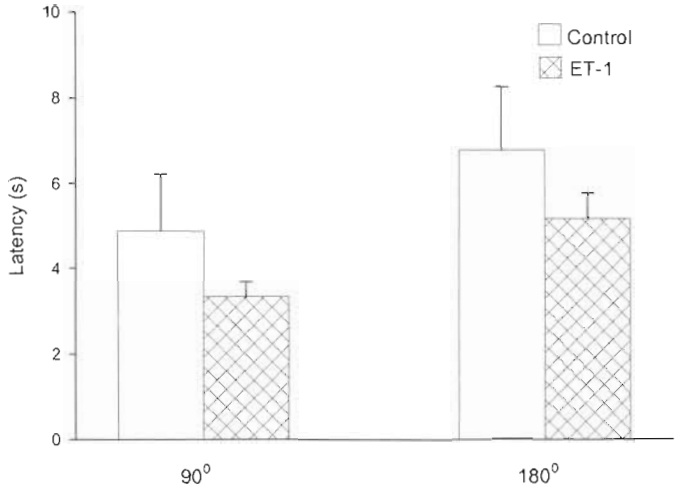

Figure 19. Mean latencies (s) to rotate 90^0 and 180^0 on the inclined plane of negative geotaxis task. Rats had been exposed to vehicle or different dose of ET-1 (20 or 40pmol) on the PD 12. The data are expressed as mean ± SEM (Kruskal-Wallis). The differences between Control vs ET-1 were not significant.

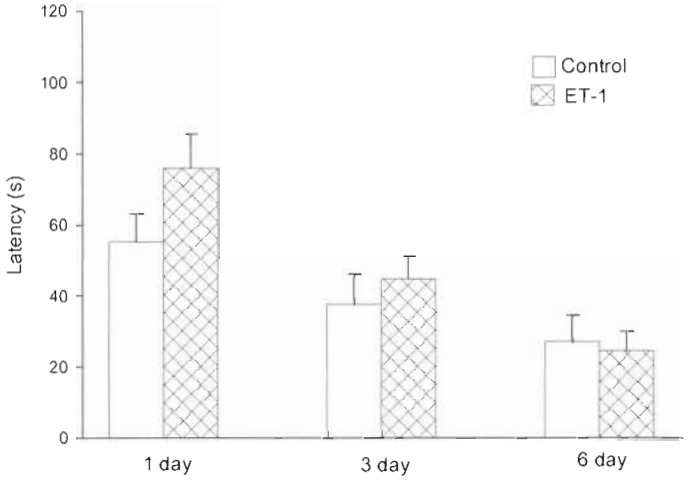

Figure 20. Wire mesh test. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 12. Data represent mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

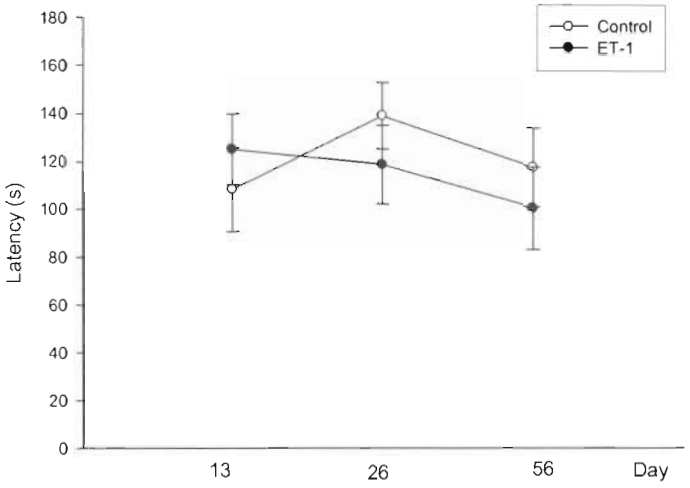

Figure 21. Rotarod test with speed 15 r.p.m. Rats had been exposed to vehicle or different dose of ET-1 (20 or 40pmol) on the PD 12.Data are expressed as mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

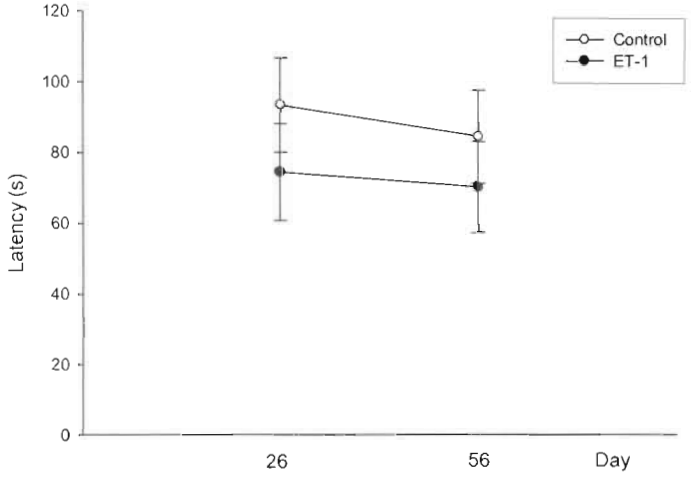

Figure 22. Rotarod test with speed 30 r.p.m. Rats had been exposed to vehicle or different dose of ET-1 (20 or 40pmol) on the PD 12. Data are expressed as mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

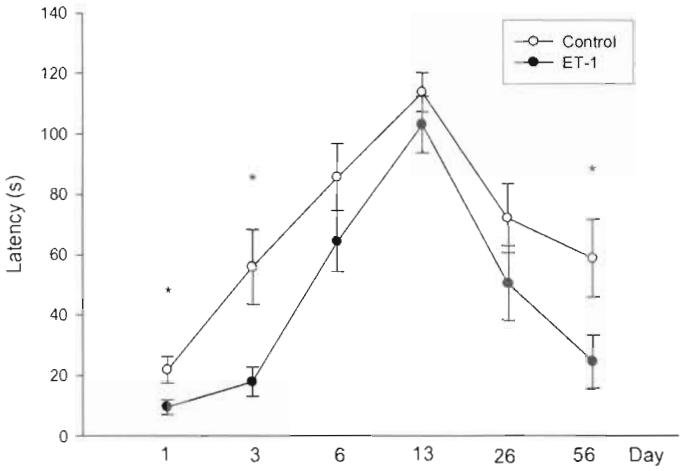

Figure 23. Bar holding test. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 12. Data represent mean ± SEM (Kruskal-Wallis (ANOVA) test). Comparison were made between controls and ischemics (after ET-1) at each time point (* indicate $p<0.05$).

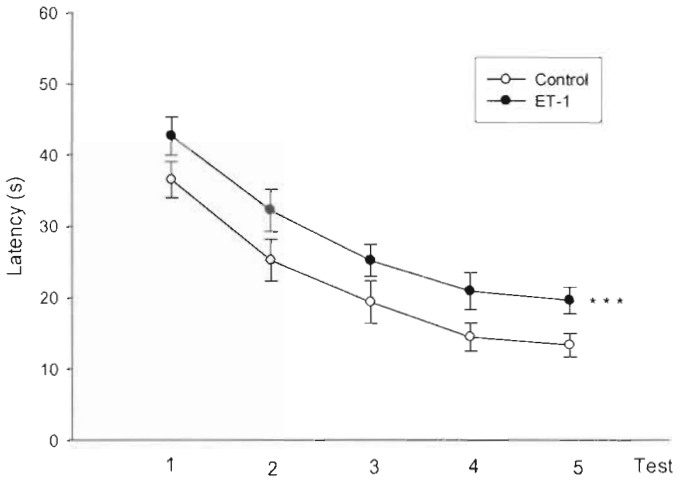

Figure 24. Learning curves in the Morris water maze 3 months after ET-1 injection. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 12 and tested 5 consecutive days for learning abilities. Data are presented as mean ± SEM (One –way RM ANOVA). Comparison were made controls and ischemics (after ET-1) at each time point (*** indicate $p<0.001$).

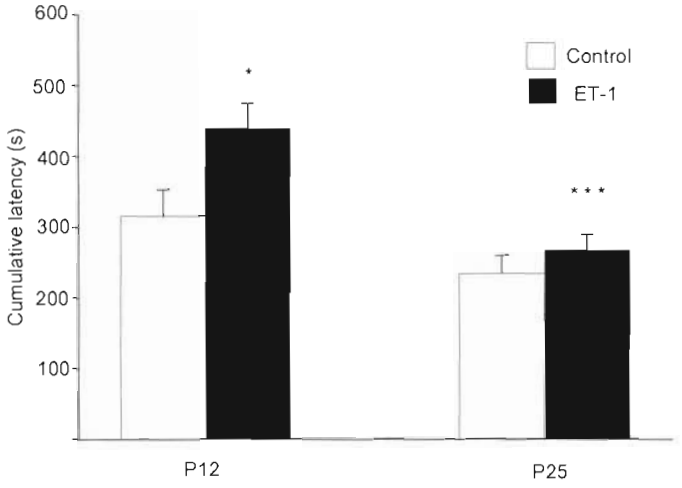

Figure 25. Performance in Morris water maze. Comparison of the best performance reached by animals in individual age- and dose-groups in this test. Results are expressed as a mean of cumulative latency ± SEM (Kruskal-Wallis (ANOVA) test). Significant differences ($p<0.05$) between controls and ischemics (after ET-1) in one age group are indicated with *. Significant differences ($p<0.001$) between the two age groups receiving corresponding dose of ET-1 with * * *.

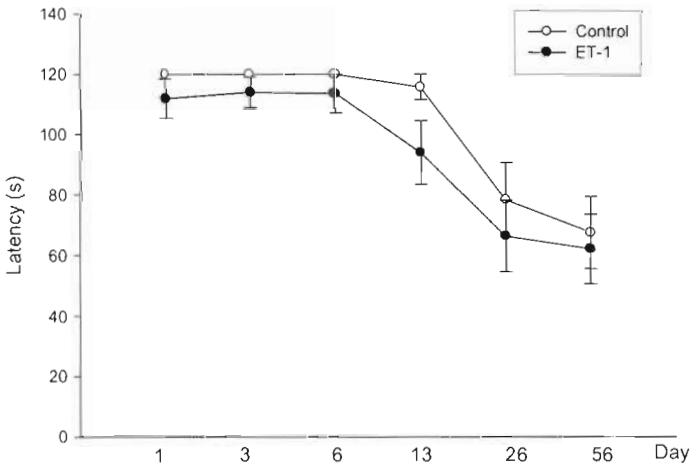

Figure 26. Bar holding test. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 25. Data represent mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

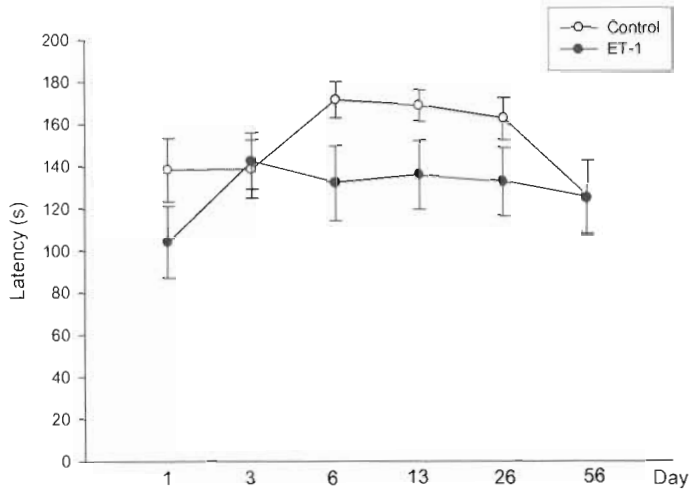

Figure 27. Rotarod test with speed 15 r.p.m. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 25.Data are expressed as mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

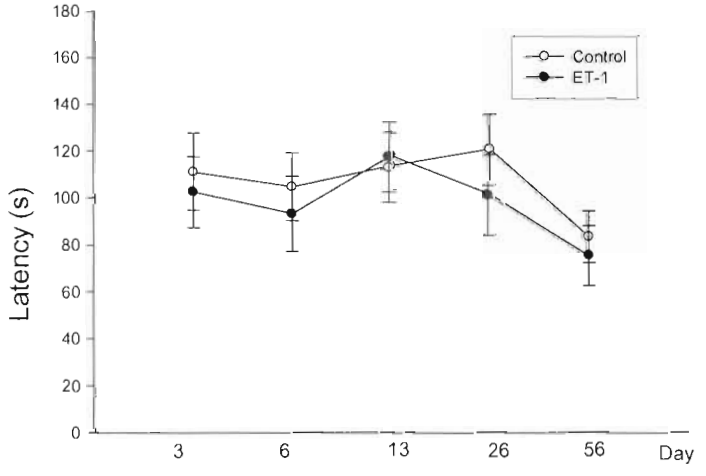

Figure 28. Rotarod test with speed 30 r.p.m. Rats had been exposed to vehicle or different dose of ET-1 (20 or 40pmol) on the PD 25. Data are expressed as mean ± SEM (Kruskal-Wallis (ANOVA) test). The differences between Control vs ET-1 were not significant.

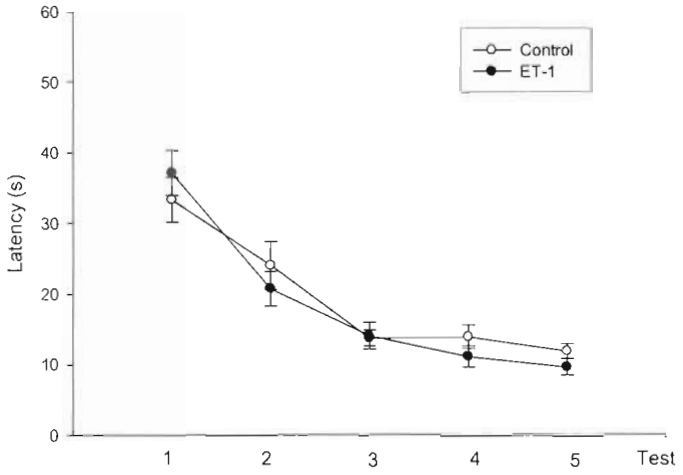

Figure 29. Learning curves in the Morris water maze 3 months after ET-1 injection. Rats had been exposed to vehicle (control) or different dose of ET-1 (20 or 40pmol) on the PD 25 and tested 5 consecutive days for learning abilities. Data are presented as mean ± SEM (One–way RM ANOVA). The differences between ischemics (all rats after ET-1) and controls were not significant.

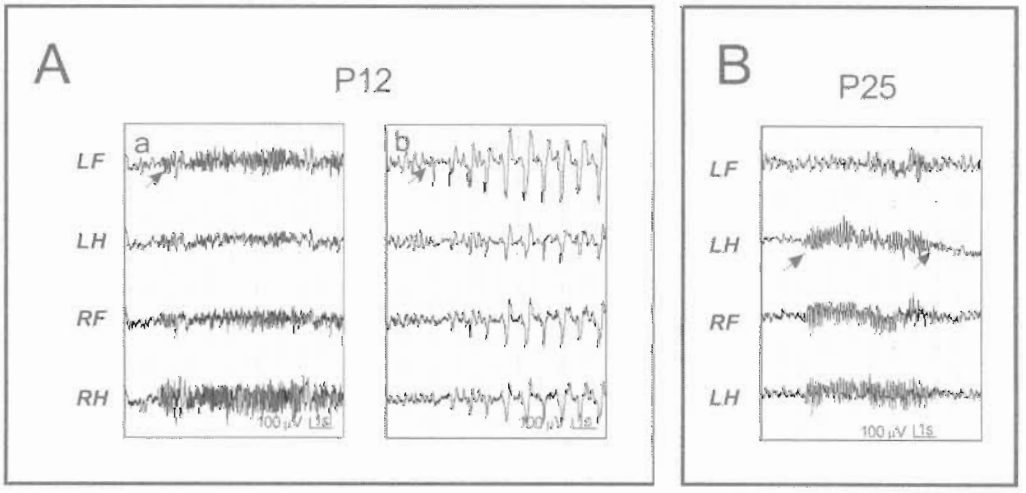

Figure 30. Examples of electroencephalographic recordings at 3 months after ET-1 injection. The beginning and the end of the electrographic seizure is indicated with arrows. **(A)** Examples of two types of electrographic seizures registered in the same animal with ET-1 (20 pmol) at P12. **(a)** A seizure formed by fast spikes (with a frequency of 6-8 Hz). **(b)** A seizure formed by huge sharp waves with a frequency between 1 and 2 Hz. **(B)** A spontaneous electrographic seizure associated with behavioral immobility in a rat with ET-1 (20 pmol) at P25.

Time marks 1 s, amplitude calibration 100µV. Individual leads: LF-left sensorimotor cortex; LH-left hippocampus; RH-right sensorimotor cortex, all in reference connection.

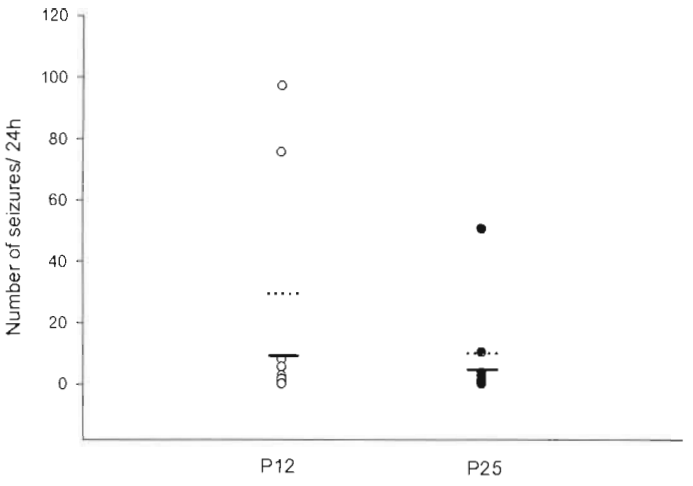

Figure 31. Number of seizures per 24 h. Data from both dose ET-1 groups were collapsed. Symbols represent values from individual animals and mean values are represented by the black line. Dotted line marks mean ± SD. Seizures were more frequent in animals with ET-1 injection at P12 (n=10) than at P25 (n=11). However, the differences between P12 and P25 animals were not significant (Kruskal-Wallis (ANOVA) test).

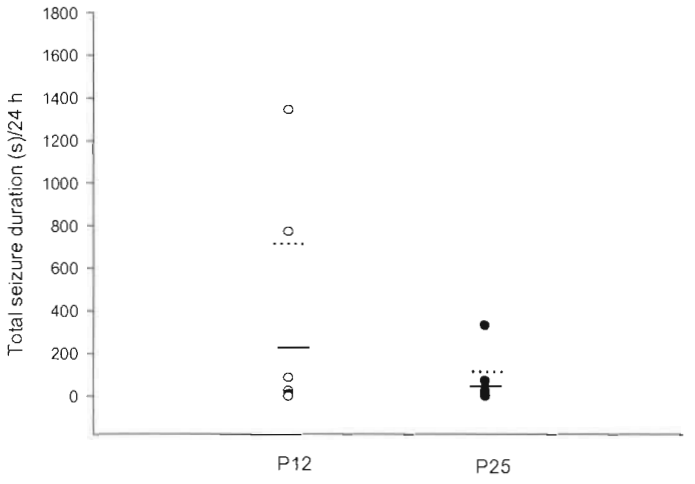

Figure 32. Total seizure duration (s)/24 h. Data from both dose ET-1 groups were collapsed. Symbols represent values from individual animals and mean values are represented by the black line. Dotted line marks mean ± SD. Total seizure duration per 24 h was longer in animals with ET-1 injection at P12 (n=10) than at P25 (n=11). The differences between P12 and P25 animals were significant (p=0.032) (Kruskal-Wallis (ANOVA) test).

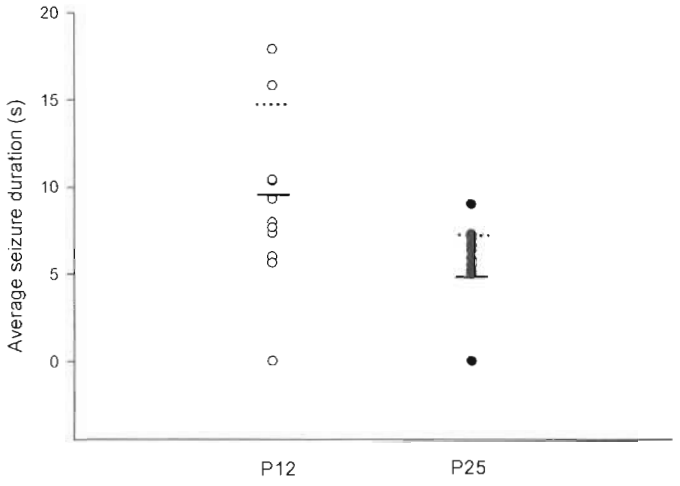

Figure 33. Average seizure duration (s). Data from both dose ET-1 groups were collapsed. Symbols represent values from individual animals and mean values are represented by the black line. Dotted line marks mean ± SD. Average seizure duration was longer in animals with ET-1 injection at P12 (n=10) than at P25 (n=11). The differences between P12 and P25 animals were significant (p=0.038) (Kruskal-Wallis (ANOVA) test).

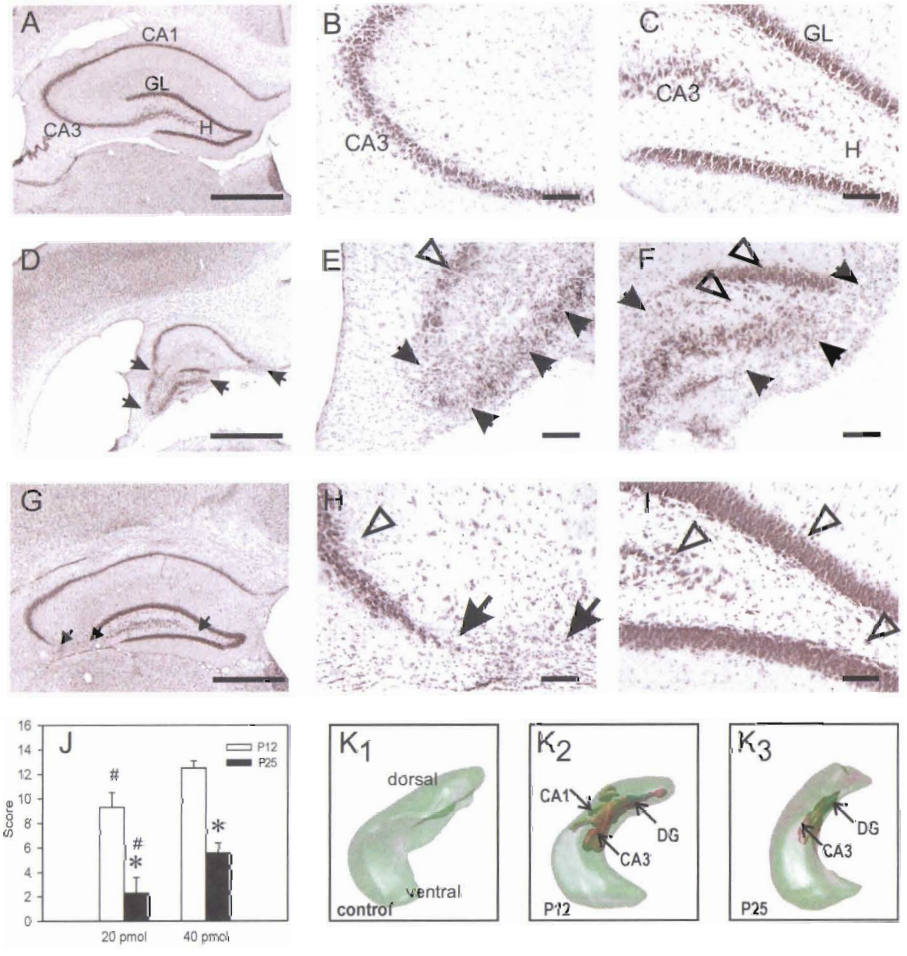

Figure 34. Pattern of the hippocampal lesion in adult rats 3 months after intrahippocampal injection of ET-1. **(A)** Digital image of the hippocampus of the control rat. Higher magnification of the CA3 field **(B)** and the dentate gyrus **(C)** taken from the same section. **(D)** Marked shrinkage of the hippocampal formation and the extensive hippocampal damage in the animal with ET-1 (40 pmol) injection at P12 (morphological score = 14.1). An extensive neuronal loss is accompanied by marked gliosis in the CA3 field **(E)** as well as in the dentate gyrus **(F)**. Higher magnification illustrates typical "patchy" pattern of distribution of neuronal loss (black arrows). Open arrows show surviving neurons. **(G)** Significantly less extensive damage in the animal with ET-1 (40 pmol) at P25 (morphological score = 2.9). **(H)** Detail of injury to CA3 field. Note a decrease of hillar neurons **(I)**. Scale bar A, D, G = 1 mm; B, C, E, F, H, I = 100 µm. **(J)** Age- and dose-related differences in the extension of the ET-1-induced lesion 3 months after the injection. Results are expressed as morphological score (mean + SEM). Significant differences ($p<0.05$) between doses in one age group are indicated with #, differences between different age groups receiving the same dose of ET-1 with *. **(K)** 3D reconstruction of the injected hippocampus of the representative control (K_1), P12 (K_2) and P25 (K_3) animal 3 months after injection. 3D models were prepared using Ellipse software (ViDiTo, Kosice, Slovakia).

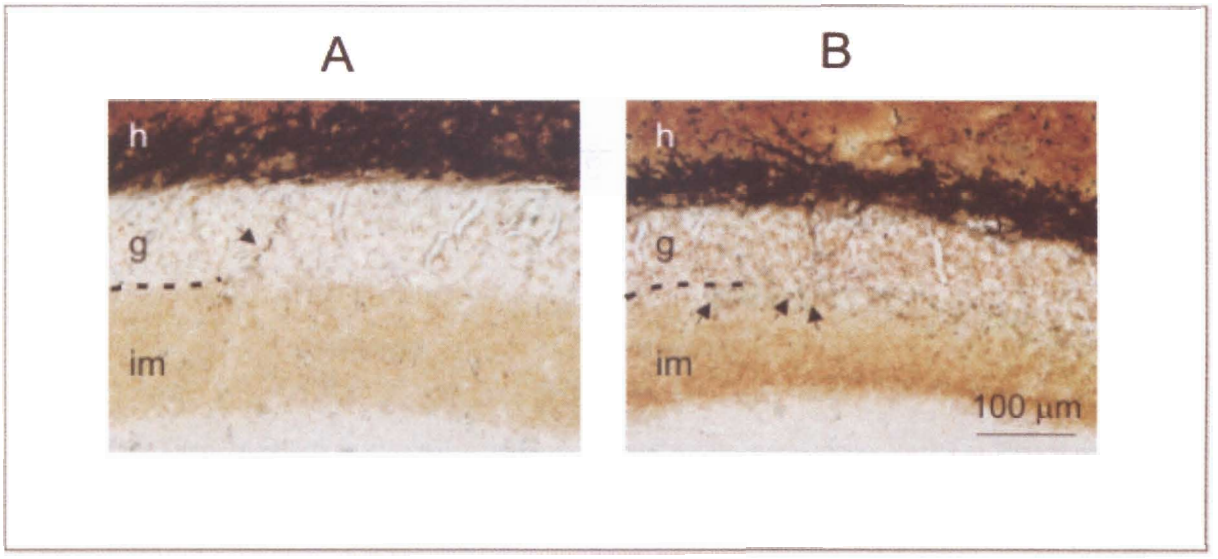

Figure 35. Bright field microphotographs of Timm stained sections demonstrating mossy fibers sprouting in the septal hippocampus (arrows) in control **(A)** and ET-1 rats **(B)** 3 months after intrahippocampal injection of ET-1 (40 pmol). Mossy fibers make tree-like arborizations (arrowheads) in the inner molecular layer corresponding to score 2 sprouting. Scale bar 100µm.

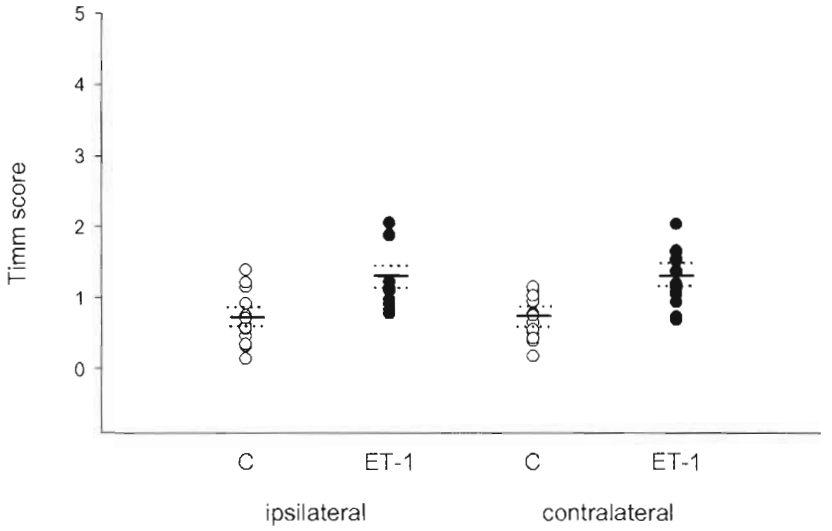

Figure 36. Timm sprouting in the ipsilateral (injected) and contralateral hippocampus expressed as a score 3 months after surgery. Rats had been exposed to vehicle (C-control) or different dose of ET-1 (20 or 40pmol) on the PD 12. Symbols represent values from individual animals. The black lines represent mean ± SEM (dotted lines).

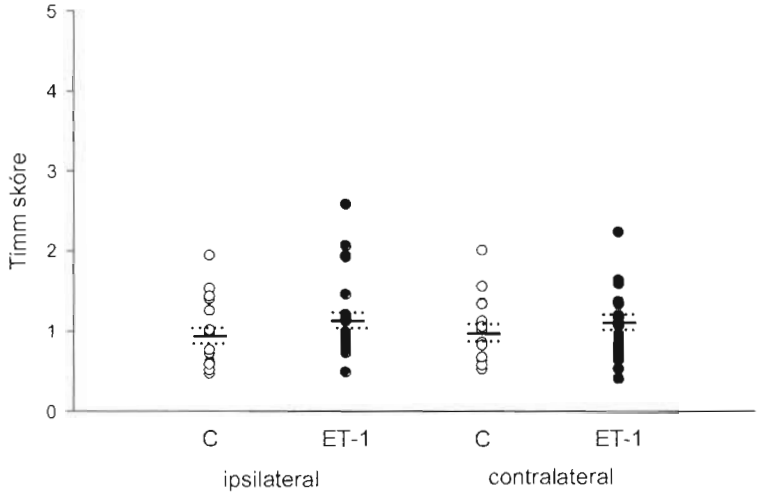

Figure 37. Timm sprouting in the ipsilateral (injected) and contralateral hippocampus expressed as a score 3 months after surgery. Rats had been exposed to vehicle (C-control) or different dose of ET-1 (20 or 40pmol) on the PD 25. Symbols represent values from individual animals. The black lines represent mean ± SEM (dotted lines).

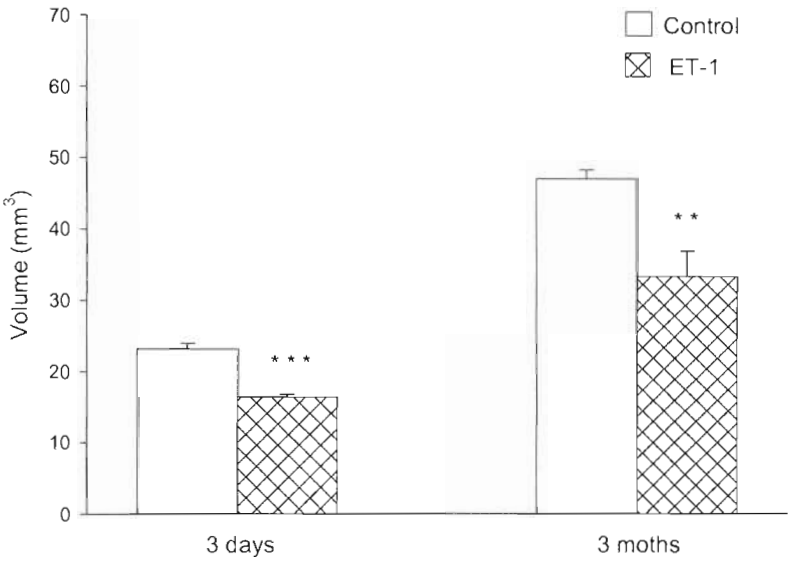

Figure 38. Bar graph indicating the volume of the injected left hippocampus 3 days and 3 months after injection of ET-1 at P12. Data represent mean ± SEM (Kruskal-Wallis (ANOVA) test). Comparison was made between ischemics (after ET-1) and controls (* * indicate $p<0.01$, * * * indicate $p<0.001$).

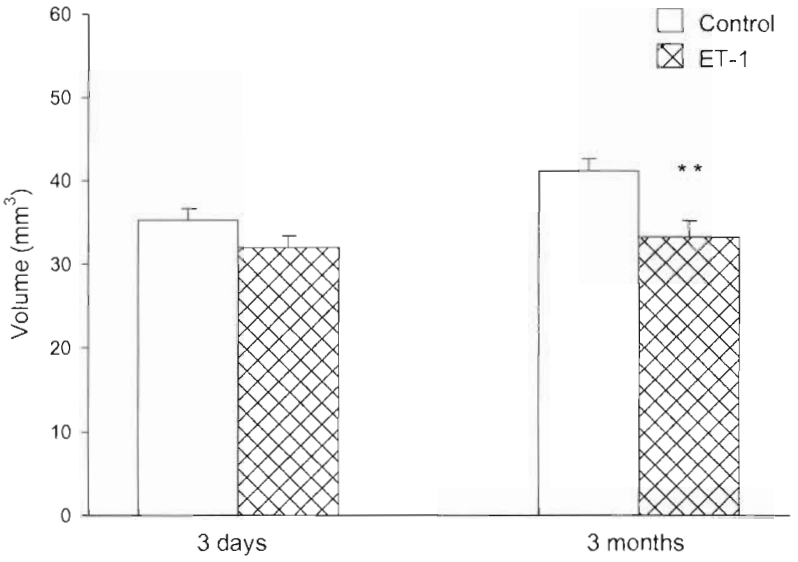

Figure 39. Bar graph indicating the volume of the injected left hippocampus 3 days and 3 months after injection of ET-1 at P25. Data represent mean ± SEM (Kruskal-Wallis (ANOVA) test). Comparison was made between ischemics (after ET-1) and controls (* * indicate $p<0.01$).

V. DISCUSSION

Present data document that ET-1 injected unilaterally into the dorsal hippocampus of developing rats induces epileptic seizures in both P12 and P25 rats. In adulthood, recurrent epileptic seizures were detected in 60% to 100% of animals of both age groups. Severity of seizures correlated in adulthood with severity of epilepsy in P25 but not in P12 group. Intrahippocampal ET-1 injection was followed only by subtle changes of motor development. Significant cognitive impairment in adulthood was found only in P12 group. When injected at P25, ET-1 did not induce any marked long-term changes of motor abilities or cognitive functions.

Methodological considerations

Previous studies demonstrated that ET-1-induced lesion of the striatum in the anesthetized rats involve local ischemia. Vasoconstrictor effect of ET-1 is dose dependent and injection of ET-1 induces prolonged and severe transient ischemia in the brain *(Fuxe et al. 1992)*. Following intrastriatal injection of 10 pmol ET-1, local CBF in the injected striatum compared to the matched area in the noninjected striatum was reduced by 40% at 1 h and 20% at 3 h in adult rats *(Hughes et al. 2003)*. As evaluated by laser Doppler flowmetry, ET-1 in a dose of 0.43 nmol produced about a 60% reduction of CBF within 20 min. This reduction was largely maintained throughout 3 h with only a small return (reduction by 40% at the end of 3 h interval). Reduction of CBF was accompanied by local increase of lactate levels with a peak increase at 40 min interval. From this time point, levels of lactate gradually declined and the control level was reached 160 min after injection *(Fuxe et al. 1992)*. The decrease of CBF flow as well as increase of lactate level was reversed by dihydralazine (53 nmol added into perfusate for microdialysis), a drug with direct vasodilatative effects on the smooth muscle cells suggesting that the development of the ET-1-induced lesion of brain parenchyma is related to a hypoperfusion triggered

by the ET-1-induced vasoconstriction. Effects of ET-1 on blood flow during brain maturation remains to be studied. In addition to an ischemic effect, the contribution of other, non-vascular mechanisms may participate in the development of ET-1-induced brain injury. Among them, in vivo ET-1 may exacerbate neuronal death indirectly through the excessive release of potentially neurotoxic mediators from astrocytes. Studies in vitro, however, demonstrated that ET-1 is not directly neurotoxic *(Lustig et al. 1992)*. Therefore, we can hypothesize that lesions caused by ET-1 are directly related to its ability to reduce CBF and represents a model of ischemic injury rather than a primary excitotoxic lesion.

Effects of endothelins are mediated by specific ET_A and ET_B receptors *(Masaki 2004)*. The application of ET-1 directly into the CNS parenchyma will activate the ET_A receptors, resulting in a potent vasoconstriction, and hence a focal atraumatic ischemia. The consequences of astrocytic, microglial or even neuronal ET_B receptor activation are likely to be secondary to the effect of the profound ischemia induced *(Corkill 2003)*.

Dreier et al. demonstrated that application of ET-1 at a concentration between 10 nM to 1 mM potently induces cortical spreading depression *(Dreier et al. 2002)*. In our study, no depression of EEG activity occurred in the injected hippocampus in any age group. This may be explained by the difference in ET-1 concentration used in our study (20–40 pmol). In addition, classical studies suggest that spreading depression cannot be induced in the hippocampus of rats younger than 15 days *(Fifková 1964)*.

For verification of the present model as a model of human developmental impairment it is critical to know how the age of rats correlates with those in humans. Such correlation is very difficult. Various parameters have been used for comparison of developmental stages between different species. Therefore, even conclusions obtained in the same laboratory might vary in relation on measured characteristic. Adlard et al. claimed that the 5-day-old rat is an appropriate model for the human newborn in term of brain maturation and their conclusion is based on the timing of peak velocity of accumulation of brain wet weight in both species *(Adlard et al. 1973)*. However, based on timing of "growth spurt" as a vulnerable period, Dobbing compared human babies from the last few weeks of gestation through the first few months of life to rats 10-12-day-old *(Dobbing 1970)*. In addition to biochemical parameters, studies on bioeletrical activity of the rat brain demonstrate that irregular

EEG activity appears at the age of 5-6 days *(Ellingson and Rose 1970; Mareš et al. 1979)* and up to P10 EEG activity is interrupted by periods of electrical silence corresponding to "tracé alternant" *(Ellingson 1964)* in preterm newborns but never seen in full-term human newborns. Sexual maturation in rats takes place between P35 and P45 in both sexes *(Piacsek et al. 1978)*. Based on these facts, we assume that 12- and 25-day-old rats may correspond with early infancy and preschool or early school age in humans, respectively.

Intrahippocampal injection of ET-1 induces epileptic seizures in immature rats

Present data demonstrate that intracerebral injection of ET-1 induces electrographic seizures. Seizures were behaviorally characterized by forelimb clonus and rotational phenomena as circling and barrel rolling. Pattern of behavioral seizures seen in immature rats was consistent with previous study in non-anesthetized adult rats *(Nagasaka et al. 1999)*. Previously, convulsion-like behavior was observed after the injection of ET-1 into the lateral ventricle of freely moving adult rats *(Chew et al. 1995; Gross and Weaver 1993)*, epileptiform activity in EEG was however not detected probably due to continuous halothane anesthesia during EEG monitoring in this study *(Gross et al. 1992)*. As suggested before halothane attenuated ischemia-induced seizures and thus recording in awake animals was necessary to detect epileptiform EEG activity *(Hartings et al. 2003)*. Early studies reported "barrel rolling" as the most typical convulsive behavior observed after central administration of ET-1 *(Chew et al. 1995)*. Our data document that barrel rolling is accompanied by electrographic seizures in freely moving animals.

Our study suggests specific scoring system for evaluation of behavioral seizures in this model. Scoring system was developed with respect to presence of specific convulsion patterns, progression of seizure severity and age-specificity (for details *Velíšková 2006)*.

Development of seizures immediately after ischemic insult was described in a model of middle cerebral artery occlusion in adult rats. Using EEG monitoring,

Hartings et al. demonstrated spontaneous seizures in animals with permanent occlusion as well as in animals subjected to transient ischemia with reperfusion *(Hartings et al. 2003)*. Recently, Yager et al. mentioned the occurrence of convulsive seizures in animals with focal ischemia induced by the injection of ET-1 to the MCA in both immature (P10) and adult (3 and 6 month) rats *(Yager et al. 2005)*. They however did not provide detailed description of seizures. In immature CD1 mice, ligation of unilateral carotid artery without general hypoxia induced behavioral seizures in 75% of animals suggesting that immature brain may be prone to ischemia-induced seizures *(Comi et al. 2004)*. In immature rats with ligation of the common carotid artery development of immediate convulsions was never described, but hypoxia-ischemia damage led to dynamic changes of susceptibility to bicuculline-induced seizures (*Cataltepe et al. 1995*).

Previously, significant age-related differences in seizure susceptibility were found in a model of global hypoxia. The severity of both behavioral and EEG responses to hypoxia conditions were most severe in P10 to P12 animals *(Jensen et al. 1991)*. Another model of temporary bilateral carotid ligation in immature rats resulted in an appearance of clonic-tonic seizures with a maximum between P20 and P30 *(Jílek et al. 1964)*. In our model (highly probable model of focal ischemia), intrahippocampal injection ET-1 induced epileptic seizures in both P12 and P25 rats, and there was no difference in seizure severity between age groups. This result is somehow surprising in view of significant difference in the severity of brain damage between both age groups. However, participation of other mechanisms additional to ischemia and their age-related changes in seizure development has to be analyzed.

In present study, animals determined for long-term follow up were injected under light halothane anesthesia and after the end of infusion cannula was removed. Rapid growth of immature rats makes it impossible to keep a cannula fixed to the skull till adulthood. Halothane was previously reported to block or attenuate seizure activity induced by middle cerebral artery occlusion *(Hartings et al. 2003)*. In the present study, convulsive behavior was seen within one hour in all age- and dose-groups. Righting reflexes were re-established within 7 min after disconnecting anesthesia and placing animals into an observation box in both age groups. It is, therefore, unlikely that brief general anesthesia during infusion significantly changes the pattern of behavioral seizures.

Transient focal ischemia induces lesion of the hippocampus

Our data demonstrates that severity of ET-1-induced lesion is higher in P12 than in P25 groups 24 h as well as 3 months after injection. Previous studies already suggested that immature brain might be more susceptible to hypoxic ischemic injury compared to mature one. This relationship is however not linear with age. In contrast to our data, Yager and Thornhill found more severe lesion in the hippocampus of P21 rats compared to P10 or P63 animals in a model of ischemic insult induced by unilateral carotid artery ligation *(Yager and Thornhill 1997)*. There are several possible explanations for such difference. First, increase of glutamate levels due to direct effects of ET-1 on glial cells *(Sasaki et al. 1997)* can exacerbate ischemia-induced damage. It was demonstrated previously that younger brain may be more susceptible to excitotoxic damage due to enhanced postsynaptic development of the NMDA receptors *(McDonald et al. 1988)*. Ikonomidou et al. found that vulnerability to NMDA-induced injury was the highest between P4 and P14 with peak at P6 *(Ikonomidou et al. 1989)*. With increasing age, excitotoxic response progressively decreased and animals older than 20 days were relatively less sensitive than P10 animals. Second, sensitivity of vessels to ET-1 induced vasoconstriction might vary in relation to age. Studies on the effects of ETs on cerebral vascular functions in immature animals are however rare. Available data confirmed vasoconstrictive activity of ET-1 even during early infancy. ET-1 topically applied constricted pial arterioles in a dose-dependent manner in concentrations 10^{-10} mol and higher in 3-day-old piglets *(Yakubu et al. 2002)*. However, both age-related and regional differences in vascular effects of ET-1 remained to be studied in details. Also, substantial differences between models might play an important role.

ET-1 injection induced volume decrease of the injected hippocampus in both age groups. In agreement with semiquantitative assessment of the hippocampal lesion stereological volumetry confirmed higher sensitivity of P12 animals to ET-1-induced brain damage. In both age groups, the extent of the lesion increased with time after ET-1 injection. Whether such increase is due to continuous neurodegeneration remains to be further studied.

Sprouting of granule cell axons or mossy fibers was used as a marker of axonal plasticity. The excessive sprouting was observed only in rats with ET-1 injection at

P12 but not at P25. Such difference may be related to the fact that damage to the hippocampus is less extensive in P25 and therefore severity of the damage is not sufficient to induce sprouting. This hypothesis is supported by the fact that sprouting intensity correlated with severity of the damage in P12 rats. In contrast, intensity of sprouting did not correlate with total duration of seizure activity in any age group. This observation supports previously published data demonstrating that mossy fiber sprouting is not necessary for the occurrence of spontaneous seizures *(Nissinen et al. 2001; Bender et al. 2003; Raol et al. 2003).* In contrast, positive correlation between extension of the hippocampal lesion and sprouting found in P12 animals suggests possibility that sprouting is an important player in recovery mechanisms after the hippocampal injury.

Transient focal ischemia in the immature brain leads to development of spontaneous seizures in adulthood

The immature brain is more prone to generate seizures. Using chemically as well as electrically induced seizures, many experimental studies proved the higher seizure susceptibility in immature than in adult brain. On the other hand, ontogenetic studies of epileptogenic effects of early brain insult bring controversial data. Numerous studies demonstrated that early brain insult like prolonged hyperthermic seizures *(Dubé et al. 2000)*, perinatal hypoxia *(Jensen et al. 1991),* repeated brief seizures induced by fluorothyl *(Villeneuve et al. 2000),* or multiple episodes of pilocarpine-induced status epilepticus (SE) *(Santos et al. 2000)* can cause permanent hyperexcitability. Studies reporting the development of spontaneous recurrent seizures as a consequence of brain insult in animals younger than 2 weeks are, however, sparse. In a model of lithium-pilocarpine-induced SE, long term video/EEG monitoring confirmed spontaneous recurrent seizures in 30% of the animals with SE at P14, whereas 75% of the animals that underwent SE at 4 weeks of age developed spontaneous seizures *(Sankar et al. 1998).* Also, our previously published study demonstrated that SE as early as at P12 can trigger epileptogenesis in 25% of animals and that number of animals developing spontaneous seizures increases with age at SE

(Kubova et al. 2004). Observation of rats with SE as adults demonstrated that all of them developed spontaneous convulsive seizures *(Hort et al. 1999; Leite et al. 1990)*.

There are two previous reports showing that injection of SE-inducing toxins into the immature hippocampus results in the development of spontaneous seizures. Babb et al. reported spontaneous seizures with a latency of up to a few months in rats with intrahippocampal administration of kainate at P7 *(Babb et al. 1995)*. Lee et al. detected temporary spontaneous seizures in P7 rats after injection of tetanus toxin into the hippocampus *(Lee et al. 1995)*.

Relatively few experimental studies have been designed to determine the relationship between ischemic injury and development of epilepsy. Published data however strongly suggest that the epileptogenic effect of stroke is highly related to the model used and to the age of animals at the time of lesion. In adult rats, cortical phototrombic brain infarction resulted in the development of epilepsy in 75% of animals within 2 months after lesion *(Kelly et al. 2001)*. In contrast, Karhunen et al. did not find recurrent seizures during a 1-year follow up after transient (120 min) MCAO *(Karhunen et al. 2003)*. In both studies, video/EEG monitoring was used to detect seizures. Therefore, it is unlikely that non-convulsive seizures remained undetected. In contrast, developmental studies suggest higher susceptibility to ischemia-induced epileptogenesis. Williams et al. detected motor seizures in 40% of rats with hypoxic-ischemic lesion induced by the permanent ligation of the right common carotid artery at P7 followed by hypoxia (8% oxygen) *(Williams et al. 2004)*. Seizures were detected by regular observation of animals in vivarium for 7 to 24 months without EEG monitoring.

In our study, continuous EEG monitoring revealed electrographic seizures in both P12 and P25 groups 3 months after ET-1 injection. Incidence of animals with spontaneous seizures was very high (60-100%) and tended to increase with the dose of ET-1. In both age groups, seizures were non-convulsive and motor seizures were never observed. Our data are consistent with data published by Romijn et al. 1994, who found spontaneous EEG seizures in 83% of rats with hypoxic-ischemic lesion. In their study, lesion was induced by permanent ligation of the right carotid artery followed by 90 min hypoxia (8% O_2 and 92% N_2) at P12-13 and EEG recording was performed 3 months after lesion. They never observed motor seizures. Both studies

were, however, relatively short, and it is possible that animals would start to have motor seizures if allowed to survive for longer time.

In the present study, there was no difference in seizure frequency or duration of seizures between P12 and P25 groups. There was, however, substantial interanimal variability in the seizure frequency and total duration of seizure activity in individual groups. In each age-and-dose group, there was one animal with seizure frequency 7 to 68 times higher than the mean number of seizures per 24 h. When the animal with an extremely high number of seizures was excluded, seizure frequency was 1 or 2 seizures per 24 h in both age groups. In another model of early insult, lithium-pilocarpine-induced status epilepticus, seizure frequency increases with age at brain insult. Rats with SE at P25 exhibited 3 month after SE higher seizure frequency compared to animals with SE at P12 (5 seizures vs. 1 seizure per 24 h; Kubová unpublished), suggesting a different role of age in epileptogenesis induced by various mechanisms. It is, indeed, clear that EEG monitoring restricted to 5 days used here leads only to rough characterization of seizure activity. Additional longer-lasting studies with repeated video/EEG monitoring at different time-points after insult are necessary to characterize focal ischemia-induced epileptogenesis and its dynamics.

In the present study, there was correlation between severity of behavioral seizures after the ET-1 injection and seizure severity 3 months later in animals with lesion at P25. These data suggest that severity of acute seizures during ischemic attack might be used to predict severity of epilepsy.

Transient focal ischemia in immature brain impairs spatial memory and learning abilities by age-depending manner

Our data show that transient focal ischemia, induced by intrahippocampal injection of ET-1 at P12 but not at P25 leads to permanent changes of learning abilities and spatial memory. In contrast, repeated testing in battery of motor tests did not confirm permanent motor deficits in any age group. Transient changes in bar holding test occurred in animals with higher dose of ET-1 at P12 suggested mild developmental

delay in motor development, whereas other motor tests used at the same age did not support this possibility. No differences between controls and ET-1 animals were observed in negative geotaxis and wire mesh tests. Previously published studies, however, demonstrated that the motor abilities necessary to pass the last two tests successfully mature relatively early. Virtually all P5 animals are able to rotate their bodies in the negative geotaxis test. By P9 the minimal time necessary to rotate 180° is reached *(Altman and Sudarshan 1975)*. Also, about 80% of P13 animals are successfully ascending in the wire mesh test *(Altman and Sudershan 1975)*. Therefore, these two tests are suitable to detect extensive motor impairment rather than mild developmental delay in animals P13 and older.

Animals with injection of ET-1 at P12 were slower to learn the Morris water maze task compared to controls, and they never reached control level. Experimental studies indicate that even brief epileptic seizures can cause learning disabilities and impairment of spatial memory *(for rev. Majak and Pitkanen 2004)*. In models of epileptogenesis induced by status epilepticus in adult rats, learning and memory retrieval was significantly impaired in spontaneously seizing animals in the water maze test *(Harrigan et al. 1991; Rice et al. 1998)*. Hort et al. documented correlation between worsening of performance in Morris water maze and occurrence of spontaneous behavioral seizures *(Hort et al. 1999)*. The learning abilities were impaired more severely in animals with frequent seizures (>1/day) than in those with rare seizures (<1/day; *Nissinen et al. 2000)*. The seizure frequency in our study ranged between 1 - 2 seizures/24 h in all age-and-dose groups when animals with extreme number of seizures were excluded. There was no difference in seizure frequency, mean seizure duration or in total seizure duration per 24 h between P12 and P25 groups. Also, the incidence of epileptic animals did not differ significantly between two age groups, however, P25 animals tended to develop epilepsy more frequently. Taken together, in spite of similar epileptogenic effects of ET-1 injection in both age groups, P12 animals were significantly worse in the spatial memory task than P25 animals. Comparison of animals with recurrent seizures with those, which remained seizure free after ET-1-induced focal ischemia, is indeed very difficult because spontaneous seizures were detected in majority of animals. Therefore, the number of seizure-free animals is not sufficient for statistical analysis. Comparison of latencies to the hidden platform between individual animals with extremely numerous seizures

and other animals with seizures in corresponding groups did not, however, show any difference. Not only seizure frequency but also pattern of seizures and seizure duration might play a role in cognitive impairment. Fully kindled animals that experienced 10-20 partial and at least 3 generalized seizures (Stage 5 according to *Racine 1972*) exhibit impaired performance in spatial tests *(Hannesson and Corcoran 2000)*. Also, duration of seizures in the kindling model is prolonged from seconds to tens of seconds and severity of convulsions progresses with repeated stimulation. In our study, the mean seizure duration was $\leq$10 s in both age groups and only non-convulsive seizures were detected. The majority of animals spent seizing less than 1 min per day. It raises a question whether such brief and mild epileptic seizures are sufficient to induce cognitive impairment. We hypothesize that age at the time of insult, rather than spontaneous seizures at adulthood, affect cognitive functions. Previously published studies suggest that subcortical damage often has more severe consequences in immature than in adult rats. Unilateral electrolytic hippocampal ablation in adult animals only modestly affects learning abilities, whereas unilateral lesion in newborns severely and permanently impairs spatial memory *(van Praag et al. 1998)*. Negative effects of unilateral hippocampal lesion during the first 2 weeks of life on learning abilities and memory were observed in the T-maze test *(van Praag et al. 1994)*. More dramatic effects of unilateral hippocampal lesion in neonates than in adults may be related to extensive postnatal development of the hippocampus *(Altman et al. 1973)*.

The cognitive decline was also previously documented in other models of ischemic stroke. Adult animals subjected to transient occlusion of the middle cerebral artery exhibit impaired performance in the Morris water maze *(Karhunen et al. 2003; Yonemori et al. 1999)*. Also, hypoxic-ischemic brain injury induced at P7 led to learning deficits. Ikeda et al. demonstrated significant learning impairments in choice reaction time task, which is related to the attention process, and in the water maze task, which examines long-term reference memory, in animals with permanent carotid artery ligation and subsequent hypoxia at P7 *(Ikeda et al. 2001)*. In these studies, late seizures were not detected and cognitive impairment was rather explained by destructive lesion than by epilepsy.

Final comment

Taken together, our data demonstrated strong epileptogenic effects of ET-1-induced focal ischemia in the immature brain. The severity of epilepsy seems to correlate with the severity of acute seizures observed shortly after ET-1 injection at least in P25 rats. Our data suggest that early focal ischemic lesion induced abnormalities in learning and memory only in the younger rats. Further studies are, however, necessary to exclude or demonstrate possible developmental delay of learning abilities in both age groups. Also, morphological pattern and extension of ET-1-induced lesion has to be studied to characterize the present model in all aspects. In future, this model can serve as a reproducible animal model for development of preventive and curative strategies in developing individuals.

VI. CONCLUSIONS

1. After ET-1-injection, seizures occurred in 83-100% animals of all age-and-dose groups and persisted for 24h except P12 rats with 10 pmol.

2. ET-1-induced lesion leads to epilepsy development in 60-100% animals of both groups used (P12 and P25).

3. ET-1 injection induces only hippocampal lesion. The extent of the lesion decreases with age at ET-1 injection and increases with used dose. Early ET-1-induced lesion did not affect motor development.

4. Early ET-1-induced lesion did not affect motor development and has only impalpable effects on motor abilities. Impairment of spatial memory was detected only in animals with ET-1 injection at P12 adulthood.

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