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Changes in motor function and seizure susceptibility after photothrombic ischemic stroke in immature rat

Doctoral dissertation

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Změny motorických funkcí a citlivosti k vyvolání epileptických záchvatů po fototrombní mozkové ischemii u nezralého potkana

Disertační práce

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Praha, Červen 2012

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DEDICATION

I would like to dedicate this Doctoral dissertation to my parents:

Meme Wilhelmina Nuusiku Jason and Tate Jolonimu Mwetulundila Jason

Who just proved to the world that you do not have to have educated parent in order to make it. There is no doubt in my mind that without their continued support and consolation, I could have never made it this far. As the very first Jason to reach this level of education, I owe it all to them. Although they were not as privileged as I am; they never failed to set a great example and pave the way to ensure that those around them might have opportunities they never had. My Father is a great believer in education, diligence, science and the pursuit of academic excellence. He does his best to help all those who want to learn. I will never forget his request to get him a degree, each and every morning as he brought us right in front of our school gates.

Tate; Meme: this is for you, all the way from Czech Republic to Namibian

"Tate; Meme: odjapo yetu oyeyi. Kalunga ne mu jambeke unnen."

Tangi Unenne Meme na Tate, Ohandi pandula wo Onshiti we tu eshi a pange ovakuluhnu vange. Vatekulange Vatekulange fiyo Opo ghatu apa ndili nena eli.

Finally, I dedicate this work to my Loving husband, who from a far is always there to listen and advice me. His daily words of support and encouragement is what pulled me though those last days. Travelling this journey with you made it worthwhile. Thank you and keep up the good work.

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ABSTRACT

Changes in motor function and seizure susceptibility after photothrombic ischemic stroke in immature rat

Perinatal stroke is a common cerebrovascular disorder affecting one in every 4000 births, typically associated with sequelae that include motor and cognitive deficits and long term comorbidities including epilepsy. We sought to determine the effect of perinatal induced stroke on motor function and seizure susceptibility in rats.

Photothrombotic model of stroke was used in rat at postnatal day 7. Firstly we induced ischemic lesions of different extends to assess the consequences of stroke on motor function, locomotion and its correlation to morphological changes after stroke. To this end, paradigms sensitive to sensorimotor changes were used; histological changes were also assessed. Secondly, with the use of pure cortical lesions, seizure susceptibility in PTZ elicited models of epileptic seizures was analysed. For seizure occurrence, latency and severity, two different concentrations of PTZ (60 and 100 mg/kg) were administered subcutaneously in two different age groups at P 12 and P 25. In addition, episodes of rhythmic EEG activity were registered at P 25 following successive 20- and 40-mg/kg doses of PTZ administered interperitonealy.

Our data depicted two kinds of lesions with different shapes and sizes relative to laser illumination. Motor performances of rats submitted to stroke were poor compared to controls; differences in motor performance were also noted between rats with small and large lesions. Cortical photothrombotic lesions induced in immature rats, affected seizures elicited by pentetrazol, later during postnatal development. Major changes were found in a model of human absences induced by a low dose of PTZ and an easy transition from EEG spike-and-wave rhythm into minimal clonic seizures.

A clear relationship between motor impairments and lesion extend was observed; indicating that brain injuries greatly affect motor function in rats. Cortical ischemic lesion during early development also had an impact on the sensitivity PTZ; decreasing thresholds and increasing susceptibility to PTZ-induced seizures, just 5 and 18 days post photothrombotic insults.

ABSTRAKT

Změny motorických funkcí a citlivosti k vyvolání epileptických záchvatů po fototrombní mozkové ischemii u nezralého potkana

Perinatální mozková ischemie je časté crebrovaskulární onemocnění, které se vyskytuje při každém 4000 porodu a u novorozence se typicky projevuje motorickým a kognitivním deficitem a dlouhodobými komorbiditami včetně epilepsie. Cílem práce bylo zjistit efekt perinatálně indukované mozkové ischemie na motorické funkce a citlivost k vyvolání epileptických záchvatů u potkana v průběhu života.

Fototrombní mozková ischemie byla vyvolána u nezralých potkanů sedmý den po narození. Indukovali jsme ischemické léze různých rozsahů, abychom mohli posoudit vliv ischemie na motorické funkce, pohyb a korelovat je s morfologickými změnami po ischemii. Byly použity motorické behaviorální testy citlivé na senzomotorické poruchy. V druhém experimentu jsme vyvolali čistě korovou ischemickou lézi a hodnotili citlivost k vyvolání epileptických záchvatů pomocí PTZ. Pro zhodnocení výskytu, latence a charakteru záchvatů dvě různé dávky PTZ (60 a 100mg/kg) byly aplikovány subkutáně ve dvou věkových skupinách P12 a P25. Rytmická EEG aktivita byla hodnocena na EEG záznamu u 25denních potkanů při dvou additivních intraperitoneálních dávkách PTZ (20 a 40 mg/kg po 20min.)

Velikost ischemické léze byla u mláďat potkana závislá na délce osvitu excitačním laserovým světlem. Motorické schopnosti potkanu po prodělané perinatální ischemii byly signifikantně zhoršeny v porovnání s kontrolní skupinou, rozdíl byl rovněž zanamenán v závislosti na velikosti léze. Kortikální fototrombotická léze u nezralých potkanů výrazně ovlivnila záchvaty vyvolané pentetrazolem v průběhu postnatálního vývoje. Signifikantní změny byly zaznamenány především u modelu lidských absencí indukovaných nízkou dávkou PTZ a dale se projevovaly snadným přechodem rytmu hrot-vlna do minimálních klonických záchvatů.

V naší studii jsme popsali jasný vztah mezi rozsahem léze a výsledným motorickým deficitem v dospělosti. Korová léze vyvolaná během časného vývoje jedince měla dále signifikantní vliv na citlivost k PTZ, snížila práh a zvýšila citlivost k vyvolání záchvatů pomocí PTZ pátý a osmnáctý den po ischemickém insult.

Contents

LIST OF ABBRIVIATIONS

Meaning	Abbreviation
Anterior posteriorly	AP
Blood brain barrier	BBB
Experimental animal	BRI
Model of common carotid artery occlusion	CCAO
Central nervous system	CNS
Control	Contr
Electroencephalogram	EEG
Extracellular matrix	ECM
Endothelial cell	EC
Exposed to laser light for 30 sec.	(BR_30 s)
Exposed to laser light for 5 min.	(BR_5 min)
Factor V Leiden	fVL
Factor-1	HIF-1
Fibroblast Growth Factor-2	FGF-2
Hypoxic ischemic	HI
Intravenous application	i.v
Laterally	L
Matrix metalloprotease	MMPs
Model of middle cerebral artery occlusion	MCAO
Minimal clonic seizures	mS
Nitric oxide	NO
Open field	OF
Postnatal days - the age	P
Pentylentetrazol	PTZ
Sham-operated controls	Sham
Tonic-clonic seizures	GTCS
Vascular Endothelial Growth Factor	VEGF

LIST OF FIGURES

Figures **Page**

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

Opening remarks

Perinatal ischemic stroke is the leading cause of cerebrovascular disorder in infants occurring around the time of birth with pathological evidence of focal arterial infarction (de Vries et al. 1997). Symptomatic perinatal ischemic stroke is experienced by about one in every 4000 term births (Estan & Hope 1997; Lynch & Nelson 2001) and is associated with high social and medical cost to society, due to long-term co-morbidity (Sran & Baumann 1988; Sreenan et al. 2000). Although, research indicates that the number of reported neonatal stroke cases are on the increase in some areas (Azzopardi et al. 2000; Edwards & Azzopardi 2000; Ferriero 2004; Ramaswamy et al. 2004; Lee et al. 2004; Miller et al. 2004; Wu et al. 2004), the incidence is likely higher than the above indicated value; possibly due to a lack of symptoms during early postnatal periods. Up to 27% of infants with these brain injuries later in life develop, numerous consequent pathologies,. Among these are behavioral and functional deficits, speech delays, hemi-paresis and hemi-sensory impairments (Westmacott et al. 2009b; Vinay et al. 2005; Barmada et al. 1979; Hattori et al. 2000). Clinically, little is known concerning long-term behavioral outcome after neonatal stroke (Westmacott et al. 2009), this fact holds true also for experimental studies. There is a shortage of stroke models focused purely on long-term neurobehavioral and functional outcomes induced at an immature age and assessed in adulthood. Existing work of this nature documented a correlation between the degree of brain injury in neonatal rats and functional deficits such as asymmetries of limb placing, foot-faults and abnormality in the postural reflex tests (Bona et al. 1997). Although neonatal stroke is increasingly being studied, most of the foundation of our understanding on functional and behavioral end points comes from research done in adult animal models.

In order to study perinatal stroke, several methods to induce ischemic brain damage have been elaborated in immature rats at postnatal day (P)7 (Rice, III et al. 1981; Ashwal et al. 1995; Renolleau et al. 1998; Vannucci & Hagberg 2004). Research evinced that rat's brain at this age is as mature as the brain of a human fetuse in the third trimester; with regard to parameters such as number of synapses, neurochemical development and cortical organization (Hagberg et al. 1997; McCutcheon & Marinelli 2009). Although hypoxic ischemic (HI) based methods are dominating in this field, they prove to be technically challenging to perform at an early age resulting in inconsistent infarct volume and high mortality rates. In contrast to these methods, photothrombosis is a less invasive method and can be performed at an immature age (Maxwell & Dyck 2005). Photothrombosis is based on thrombus-producing photochemical reaction of photosensitive dyes activated by green laser light that typically produces a consistent infarct of a specific location and size (Karhunen et al. 2007; Brant D et al. 2009; Watson et al. 1985; Kelly et al. 2001; Ginsberg et al. 1988; Futrell et al. 1988; Dietrich et al. 1988).

In the following studies, we used the Bengal Rose model of photothrombotic stroke to induce two ischemic lesions of different extends, at postnatal day (P) 7. Paradgms sensitive to sensorimotor changes caused by various brain injuries were used to assess the consequences of stroke on motor function responses, such as posture and motor coordination in adulthood. Four simple behavioral tests (bar holding, rotarod, inclined grid, and ladder rung walking) were used for this purpouse. Moreover, locomotor activity expressed as distance moved in the open field (OF) was monitored. This evaluation of movement may also help distinguish the levels and severity of motor impairment post stroke. Descriptions of motor patterns used for task performance can help better quantify impairment levels post stroke. Kinematic variables provide detailed measures at 2 levels: motor performance (end point error, velocity, etc) and movement quality (joint ranges, trunk movement). Motor performance variables may

better identify motor control deficits than clinical outcome measures alone (Subramanian et al. 2010).

Epilepsy is a neurological condition characterized by spontaneous recurrent epileptic seizures. These seizures often appear as a consequence of numerous pathologies including stroke, resulting in brain damage and neuronal hyperexcitability (Sahin et al. 2003). Initial brain damage that leads to epilepsy is frequent in the early stages of brain development and initial seizure induction is highest in the first month of life (Hauser et al. 1993).

We applied photothrombosis in immature rats at postnatal day 7, to analyze seizure susceptibility in a Pentylentetrazol (PTZ) elicited model of epileptic seizures as a consequence of stroke, five and eighteen days after induction of stoke.

PTZ a GABA-A receptor antagonist, that effortlessly passes though the blood-brain barrier (BBB) is widely used to induce behavioral seizures and allows for the assessment of brain excitability (Klioueva et al. 2001). To analyze changes of post stroke susceptibility to PTZ–induced seizures in immature rats, we evaluated three types of epileptic seizures facilitated by a systemic administration of PTZ; namely, minimal clonic seizures (mS) that are generated in the basal forebrain, generalized tonic-clonic seizures (GTCS) generated in the brainstem (Browning & Nelson 1985) and nonconvulsive seizures (rhythmic EEG spike-and-wave activity) with cortico-thalamo-cortical mechanisms of generation (Snead, III 1992). All these models are routinely used in our laboratory and their development in postnatal rats was described ; Tchekalarova et al., 2010; Velisek et al. 1992). Rat pups were studied at two different ages - 12 and 25 days corresponding to human early postnatal stages and school going periods respectively (Dobbing 1970).

OVER VIEW OF ISCHEMIC STROKE

Transient or permanent interruptions in cerebral blood flow mostly caused by the occlusion of a cerebral artery either by an embolus or by local thrombosis, leads to a debilitating neurological condition termed stroke (Hossmann 2006). This conditions results in damage to neuronal networks and impairments of sensation, movement or cognition (Murphy & Corbett 2009).

Stroke is typically thought to be a disease of the elderly but it also commonly occurs in neonates and children. It is among the top ten reasons of death in children, in industrialized countries (Sola et al. 2008). Studies have shown that the number of reported neonatal stroke cases are on the increase in some areas (Vannucci & Hagberg 2004; Edwards & Azzopardi 2000). The precise timing of this injury in neonates cannot be determined, but time of diagnosis is usually within the first few days of life. Some neonatal strokes cases are not captured in these perinatal period; due to a variety of reasons including the facts that, some infants with stroke may not manifest symptoms to elicit imaging studies, or the studies that are performed are not sensitive enough to detect acute ischemic stroke.

DEFINITION

Perinatal ischemic stroke commonly refers to neurologic signs and symptoms, that are due to vascular causes around the time of birth and can be divided into three subtypes: ischaemic stroke, either arterial or sinovenous ischemic stroke and haemorrhagic stroke, (Govaert et al. 2009). Stroke was defined during a workshop on perinatal ischemic stroke as "a group of heterogeneous conditions with focal disruption of cerebral blood flow secondary to arterial or cerebral venous thrombosis or embolization, occurring between 20 weeks of fetal life through the 28th postnatal day, confirmed by neuroimaging or neuropathology studies" (Raju et al. 2007)

Perinatal ischemic stroke is a major cause of brain injury and remains a leading cause of cerebral palsy, chronic morbidity and mortality in children (Raju et al. 2007). Of all perinatal stroke survivors, approximately 75% suffer a range of cognitive, behavioural and motor impairments. Among these are sequelae such as epilepsy, cerebral palsy, hemiparesis, learning disabilities and visual-field deficits, as well as mental retardation (Delsing et al. 2001). Over the last 30 years, this knowledge has led to increased awareness of this neurological condition. Although the importance of perinatal stroke is increasingly being recognized, there is still little known about the long-term neurological, functional and behavioural outcome of this pathology from a clinical and especially a biomechanical point of view, specifically the mechanical interactions with biological systems, which influence functional kinematics.

CLASSIFICATION

Infantile developmental periods, refers to the time frame that extends from the middle of pregnancy (referred to as fetal life) through birth, right into the first month of life. Infantile stroke is therefore classified according to timing of stroke using common terms that include overlapping time frames:

Fetal stroke – from 8 weeks gestation to delivery (>36 weeks defining term)

Cerebral injuries detected in utero before the onset of labour, or with in the first week after birth and presenting clear signs of tissue loss are termed 'fetal stroke'.

Perinatal stroke – from 28 weeks gestation to 7 days after birth

Strokes occurring during this period lack acute symptomatology. They are therefore only diagnosed retrospectively, with emerging hemiparesis or seizures usually occurring between 4 and 8 months (Golomb et al. 2001).

Neonatal stroke – from birth up until 28 days of life

Neonatal stroke is divided into two types based on the age of symptom onset: early neonatal stroke and late neonatal stroke. Early neonatal stroke occurs in the first three days, probably related to labour or parturition, including early infection onset, placental embolism, birth trauma and diffuse hypoxic–ischaemic encephalopathy. Late neonatal stroke occurs between four and 28 days, likely related to disorders of late neonatal period; which includes cardiac disease, extracorporeal membrane oxygenation, venous thrombosis with embolism, post-natal infection or other events after birth (Govaert et al. 2009; Kirton & DeVeber 2009)

Infantile stroke is also classified according to observed symptoms and time of diagnosis. Some asymptomatic infants, not thought to be neurologically ill as neonates, are usually

diagnosed months after observed asymmetry of reach and grasp, failure to reach developmental milestones, and/or post-neonatal seizures (Nelson & Lynch 2004).

Finely, stroke in these periods is also classified by the type of vessel affected in to: Arterial Ischemic Stroke, which refers to thrombotic occlusion of cerebral arteries and Cerebral Sinovenous Thrombosis referring to occlusion of cortical or deep cerebral veins or venous sinuses.

From the above classification, four clinically relevant infantile stroke syndromes can be defined: symptomatic neonatal arterial ischemic stroke, symptomatic neonatal cerebral sinovenous thrombosis, presumed perinatal ischemic stroke, and periventricular venous infarction (Kirton & DeVeber 2009).

EPIDEMIOLOGY

The incidence of perinatal stroke is frequently underestimated (Derugin et al. 2000), the true incidence remains poorly defined and has been estimated that its rate is as high as the annual incidence of large-vessel ischemic stroke in adults (Wu et al. 2005). The exact number of infants suffering this pathology and are asymptomatic in the infantile period is unknown (Van Miller 2000). Clinically perinatal stroke can be silent or neurologically catastrophic. Depending on the time of diagnosis; perinatal stroke usually is presented by seizures (Raju et al. 2007). It may also be subtle; recent population- based studies suggest that many go undiagnosed in the perinatal period (Lynch 2009). Despite this, the incidence of symptomatic perinatal stroke has been estimated as one in every 1600 to 5000 births. (Estan & Hope 1997; Lynch & Nelson 2001; Raju et al. 2007); of all strokes occurring in infants, 55% are of ischemic origin (Williams et al. 1997). Cerebrovascular injuries occurs in about 20% to 30%

of infants born at the 35th gestational week is much higher than in term infants or people older than 65 years (Williams et al. 1997; Yager & Thornhill 1997).

RISK FACTORS

Various studies demonstrated a major difference between adult and neonatal stroke etiologies and risk factors that leads to this debilitating condition. Although the etiology of stroke in adults is well understood and defined; etiology of neonatal stroke is not fully elucidated. Despite this, a wide range of risk factors unique to this developmental stage has been identified, including a complex interplay between environmental factors/interactions and genetic risk factors as reviewed by (Lynch 2009) and summarized in Figure. 1.

The major risk factors associated with stroke in this period are cardiac disorders that include congenital heart disease, blood disorders such as prothrombotic disease; infection, trauma, drugs, maternal and placental disorders; as well as perinatal asphyxia which can be caused by abruption placenta, contracture of the uterus, vena cava occlusion syndrome and compression of the umbilical cord (Aden 2009; Roach et al. 2008; Lynch 2009; Berger & Garnier 1999). Apart from these factors, evidence exists suggesting that coagulation disorders may be responsible for a greater proportion of infantile stroke. Lynch pointed out that these disorders were identified in up to half of the infants with cerebral thromboembolism (Lynch 2009). Furthermore, risk factors contributing to stroke among neonates may be multiple, incorporating complications that occur before, during, and after delivery (Sola et al. 2008). The diagram below illustrates several maternal and neonatal disorders that have been reported in infants with perinatal stroke.

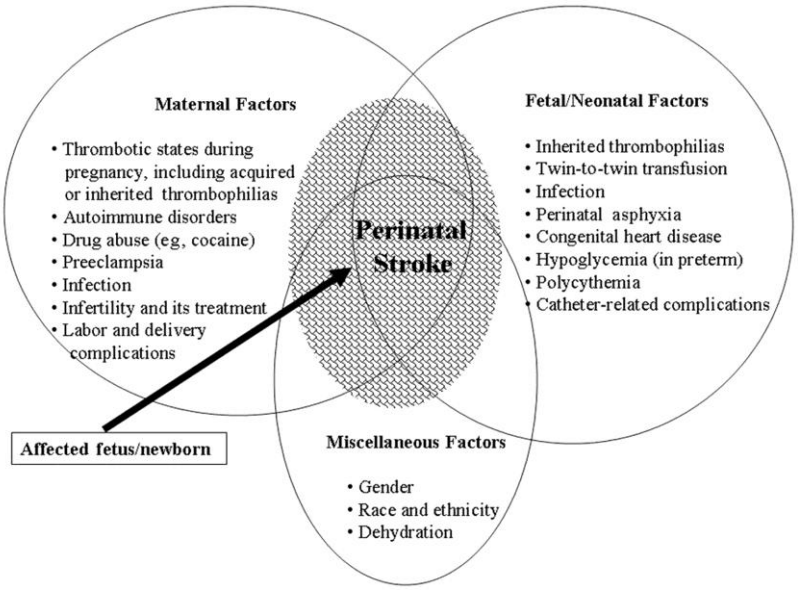

Figure. 1. Conceptualization of risk factors for perinatal ischemic stork. Taken from (Raju et al. 2007)

PATHOLOGY OF STROKE

The disturbances in cerebral circulation leading to focal brain ischemia and brain injury develop from a complex series of pathophysiological events (for full review, see (Dirnagl et al. 1999) that evolve in time and space. This involves interplay of many different cells and tissues such as neurons, glia, endothelium, and the immune system. In short; the major pathogenic mechanisms leading to stroke, include excitotoxicity, periinfarct depolarizations, inflammation and cell death that involves necrotic and apoptotic mechanisms.

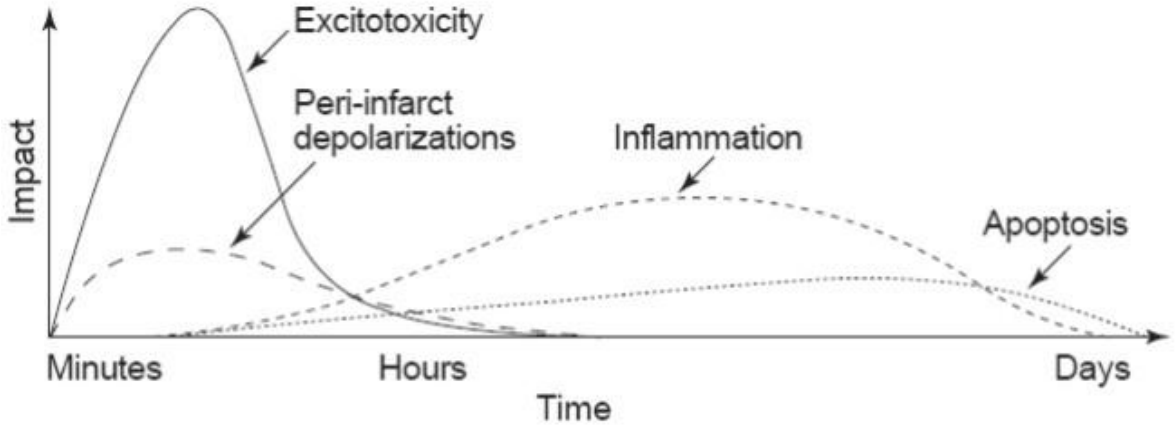

Figure. 2. Putative cascade of damaging events in focal cerebral ischemia. Shortly after focal interuption of blood supply, excitotoxic mechanisms may damage neurons and glia lethaly. Likewise, excitotoxicity usully triggers a number of events that may further contribute to the demise of tissue. This inclueds peri-infract depolarization, mechanisms of inflammation and apoptosis - programmed cell death. The x-axis indicates the evolution of this cascade over time, while the y-axis illustrates the impact of each element of the cascade on final outcome (Dirnagl et al. 1999)

During a stroke, oxygen- and energy-hungry neurons that are deprived of their normal metabolic substrates cease to function in seconds and show signs of structural damage (Murphy & Corbett 2009). As energy-dependent processes fail, neurons are unable to maintain their normal trans-membrane ionic gradients, resulting in an ion and water imbalance that leads to apoptotic and necrotic cell death cascades and ultimately, the impairment of sensory and motor function. Inadequate blood supply to the brain restricts the delivery of substrates, particularly oxygen and glucose, and results in impairment of energy dependent ionic pumps, increased release of glutamate and calcium, and potassium leakage. Excessive calcium overload exceeds the capacity of its tight regulation systems in cells and water follows passively. This is followed by mitochondrial damage and activation of numerous secondary events that impact the development of tissue damage profoundly, such as activation of proteases, leading to digestion of cell proteins, activation of lipases, leading to digestion of cell membranes, activation of endonucleases, leading to DNA degradation, activation of phospholipase A2 and cyclooxygenase generating free-radical species and NO

that overwhelm endogenous scavenging mechanisms, producing lipid peroxidation and membrane damage; all of which induce cell death (Dirnagl et al. 1999).

Activation of glutamate receptors is a major factor involved in initiating ischaemic cell death. Glutamate is an excitatory neurotransmitter, its receptors are said to be the gateway to excitotoxicity; the most important trigger and executioner of tissue damage in focal cerebral ischemia. Excitotoxic mechanisms can cause acute cell death (necrosis) but can also initiate molecular events that lead to a delayed type of cell death known as apoptosis. In addition, the intracellular signalling pathways activated during excitotoxicity trigger the expression of genes that initiate post-ischaemic inflammation, another pathogenic process that contributes to ischaemic injury (Dirnagl et al. 1999).

Ischaemic neurones and glia depolarize due to the following reasons: a shortage of energy supply, the release of K1 and the release of glutamate. In the core region of the affected brain tissue, cells can undergo an anoxic depolarization and never repolarize. The area surrounding the ischemic core with preserved perfusion is known as penumbra; cells in this area can repolarize. The same cells can depolarize again in response to increasing glutamate or K1 levels, or both. Repetitive depolarizations, otherwise known as ‘peri-infarct depolarizations’ occur. As the number of depolarizations increases, the infarcts grow larger. In addiation, according to animal studies, if blood supply is not restored and the tissue protected metabolically within 6 hours, the penumbral area deteriorates and contributes to centrifugal enlargement of the ischemic core (Ginsberg 1997; Lee et al. 2006).

PATHOPHYSIOLOGY OF PERINATAL STROKE

The pathogenesis of cortical brain injury in early developmental periods includes antenatal determinants, fetal and/or maternal (Chabrier et al. 2011) usually resulting in an acute reduction of the uterine or umbilical circulation, brought about by severe intrauterine asphyxia (Berger & Garnier 1999) or following thromboembolism from an intracraninal or extracranial vessel, from the heart or from the most commonly suspected source, the placenta (Nelson & Lynch 2004). This followed by a severe lack of oxygen leads to an activation of the sympathetic–adrenergic nervous system with cardiac output redistribution in favour of central organs such as the brain, heart and adrenals. Persisting asphyxic and/or thromboembolic insults make the maintenance of circulatory centralisation, and cardiac output impossible and the extent of cerebral perfusion decreases. Due to the acute reduction in oxygen supply, oxidative phosphorylation in the brain halts. The Na^+/K^+ pump at the cell membrane becomes energy depleted and unable to maintain the ionic gradients. In the absence of a membrane potential, large amounts of calcium ions flow through the voltage-dependent ion channel, down a steep extra-cellular –intra-cellular concentration gradient, right into the cell. Currently, it has been suggested that excessive increase in levels of intracellular calcium, the so-called calcium overload, leads to cell damage through the activation of proteases, lipases and endonucleases. Apart from the influx of calcium ions into the cells via voltage-dependent calcium channels during ischemia, more calcium also enters the cells through glutamate-regulated ion channels. Glutamate is released from presynaptic vesicles during ischemia following anoxic cell depolarisation. The acute lack of cellular energy, that arising during ischemia induces almost complete inhibition of cerebral protein biosynthesis. Protein biosynthesis only returns to pre-ischemic levels after the end of ischemic period, in the non-vulnerable regions of the brain; while in the more vulnerable areas it remains inhibited. This protein synthesis inhibition, hence, appears to be an early indicator of subsequent neuronal

cell death; followed by a second wave of neuronal cell damage occurring during the reperfusion phase. The latter is presumed to be caused by the post-ischemic imbalances between the excitatory and inhibitory neurotransmitter systems, release of oxygen radicals, synthesis of nitric oxide NO, inflammatory reactions as well as programme cell death, known as apoptosis (Berger & Garnier 1999; Jensen et al. 2003).

During Infantile cortical injury in the mature fetus, the most severely affected territory is the parasagittal aspects of the cerebral cortex and the basal ganglia. The middle cerebral artery regions is presumed to be the location that most perinatal stroke occurs with a predominance of left hemisphere lesions. The latter, might be due to the difference in hemodynamic from a patent ductus arteriosus or the involvement of the left common carotid. A difference in the distribution of cerebral injury has been noted with gestational age. Multifocal and subcortical lesions (Benders et al. 2007), that involved the certical or lenticulostriate branches of the middle cerebral artery where identified in preterm infants while occlusion of the main branch were noted in full-term infant (de Vries et al. 1997).

DIFFERENCES IN PERINATAL AND ADULT ONSET OF STROKE

Although perinatal stroke shares many features with strokes occurring later in childhood or adulthood, significantly important differences among them have been shown. For instance, various factors such as the fVL and prothrombin mutations are associated with arterial thrombotic events in the newborn, while in adults they are associated with venous disease rather than arterial disease. Coexisting infection, seems to predispose infants and children to arterial stroke whereas, in adults venous thromboembolic disease are affected much less by inflammation. It is therefore important to consider the fact that, the neonatal coagulation system is immature and more susceptible to clot formation leading to stroke. Furthermore,

maternal and placental pathology may also be relevant to stroke in the perinatal periods (Nelson & Lynch 2004).

Over the last few years, several papers have reinforced the importance of increased knowledge of perinatal stroke. Cerebral stroke in these periods is different in many important ways from cerebral stroke in older children and adults, clearly distinguished by a broad spectrum of multiple risk factors. Several reports showed that the immature brain reacts differently to ischemia than does the mature brain, (Yager & Thornhill 1997; Chen et al. 1999; Aden et al. 2002; Ditelberg et al. 1996; Saucier et al. 2007) with many developmental and functional differences between them (Aden 2009).

Stroke type varies according to age; remarkable differences in stroke presentation among adults, children and neonates have been noted. In Western countries for instance, the percentile rate of ischemic stroke among adults is estimated to be 80% to 85%, in children is 55% while during perinatal periods, approximately 80%, the remainder of strokes are due to hemorrhage (Roach et al. 2008).

Perinatal ischemic stroke is greatly related to abnormalities in the coagulation system rather than to fibrinogen activation, as commonly seen in adult stroke. Neonates seem to be at higher risk for stroke than older children which may be because of the fact that physiological processes during pregnancy are of prothrombotic states, leading to maternal coagulation activation and hypercoagulability (Aden 2009). Furthermore, thrombotic episodes on the fetal side of the placenta can potentially lead to an embolic phenomenon in the fetal brain because of the patency of the foramen ovale and the right-to-left direction of blood flow in the fetal ductus arterious (Raju et al. 2007). In addition, the inflammatory response of the neonatal brain is said to be robust, and differs in its response to free radical scavengers when compared with the adult brain. The mechanism by which it occurs includes: thromboembolism from an

intra or extra-cranial vessel or from the heart, acute, transient, or progressive arteriopathy and other rare causes (Lynch et al. 2002).

Ischemic perinatal stroke, when compared with strokes in other age groups, has therefore, many unique features. Some studies analyzing perinatal ischemic stroke outcomes, also demonstrate that neonatal stroke has a low mortality rate and its prognosis is more favorable than that of older children and adults, (Boardman et al. 2005; Lynch et al. 2002); this is mostly due to plasticity of the immature brain. Considering these favorable prognosis of the young brain and the unique features of the immature brain, it becomes even more evident that outcome of children after stroke should be different from that of adults. The most common impairments after neonatal stroke include hemiparesis, language delay, behavioral problems, and epilepsy (Aden 2009).

CONSEQUENCES AND OUTCOMES OF STROKE

Perinatal ischemic stroke leads to considerable clinical and socio-economic consequences for both the affected children and their families, and the health system at large. The outcomes are variable depending on severity and anatomic location (Wu et al. 2005). Due to the fact that, most perinatal ischemic stroke cases result in focal neurologic deficits that tend to emerge only after early infancy with new deficits that continue to evolve over several years of childhood (Raju et al. 2007); long-term follow-up with standardized measures is therefore imperative (Raju et al. 2007). In addition, subsequent care in these patents requires a high level of multidisciplinary commitment and co-operation between child-neurologists, pediatricians, physiotherapists, speech-therapists as well as psychotherapists, and other specialists. (Jensen et al. 2003; Berger & Garnier 1999).

According to Raju et al. (2007) accurate long-term outcome data for ischemic perinatal stroke are difficult to summarize due to varying characteristics of the cohorts, definitions, measurements, and the duration of follow-ups. Nevertheless a broad conclusion has be drawn as follows: Neurologic deficits or epilepsy occur in 50% to 75% of survivors, with sensorimotor deficits being the most common; ranging from mild hand weakness to severe quadriplegia. More than 80% of infants with perinatal ischemic stroke have hemiparesis which is increased when cerebral infarction involves the cortex and ipsilateral basal ganglia and internal capsule. The rates of motor disability and cerebral palsy have ranged from 50% to 60% among the largest series of perinatal stroke. Cognitive, language, vision and behavioral disorders are estimated to occur in 20-60% of all survivors (Kirton & deVeber 2009) and mortality rates ranged as high as 22% among hospital-based studies. Lastly, a long-term (over a 30 year period) perinatal stroke studies review, reviled that, 40% of infants were considered neurologically normal, 57% had motor and/or cognitive deficits, and 3% died from the stroke (Nelson & Lynch 2004; Lynch 2009).

Epilepsy as a consequence of perinatal stroke

Brain injuries including stroke are commonly known to trigger epileptogenesis, a process leading to the occurrence of spontaneous epileptic seizures (Friedman et al. 2009). Epilepsy, according to the International League against Epilepsy, is defined as the occurrence of at least one epileptic seizure, which is a transient occurrence of signs and symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Furthermore epilepsy is characterized by unpredictable repeated seizures caused by aberrant electrical discharge in the brain, triggered by numerous pathologies including stroke. With a prevalence of about 0.5–2% of the population or 5 million people worldwide epilepsy is the second most common neurological disorder. Population studies show that seizure incidence is highest in the first

month of life. According to Hauser et al. (1993), initial brain damage that leads to epilepsy is frequent in the early stages of brain development and initial seizure induction is highest in the first month of life. Up to 27% of infants with neonatal seizures, possibly rooted in perinatal periods, develop epilepsy and/or cognitive and behavioral deficits in later life (Vinay et al. 2005). Despite this, Researchers in this field do not pay particular attention to epiphenomenon, long term electrophysiology and behavioral patterns associated with perinatal brain injuries. Furthermore, the mechanisms underlying the latent period preceding the occurrence of spontaneous epileptic seizures (epileptogenesis) are poorly understood.

Generally, clinical research appears to be unable to resolve all these arising queries concerning possible consequences of neonatal stroke and the mechanisms responsible for their harmful effects. Since it is obviously difficult to obtain the precise pathologic and pathophysiologic correlates of affected tissue from patients directly after stroke, it is necessary to address these queries by turning to experimental models. We made use of two such models: experimental models of human stoke and experimental models of human epilepsy.

EXPERIMENTAL MODEL OF STROKE

Human stroke is heterogeneous in its causes, anatomic sites, and in its manifestations. Therefore it is imperative to explore and understand the mechanisms including biomechanical aspects, leading to brain tissue damage post stroke; from a molecular, cellular, tissue, and whole body point of view (Woodruff et al. 2011). To this end, great attempts in search for different approaches and methods to study ischemic brain injuries have been made (Woodruff et al. 2011). Studies were conducted in a wide range of in vitro and in vivo stroke models with a common aim to mimic pathophysiological conditions of stroke in order to discover and

develop useful therapies for the treatment and prevention of cerebrovascular diseases. In addition, these models of stroke served as a tool to evaluate the safety of these therapies, enabling the design of safe and efficient clinical trials (Leadley et al. 2000).

Modelling human neurological conditions has never been an easy task, primarily because same neurological disorder may have different manifestations across different experimental models. The in-vitro studies attempting to mimic human stroke are extremely important as they provide reliable, technically straightforward and physiologically relevant model, useful for the study and screening of potential therapeutic targets of neuroprotection in stroke and to study the molecular mechanisms involved in brain ischaemia (Camos & Mallolas 2010). Despite this, the in vitro cellular models of stroke will always be limited to certain specific mechanisms, pathological and treatment outcomes of stroke. Due to the isolation of certain factors, while neglecting all other elements of the brain environment, these models can never completely replace in vivo models of stroke.

The in vivo studies on the other hand, take advantage of the organism as a whole. These types of models remain a fundamental part of worldwide efforts to guide the search and the development of more effective diagnostic and rehabilitative therapies of human stroke. They can be used to study the major targets of human neuroprotective therapies—reperfusion injury, delayed apoptotic cell death, and inflammatory cascades or the cellular elements of neural repair as with in vitro models. In addition, they can easily be shifted to study cell death and repair over time (Carmichael 2005). It is therefore, imperative to bear in mind that a perfect model be it in-vitro or in-vivo can never exist. There seem to be a general agreement that animal conditions rarely reproduce the entire human condition faithfully. According to Howells et al. (2010) no single animal model is able to encompass all variables known to affect human ischemic stroke. Nevertheless, animal models are the major source of our

knowledge to date, and their relevance to human stroke, depends on their sensitivity, reproductively and reliability.

In the attempt to answer queries as to how data from animal models could be used to help design clinical studies and reliably predict the clinical outcomes of novel therapy, a brief review of the relevance of animal stroke models to humans stroke is necessary.

THE RELEVANCE OF ANIMAL MODELS FOR HUMAN STROKE

The ongoing debates on the usefulness of animal models in the stroke field of research, has lead scientist to conduct studies on laboratory animal mainly in rodents models. Generally, the key to successfully model human neurological symptoms, such as those associated with stroke, is to first identify functional similarities of these neurological impairments (Kleim et al. 2007). To this end, animal models have proven to be the most reliable and serve as an indispensable tool. Researchers in the field, found that work with these animal models of stroke, could be used to answer a variety of questions including queries about behavioral dysfunctions and their underlying neural substrate. This in turn could be used to study both motor impairment and recovery associated with various neurological disorders. Animals with a known and reproducible dysfunction or damage may help in the understanding of brain functions, dysfunctions as well as their effects on behavior. In behavioral neurosciences for instance, animal models make it possible to investigate brain–behavior relations, with the aim of gaining insight into normal and abnormal human behavior (van der Staay 2006). In addition, models of this kind supplied valuable information to address specific questions concerned with the pathologic event following stroke, the development of novel therapies, the provision of pharmacodynamics and pharmacokinetics. Safety data was also obtained and can be used to design safe and efficient clinical trials.

According to van der Staay, "An animal model with clinical relevance in the behavioral neurosciences is a living organism used to study brain–behavior relations under controlled conditions, aimed to gain insight and enable the predictions of these relations in humans" (van der Staay 2006). It is imperative however, to bear in mind that a perfect model can never exist. There seem to be a general agreement that animal conditions rarely reproduce the entire human condition faithfully. Howells et al. (2010) convincingly pointed out the fact that, no single animal "ideal model" is able to encompass all variables known to affect human ischemic stroke, as human stroke itself is a divers condition (Durukan & Tatlisumak 2007). Nonetheless, animal models of stroke continue to be crucial in the discovery and development of a number of therapies that are now successfully being used for the treatment and prevention of cerebrovascular diseases.

THE USE OF RATS IN EXPERIMENTAL STROKE

Rodent stroke models provide the experimental backbone for the determination of cell death and neural repair mechanisms, the initial testing of neuroprotective compounds and for the development of novel adjuvant therapies that may enhance recovery and limit impairment (Boyko et al. 2010). Generally no laboratory animal has been studied in greater detail than the rat, and are the most widely used animal in stroke research (Howells et al. 2010; Cenci et al. 2002). This is largely due to the basic homology that exists between the brains of rodents and humans. These include similarities of the cerebrovascular anatomy consisting of the circle of Willis and physiologic; similarities of ischemic mechanisms between the two were also identified Relevance of Rodent Models of Stroke (Hachinski 1996; Durukan & Tatlisumak 2007)

At present, the Wister Kyoto seems to be the best choice (Howells et al. 2010). These animals bear a humbling resemblance to humans. Every cell type in the human brains has a counterpart in a rodent's brain (Howells et al. 2010). A careful comparison between these species reviled a developmental correlation see illustration below. For instance, the rat's brain at P7 is comparable to human premature or full-term infants. (Sreenan et al. 2000). Furthermore, a majority of structural and functional milestones occurring during the first week of life in the rat's hippocampus take place during the third-trimester, gestational period in human. Using the same approach, the first year of human life correspond roughly to P7 – P14 rat, whereas early preschool years might correlate with the rat's third week of postnatal life (Benders et al. 2007).

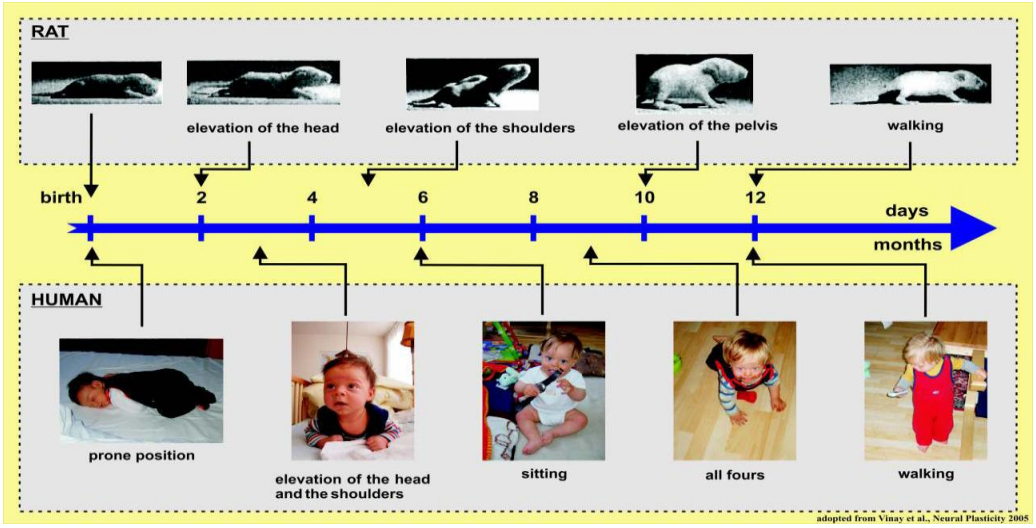

Figure.3. Development of the human baby and rat pups: by Asso. prof Jakub Otahal MD, Phd.

This close resemblance of rodents to the higher species is widely used in various studies (Ginsberg & Busto 1989). Similarities in functional deficits seen in rodents following focal and global cerebral ischemia have been correlated with those typically seen in stroke patients (Squire 1992; F.Josef van der Staay 1998). Detailed analysis of limb movement for instance, shows very similar motor components in human upper extremity and rat forelimb movement

during reaching behavior (Cenci et al. 2002). Extensive knowledge, like the above, of the anatomical and neurophysiologic organization of the rodent motor system facilitates the identification of the neural mechanisms underlying motor damage and recovery.

Gharbawie & Whishaw (2006) made a convincing case concerning the ability of rats to reach and grasp food with a single paw; stating that it enables these species to serve as a model for exploring neural networks of skilled limb movements. These species-specific motor behaviors, led to the development of a battery of sensorimotor tests that can be used to measure various aspects of both motor impairment and recovery after ischemic insult.

METHODS OF STROKE INDUCTION

Opening remarks

Several models in different species with difference in their susceptibility to the various types of ischemic insults and mirror the pathology of stroke in humans, are currently known and elaborated in the immature rats at P 7 (Rice, III et al. 1981; Ashwal et al. 1995; Renolleau et al. 1998).

These methods of stroke induction, prove to be technically challenging to perform at an early age, not only in associated with the small size of blood vessels, but they also result in inconsistent infarct volume and high mortality rates (Watson et al. 1985).

The basic concepts of stroke is derived from studies carried out in adult experimental models of stroke and was assumed that the mechanism, development and outcomes of neonatal stroke could be derived from those of adult stroke. However, as elaborated earlier, considerable reports showed that the immature brain reacts differently to stroke than does the mature brain (Yager & Thornhill 1997; Chen et al. 1999; Aden et al. 2002; Ditelberg et al. 1996; Saucier et

al. 2007) with many developmental and functional differences between them (Aden 2009). There is therefore an obvious need for valid, age appropriate animal models of stroke.

A number of animal models based on diverse methods of induction of stroke have been developed table presents a brief summary of methods used to induce thromboembolice ischemic stroke. These methods include focal and systematic administration of chemicals for the induction of stroke. The most frequently used neonatal stroke model of long-term neurobehavioral and functional outcomes is hypoxia-ischemia (HI) (Almli et al. 2000; Bona et al. 1997; Arteni et al. 2003; Balduini et al. 2000; Balduini et al. 2003; Northington 2006) for summary of the pros and cones of these models see the table below. Although these models are the most commonly used in the field to induce cerebral ischemia, Brant D. Waston and Prado argues that they do not produce stroke *per se.* They state that, photothrombotic stroke simulates more accurately the occlusion –initiating events in human stroke, making it a more realistic model in its ischemic thromboembolic from. This model is also said to have long-term survival rates (Brant D et al. 2009; Kelly et al. 2001) and variable behavioral outcomes as in humans post stroke (Alaverdashvili et al. 2008). In addition, to specificity of location and lesion size, it also provides the opportunity for long-term evaluation of functional and neuropathological endpoints.

In this study we make use of the photothrombotic model of stroke to induce ischemic cerebral infraction.

Photothrombotic Model Of Stroke

Photothrombosis is based on thrombus-producing photochemical reaction of photosensitive dyes activated by laser light. This activation forms free radicals and endothelial cell damage,

aggregation of platelets and eventually occlusion of the vessels (Brant D et al. 2009; Watson et al. 1985; Kelly et al. 2001; Ginsberg et al. 1988; Futrell et al. 1988; Dietrich et al. 1988) typically producing a consistent infarct with specific location and size (Karhunen et al. 2007). Although neonatal stroke is increasingly being studied, much of the foundation for our understanding of functional and behavioral endpoints comes from research in adult stroke models (for a review,(Watson & Prado 2009) see It is therefore, critical to choose an ideal animal model of neonatal stroke for investigating pathophysiologic mechanisms(Sola et al. 2008) and functional out comes. In order to mimic perinatal brain injuries in humans, (Maxwell & Dyck 2005) modified and refined adult models of stroke, for the use in neonatal mice. Currently, the available models of focal stroke and hypoxic- ischemia (HI) demonstrates the need for a variety of age-appropriate models. This will enable investigators to study age-specific vulnerability and age-related differences in susceptibility of the immature brain to stroke. Previous works have documented a correlation between the degree of brain injury in neonatal rats and functional deficits (Bona et al. 1997; Wu et al. 2005; Felt et al. 2002).

Model	**Advantages (+) / disadvantages (-)**
Photochemically induced focal cerebral thrombosis	+ precise location and size of infarcted cortical area + minimal invasive procedures + simulates more accurately occlusion –initiating events in human stroke +high reproductiviy +low mortality rate +long-term survival rate +possibility to study long-term pathologyphysiologcal and functional outcomes + possibility to study anti platelet and thrombolytic therapy - end-arterial occlusion, which is resistant to therapies based on enhancement of collateral perfusion - differs in some respects from human stroke, e.g. no penumbra
MCA occlusion: transient	+ probably the most widely used experimental stroke model + possible to assess reperfusion damage following recanalization

	+ possible to have drug penetration to the occluded area - variation in infarct size
MCA occlusion: 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 infarct size - some models need craniectomy
Miscellaneous models of cerebral embolism and thrombosis	- random and unpredictable location and size of the lesion

Table 1: The pros and corns of some models of perinatal stroke in rats:

(Ginsberg & Busto 1989; Watson & Prado 2009)

Ptz-Induced Model Of Epilepsy

Animal models of human epilepsy and epileptic seizures are generally used to analyze the basic neuronal mechanisms of brain functions and epilepsy-related phenomena. They are essential for research design, human epileptic diagnostics, therapeutics and prevention (Engel, Jr. 2006; Engel, Jr. 2006). According to these authors, recent understanding of inhibitory control of brain excitability has been greatly derived from studies conducted in models of epilepsy. In the present thesis, we make use of a PTZ elicited model of epileptic seizures to analyze seizure susceptibility as a consequence of stroke.

Pentylenetetrazol (PTZ) a tretrazol derivative is mainly used in screening antiepileptic drugs because of its convulsant actions observed in mice, rats, cats and primates. PTZ presumably achieves this action by impairing GABA-mediated inhibition at the GABA receptor (Asla Pitkanene et al. 2006). Models of human epilepsy that makes use of this drug are known elicit generalized seizures, both absence and generalized tonic clonic seizures when used to induce recurrent seizures. A single systemic dose of this drug can induce seizures and when

administered in sufficient amounts, results in status epilepticus (Nehlig & deVasconcelos 1996).

MOTOR FUNCTION TESTS USED TO ASSESS STROKE OUTCOMES

Motor deficits are said to be objective end points of rat stroke models, which can be quantified to some extend and evaluated by a number of easy and quick methods to test gross and refined sensorimotor functions (Durukan & Tatlisumak 2007). The choice of an appropriate battery of sensorimotor tests that will be sensitive enough to detect a range of motor impairments across a significant time span is of great importance (Kleim et al. 2007).

MOTOR FUNCTION TESTS

Several studies have investigated motor and behavioural sequela in rat models of cerebral ischemic stroke assessing the effects of stroke in living organisms, (Borlongan et al. 1995; Pearse et al. 2005; Wakayama et al. 2007). This is carried out by means of a battery of simple tasks ranging from measures of gross motor performance to fine object manipulation and kinematic movement analysis. In particular, deficits of weight supported stepping, paws coordination, endurance, muscle strength, skilled walking can be analysed (Kleim et al. 2007; Mikulecká & Mareš 2002; Ticha et al. 2011). Some of the most popular tests used to examine the effects of focal stroke on refined sensorimotor function include: limb placing, beam walking, grid walking, rotarod, bar holding, sticky label test, the staircase test, just to mention a few (Hunter et al. 2000; Durukan & Tatlisumak 2007).

In the present study the use of simple tests, to assess sensorimotor and locomotion after photothrombotic stroke, was as follows: Bar holding test, Rotarod test, Inclined grid test, ladder rung walking tests and Open field. These tests originally described by Bolles and Woods (Woods 1964) are routinely used in our laboratory (e.g. (Mikulecká & Mareš 2002).

THE BAR HOLDING TEST

Adopted by (Diener & Bregman 1998), the bar holding test was developed in order to assess neuromuscular function, in particular strength. It is routinely used in neuroscience studies, in teratological and toxicological screening with the aim to measure sensorimotor performance and muscular tonus of experimental animals. This test utilizes the natural grasping function of the paw and can be used to asses both forelimb and hind limb function. Generally it is mainly employed for testing forelimb muscle function in animals with cerebrovascular as well as cervical spinal cord lesions (Pearse et al. 2005).

The stimulation is provided using a wooden bar 25 cm long, 1 cm in diameter, and suspended 25 cm above a padded soft surface. An animal is to grip a horizontal bar with the volar surface of the forepaw, and the presence or absence of grasping and the release time in seconds are evaluated. The time the rat holds onto the suspended rod is measured and recorded. (Pearse et al. 2005) recommends that, testing should be done repeatedly; we repeated it for three consecutive times, and the mean values were calculated. Pearse et al, further states that in severely injured animals, this test provides only dichotomous yes/no data. Normal or mildly impaired animals might pull themselves on top of the hanging rod, rendering such a trial immeasurable (Pearse et al. 2005). Although these authors believe this test should be used in combination with other tests like Rotarod test, obtained data are precise and unique—not many tests are able to measure limb muscle strength using such a simple method.

THE ROTAROD TEST

Originally described by (Dunham & Miya 1957), the accelerating motor-driven treadmill is a performance test based on a measure of the ability of rodents to coordinate and balance on a rotating drum (Hamm et al. 1994; Mizoguchi et al. 2002). Two relevant behavioural strategies, grasping and walking used by rats to maintain their equilibrium can be noted. Grasping behaviour demanded the animals to grasp on the Rotarod while passively (motionless) being rotated. Walking behaviour required asynchronous or synchronous walk upon the rotating rod. These abilities are often used as measures of impaired motor functional phenotypes as well as testing motor and learning skills in rodents. The performance is measured by the duration that an animal stays up on the drum as a function of drum speed. The major end point measures are: fatigue resistance - the ability for the animal to maintain on the rotating drum, measures of the time/latency an animal is able to walk on the rotating drum and a qualitative examination of walking movements.

Recent evidence in rats indicates that this test is suitable for the assessment of motor impairment after focal cerebral ischemia (Rogers et al. 1997; Rogers & Hunter 1997) and traumatic brain injury (Hamm et al. 1994) . It has been also shown to detect behavioral deficits in Wistar rats up to three weeks after stroke, specifically, 2-h after MCAO (Zhang et al. 2000). Among several behavioral tests that measure motor performance, the Rotarod, is easily quantifiable; recording the time it takes the animals to fall off the rod that is markedly smaller than their body length in diameter.

There are several factors, such as motor coordination, body weight, learning and cardiopulmonary endurance that can influence the motor performance of the animals on the rotarod (Thal et al. 2008).

THE INCLINED GRID TEST

As described by (Marshall 1982), the inclined grid test evaluates the animal's geostatic ability, immobility, catalepsy and the latency to turn to 180^0 as function of time on a grid that is incrementally raised to increasing angles. Performance on the inclined grid was shown to be sensitive and reliable as an index of animal strength (Gale et al. 1985). This performance was also correlated with the integrity of the rubrospinal tract and other non-pyramidal pathways (Fehlings & Tator 1995). In this study we us a modification of this methods with a grid (75 cm with x 100 cm height) made of stainless bars (diameter 5mm) consisting of a 13x17 matrix of 5x5 cm holes. The rats were placed on the inclined plane with their heads facing down wards. The angle of inclination was then gradually increased towards the vertical position until the rat can no longer remain in place at the starting position. The latency to turn on the grid to 180^0 was measured for a maximum of 60 seconds. There consecutive trails were carried out and the mean of three, turning latencies was recorded.

THE LADDER RUNG WALKING

The ladder rung walking test is used to assess motor and locomotry performances specifically, skilled walking, measuring forelimb and hind limb placing, stepping, and inter-limb co-ordination and balance (Metz & Whishaw 2002). This task is said to be sensitive in the detection of long lasting changes in sensorimotor performance after cerebral or spinal cord injuries. Riek-Burchardt et al. characterised short term - 7days following surgery and long lasting sensorimotor impairments of the contralateral forelimb placing after induced stroke.

This team also found a correlation between sensorimotor function and cerebral injury with the use of this test (Riek-Burchardt et al. 2004).

Various tasks can objectivly be tested by means of the ladder rung walking test. The foot fault task is among the most effective way for assessing forelimb and hindlimb function (Sedy et al. 2008). The typical approach is to place the animals on an elevated ladder; the rats spontaneously walk from a starting location to a goal along the ladder (Hernandez & Schallert 1988). Detailed quantitative and qualitative analysis of the rats' stepping pattern is possible with the use of a video camera positioned below the grid. The present study assessed spontanious locomotion, the latency to cross the horizontal ladder, on which the spacing of the rungs were periodically changed, and the ability to compleat the above mentioned tasks. According to (Metz & Whishaw 2002), these changes in rung spacing prevent animals from learning the absolute and relative location of the rungs; thereby minimizing the ability to compensate for impairment through learning.

Metz and Whishaw, pointed out that, the ladder rung walking test is sensitive to normal aging; older rats were found to be more impaired in rung walking than younger rats. It is also sensitive in the quantification of skilled locomotor movements. These authors also managed to describe three kinds of chronic impairments in limb, use after lesions to the motor system. Firstly, deficits in ipsilateral and contralateral limb placing on the rungs, secondly, deficits in digit use during grasping the rungs, and thirdly, inter-limb co-ordination and balance deficits.

The ability for this test to discriminate between lesions of the motor system due to brain or spinal cord injury, or age-associated impairment as well as the assessment of loss and recovery of function is dependent on the observed deficits in limb placing, stepping and co-ordination, displayed by the animals (Hernandez & Schallert 1988; Metz & Whishaw 2002) . Performance in all cases are highly sensitive to cerebral infarction (Sedy et al. 2008).

OPEN FIELD TEST

A simple test originally described by (Bignami 1996), the open field test is used to assess exploratory behaviour in rats placed in an open field arena (100×80 cm) and monitored individually for a period of 5 min (Bignami 1996; Metz & Whishaw 2002). This test is sensitive to a wide range of injuries, including stroke. Generally, the open field test is a good measure of gross motor behavior in rat after sensorimotor system injuries (Koesler et al. 2008; Nowak 2008). Exploratory activity in the open field is said to be sensitive especially to individual differences among animals with a low locomotor capacity; (Koesler et al. 2008; Nowak 2008) further states that even severely damaged rats can show significant locomotor activity in this test. Motivational factors such as anxiety influences spontaneous exploratory activity, which in turn can cause freezing behavior in rodents, reducing the rate of exploratory activity in these animals (Gerlai & Clayton 1999).

In the present study, the open field (OF) test was performed in a square arena (45x45x30 cm), with a camera installed above the arena. Rats were placed individually in the center of the arena after which locomotor behavior was recorded automatically by a computerised system (Etho Vision Noldus Information Technology) for 5 min. Locomotor activity expressed as distance moved (cm) was calculated.

Summary Of Tests Used To Assess Motor Function After Stroke

Behavioral test	**Behavioral task**	**Behavioral assessment**
Bar holding test	Ability to use forelimb strength to stay hanging on a horizontal bar	Forelimb muscular strength and latency to maintain hanging on the bar
Beam-walking test	Walking on a beam	Ability to maintain balance and hind limb

		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
Inclined grid test	Turning on an inclined grid	Latency in turning on an inclined grid
Ladder rung walking tests	Walking on a elevated ladder from a starting location to a goal along the ladder	Forelimb and hind limb function and coordination stepping patterns, foot faults
Montoya's staircase test Motoya:	Reaching and grasping of food pellets	Fine movements of forepaws
Pasta Matrix Reaching test	Reaching trough a high vertical slot for pasta pieces on a shelf attached to the outside wall	The number of obtained pieces of pasta
Prehensile traction test	Prehensile traction	Time of traction
Running wheel or Rotarod test	Running or walking on a rotating rod Or wheel	For walking – latency to maintain on the rod For running - forelimb slips during running
Sensory inattention test	Orientation to sensory (visual, olfactory or tactile) stimuli	Tendency to orientate and investigate impinging stimuli
Sunflower seed opening	Latency to manipulate, open and consume sunflower seeds	Bilateral object manipulation and motor impairments

Swim task	Swim from one end of the aquarium to a platform on the other end	Forepaw inhibition behaviour while swimming
Single pellet reaching	Reaching and retrieving single food pellets	Number of successful reaches and retrieved pellets
Vertical screen test	Ability to stay on a vertical screen	Forelimb and hind limb muscular strength
Water-maze test	Memorizing the location of a hidden platform	Spatial learning

Table 2: A summary of the typical tests used to measure forelimb impairments in rats post stroke (Kleim et al. 2007)

OVERVIEW OF MICROVASCULATURE

All tissue, healthy or pathologic, depends on their vascular blood supply system (vascular network) for adequate supply of nutrients, and an effective removal of waste metabolic products. Water and solutes are carried by blood via micro-vessels and exchanged across vessel walls to the surrounding tissues (Pries et al. 1996). The development of any tissue in an organism should be accompanied by an appropriately structured blood supply system. The flow of blood in these micro-vessels, with diameters in the order of 100μ m or less is called the microcirculation. The circulatory function of blood vessels (micro or macro) is highly dependent upon the architectural structure of these vasculature, their vascular networks (Annemette Lokkegaard 2004) and their mechanical behavior. The mechanical and biophysical behaviors possessed by microvasculature during the pumping and flow of blood and its regulation has been extensively reviewed and studied for many years from cellular and molecular levels. To this end investigators made use of various in-vivo and in-vitro experimental and theoretical models (Pries et al. 1996; Sugihara et al. 2004). These studies reviled that tissue adaptation, remodelling, degradation and repair are regulated by mechanical forces (Ateshian & Friedman 2009). For instance, the overall blood flow of a given perfusion pressure is determined by vascular hydrodynamic resistance; which is dependent upon the passive and active mechanisms governing micro-vascular diameters, the apparent viscosity of blood flowing in them and on their number, size, arrangement and their interconnections (Pries et al. 1996). These networks are crucial to the overall function of all tissue and organs; their structures and densities are closely related to tissue metabolic demands and function (Annemette Lokkegaard 2004). Change in tissue function can therefore be detected by changes in vascular properties and vice versa. This reflects important changes that are relevant in the understanding of physiological and pathophysiological states (Annemette Lokkegaard 2004). Through the use of experimental evidence it is reasoned that a greater

understanding of the role of mechanical forces modulating tissue and organ function; in health and disease offers enormous rational improvements in diagnostics, therapeutics and prevention. Research indicates that for these and many other obvious reasons, including the fact that many aspects of tissue function are regulated by mechanical forces; quantification and evaluation of vascular network and their mechanical properties is essential (Vermeulen et al. 2002; Vermeulen et al. 2002). This is possible though understanding the criteria by which microvascular networks are defined as well as the characterization of their mechanical properties in relation to their structure (Ateshian & Friedman 2009).

DEFINITION AND DESCRIPTION

Microvascular network is defined by means of their structural, physical and mechanical properties This includs vascular length, number of connections, number of vessels, vascular diameters and surface area. The density number is also part of this definition and is described as the number of profiles per area in the microscope – a measure that can be used to describe the length of vessels (Annemette Lokkegaard 2004). The description of microvascular network is important in both health and pathology. It serves as a reliable measure to quantify and evaluate changes occurring in the microvascular network leading to the development of efficient treatment strategies. To this end a fundamental knowledge of the basic components that make up these network is essential.

MICROVASCULAR STRUCTURE AND NETWORK

Micro-vessels generally have interconnected tubes (vessels) that are morphologically long and narrow, forming networks in tissues. These micro-vessels make up a large amount of connections; their number is much larger when compared to those of larger vessels. The network of these vessels are typically said to be extensive in a variety of organs. One large artery supplies a large number or micro-vessels; microvascular network can therefore be considered as interconnected structure, connected to the overall circulation (Annemette Lokkegaard 2004). Generally the circulatory exchange system of blood vessels can be simplified as follow: vessels that deliver oxygenated blood to tissues namely: arteries, arterioles, and capillaries, and those that that return blood with metabolic by products such as carbon dioxide for gas exchange namely: veins and venules. The difference between veins and arteries is depends upon the size of their layers (intima, media and adventia) as well as the materials that make them up. The basic structure that makes up the inner lining of these vessels is called the vascular endothelium, which consists of a monolayer of endothelial cells (Sumpio et al. 2002). Vascular endothelium is the main regulator of material exchange providing an anticoagulant barrier between circulating blood and surrounding tissue while its cells (endothelial cells) act as a selective permeability barrier (Sugihara-Seki & Fu 2005c; Sumpio et al. 2002). Maintenance of vessel wall and circulatory function is therefore highly dependent on endothelial cell structure and functional integrity.

MICROVASCULAR FUNCTION

The principal function of microvasculature in a perfused tissue is transport and exchange of material, making sure blood is available in close proximity with each point in the tissue (Pries et al. 1996). The transport function of microvasculature is a typical issue of concern that has been investigated for over 50 years (Sugihara-Seki & Fu 2005). According to Sugihara, the paramount question regarding structure–function of micro-vessel walls and the properties of cells forming the wall still remains ambiguous. Nonetheless, the importance and functional description of endothelial cells is well elaborated in and elegant study by (Sumpio et al. 2002) According to this authors, endothelial cell are unique multifunctional paracrine and endocrine organs reacting with physical and chemical stimuli to regulate hemostasis, vasomotor as well as the immune and inflammatory responses. They function as selective permeability barrier controlling the transfer of small and large molecules between vascular wall and the circulatory blood (Sumpio et al. 2002). Microvasculature networks have been shown to have the ability to cause flow resistance in these circulatory systems; the mechanics by which this is established enables the control of selective distributions of blood to the tissues and greatly influences material exchange in the circulatory system (Sugihara-Seki & Fu 2005b). This blood flow is regulated by local adjustments, to match the changing metabolic needs of tissues, through the intrinsic activity of vascular smooth muscle cells and by the central nervous system. At a capillary network for instance, hydrodynamic pressures likewise vascular wall compliance and resistance are among the basic mechanisms regulating smooth muscles tone.

Microvascular function is influenced by both physiological as well as pathological alterations. These alterations may occur as structural changes i.e. changes in mechanical properties such as changes in length, diameter, surface area, density and shear stress on cell membran (Sugihara-Seki & Fu 2005; Annemette Lokkegaard 2004). In a healthy mature brain, blood

vessel formation is tightly down regulated. However, during pathologic processes, these alternations, results in an impairment of tissue exchange system, leading to a deprivation of important elements (nutrients and oxygen) and an accumulation of toxic metabolic by products posing as risk factor for the development of conditions such as ischemic stroke. In the presents of cerebral ischemia, a condition in which the brain or part of the brain dose not reactive adequate blood flow to maintain normal neurological function, for instance, changes such as reperfusion injury and regeneration, brain plasticity and functional recovery occur. This response to ischemic injury leads to multiple rapid responses in the ischemic region especially the core. A note worthy response to ischemia is the local breakdown of Blood Brain Barrier, which allows transduction of blood-derived fluid, cytokines, or even cells, and triggering inflammatory response to injured brain tissue. According to del Zoppo & Mabuchi (2003), blood brain barrier leakage results mainly from the actions of bradykinin, VEGF, thrombin, and increased active MMPs, and released proteases from activated leukocytes.

One of the most significant responses in brain microvasculature after ischemia is angiogenesis; angiogenic factors are increased together with related progenitor cell homing, resulting in significant formation of microvascular. Before we embark on this vascular response, let us take a closer look at the governing mechanical properties of microvasculature.

BIOMECHANICAL PROPERTIES OF LIVING CELLS

The link between biomechanics and pathologies of living cells has gained substantially attention, with considerable growth, in the past decade. Numerous pathophysiological processes are accompanied by changes in mechanical properties of tissues and all living cells, which require analysis at a cellular and sub-cellular level. Therefore the relationship among cell structure, biomechanics and disease states has become of particular interests to the growing community of research in the field (Subra Suresh 2007).

All living forms on earth are physical entities, and as such are subjected to constant mechanical stimuli throughout life. They posses mechanical - structural and physical properties enabling them to withstand internal physiological, mechanical as well as external environmental forces (Lim et al. 2006; Tung-Wu Lu 2011). These properties at a cellular level, determine the structural integrity of tissue, while the transmission of mechanical load from tissue to individual cells can influence their physiological function.

Mechanical properties of living cells can be increased or decreased by both mechanical load and by chemical agents often resulting in structural changes such a stiffness of cell membrane or vascular wall (Lim et al. 2006; Tung-Wu Lu 2011) explains that biomechanical properties of living tissue are highly affected by applied loads and deformation. The mechanical response to these loads, transient and dynamic, is variformed and significantly dependent on the magnitude, direction and distribution of the applied forces. The cells in these tissue (subjected to load) are able to sense mechanical forces or deformations and transducer them into biological responses (Lim et al. 2006; Tung-Wu Lu 2011). Reviewed studies by Tung-Wu Lu (2011) indicate that mechanics has a great effect on the form, motion and function of biological systems. According to Lim et al. (2006) mechanical stimuli that changes the shape and structural integrity of cells greatly influences numerous biological processes, including

growth, differentiation, migration and apoptosis. He added that, any alterations of this kind (structural and mechanical) may result in impaired physiological function and lead to diseases by undermining the physical integrity and biological function of tissue. Tung-Wu Lu (2011) supports this notion by stating that changes in mechanical properties alters mechanical interactions resulting in tissue injury, causing degeneration and loss of function (instability and disability). Characterization of mechanical properties of tissue in relation to structure; likewise quantitative evaluation of mechanical properties and responses of cells when subject to stimulation and/or perturbation lead to a better understanding of biological function. Herein lays the reason why biomechanics – the application of mechanics to biology is crucial.

BIOMECHANICAL PROPERTIES OF VASCULATURE

Vasculature according to Tung-Wu Lu (2011) and Y.C.Fung (1993) has mechanical properties that can with stand their subjected stress, during pumping of blood. These properties are a function of the underlying tissue structure as mentioned above. Blood vessels are soft collagenous tissues containing a good deal of elastin, for this reason their stress-strain behavior resembles that of other soft collagenous tissues such as ligaments and tendons. Therefore their behavior under cyclic stress can be approximated as pseudoelastic, nonlinear material; implying a model of hyperelasticity. Like all living cells and tissues, these vessels like to live in a homeostatic stress/strain range. Stress/strain values outside this homeostatic range will lead to adaptation and changes in the tissue structure.

Structural function in soft collagenous tissues such as blood vessels is characterized according to their structural components, for instance, the ratio between collagen and elastin. It is well

known that tissue constituents affect mechanical behavior of blood vessels. This knowledge is due to the analysis of tissue mechanical behavior using a typical non-linear soft tissue stress strain curve. This type of analysis, when conducted by Roach and Burton in 1958, proved that tissue structure contributes greatly to the mechanical function of blood vessels. These authors found that the collagen in blood vessels contributed mainly to the linear region of the nonlinear stress-strain curve while elastin contributed mainly to the toe part of the stress-strain curve (Tung-Wu Lu 2011; Y.C.Fung 1993).

Primary, ways by which blood vessel tissue structure changes is through aging, disease, and change in mechanical load; or a combination of these factors. Conditions of elevated mechanical load on the blood vessels, exposes these vasculature to high stress levels, altering blood vessel structure and consequently their mechanical properties. This is apparent during fluid stresses and strains exerted on the cells. These stresses play integral roles in the regulation of vascular endothelial cell function. These cells have been widely recognized to exhibit changes in morphology, structure and functions, in response to fluid stresses (Sugihara-Seki & Fu 2005). For instance, endothelial cells can become antithrombotic or prothrombotic depending on the magnitude and duration of the shear forces applied (Grabowski 1995). According to (Holme et al. 1997; Noren et al. 2000) in regions of irregular geometry, endothelial cells become prothrombotic, which may lead to platelet aggregation forming thrombus that may lead to stroke.

THE FORMATION OF THROMBUS LEADING TO STOKE FROM A MECHANICAL POINT OF VIEW

The process by which blood clots form within blood vessels is known as thrombosis, a direct cause of stroke. The analysis of the relationships between localized shear rates/stresses and the formation of thrombus in micro-vessels resulting in stroke are of paramount importance. Studies conducted in this field indicate that both mechanical and biochemical factors contribute greatly to this process but their combined contribution to this process is not well elaborated. Three possible ways venous thrombi may occur where identified. Firstly, venous thrombi may develop by means of a primary lesion in the endothelium that produces an inflammatory reaction and then thrombosis occurs. Secondly, through abnormality of flow such as the slowing of blood flow, resulting in the adhesion of formed elements to the intima which leads to thrombosis. Lastly, due to an increase of blood coagulability from changes in the physical and/or chemical properties of plasma (Hume 1970).

Biomechanical factors can affect the activity of platelet coagulation factors responsible for the formation of thrombus near an injured/damaged vessel. For instance, (Muga et al. 1995) evinced that hydrodynamic forces and geometric changes alone can induce thrombosis without the addition of chemical agonists. (O'Brien J.R 1990) found that shear stress alone induced platelet aggregation in a manner similar to that observed in injured vessel walls; leading to increased shear stress, attachment of platelets to the vessels wall as well as the rate of platelet aggregation (Turitto & Hall 1998). Endothelial cell injury, activated by mechanical factors such as shear stress and load is a hallmark for many pathologic states including thrombo-embolic stroke, and thrombosis (Sumpio et al. 2002). For example, in an event of injury, i.e. an opening of gaps between endothelial cells, invoked by local factors in the endothelium; allowing platelets to adhere to the gaps where basement membrane is exposed,

forming thrombi as a consequence. Also, endothelial cell will reacts with these local physical and mechanical stimuli, regulating hemostasis, vasomotor tone, immune and inflammatory responses and making them pivotal in angiogenesis and vasculogenesis.

ANGIOGENESIS

Angiogenesis, the formation of new blood vessels from existing ones, is critical both in health and pathology, mainly for development, repair, tumorigenesis, and in artificial construct design. In addition, it is an important descriptor of changes in tissues especially when it comes to detecting pathological states where pro-angiogenic or anti-angiogenic therapy has become a possibility (Annemette Lokkegaard 2004). Angiogenesis is said to be the determining factor in the development of cancer prompting the search and development of efficient therapies. This process is regulated by soluble factors, cell-matrix interactions, and mechanical loading. During physiologic demand sprouting endothelial cells degrade their basement membrane and the surrounding extracellular matrix (ECM) by means of matrix metalloprotease (MMP) activity that migrate along the ECM components, and deposit new basement membrane to form patent capillary network. New capillary buds are said to start appearing in the ischemic bed 3 days after middle cerebral artery occlusion (MCAO) and activating angiogenesis was induced 7 days after focal cerebral ischemia in MCAO models in mice (Fan & Yang 2007). Likewise Sbarbati et al. (1996) reported the formation of new vessels, just two weeks after ischemic injury. According to (Fan & Yang 2007), the underlying mechanism of ischemia-induced angiogenesis is largely due to hypoxia-inducible factor-1 (HIF-1), which stimulates expression of VEGF, integrin $\alpha v\beta 3$, and erythropoietin. These processes is further facilitated by Tie-angiopoietin system alteration (Fan & Yang 2007). Similary, biomechanical factors has been proven to be one of the governors of

angiogenesis. In pathological tissues, the angiogenic process is often abnormal, leading to the development of distorted vascular beds, characterized by an excessive proportion of blood vessels and with abnormal morphology and flow characteristics.

RELEVANCE OF ANGIOGENESIS

Angiogenesis is an important descriptor of ongoing changes in tissue, be it during pathologic or physiologic states (Annemette Lokkegaard 2004). The investigation and understanding of the type of angiogenesis is therefore essential; particularly, whether the angiogenesis involves branching or elongation of vessels, sprouting or intussusceptive growth. Information about the types and differences in tissue can be gain by the mare knowledge of the number of branches in microvascular network. It is also of great importance to know the number of micro-vessels in a possibly non-expending network. According to Annemette Lokkegaard (2004), both the number and length of microvascular network connections are essential for the description of microvascular function and properties. This knowledge may provide novel search and development of treatment for conditions that are dependent on microvascular network and angiogenesis. Among these conditions is cancer; due to the fact that angiogenesis has been show to be one of the determining factors in the development of cancers (Annemette Lokkegaard 2004); the need to continuously refine Ani-angiogenic therapies for the treatment of some cancers is ever growing. On the contrary, Bosomtwi et al. (2008) writes that angiogenesis was found to be the primary factor associated with improved recovery after conditions such as stroke. He states that treatment of stroke with neurorestorative therapies leads to an enhancement of angiogenesis implying that treatment of these kinds of conditions require pro-angiogenic therapies rather than anti-angiogenic therapies used in conditions such as cancers.

BIOMECHANICS AND ANGIOGENESIS

Physiologic organ morphology is achieved by migration and orientation of cells under the influence of chemotaxis, haptotaxis, and mechanotaxis. Both externally applied and internally generated mechanical forces influence cell migratory, proliferative, and secretary activities and matrix orientation (Stopak & Harris 1982; Korff & Augustin 1999).

Angiogenesis, the growth of vasculature form pre-existing blood vessels, is present during both physiological (wound healing) as well as a pathological (hypoxia, tumor growth) need of blood vessel generation, is fully understood as a result of an imbalances in pro-angiogenic and anti-angiogenic factors. Until recently, this process has been thought to occur solely against a chemical gradient of growth factors. Although considerable information is known regarding the biochemical cues relevant for angiogenesis; with negligence to the importance of mechanical forces involved and their influences on neo-vascularisation.

Physiological aspects concerning the development of vasculature and its functional regulation observed in normal processes such as embryonic development, wound healing etc. has been widely studied but with some amount of loop holes. According to Kilarski et al. (2009) assumptions that the mechanisms involved in physiological processes of vascular formation are the same as those in pathological angiogenesis is a great over-simplification (Kilarski et al. 2009). He writes that embryonic neovascularisation for instance is a genetically controlled *de novo* vessel formation with surrounding tissues taking place in a well-organized and reproducible way. On the other hand however, non developmental neovascularisation during wound healing and tumor growth, takes place in an already differentiated tissue, usually

stimulated by unpredictable wound induced secretion of local factors; it occurs in a chaotic environment which is difficult to observe a well-defined gradient of growth factors.

The biomechanical concept of the regulation of tissue vascularization represents non developmental neovascularization whereby translocation initiates initial rapid formation of functional blood vessels in the granuation tissue. Mechanical or tensial forces generated by proto-myofibroblasts, thus mediates the contraction of provisional matrix and surrounding cells lead to vessels expandtion as granuation tissue growth. On the other hand, angiogenic neovascularization represents embryonic developmental vascularisation that is dependent on cell proliferation and migration. This slow process is nonexplinatory for the appearance of fully functional vessels in granuation tissue 24 hours later. It therefore seems that these tow e processes are driven by different mechanisms and cannot be compared.

MECHANICAL DYNAMICS OF TISSUE DURING ANGIOGENESIS

Mechanical forces affect the behaviour of nearly all life forms on earth (Garvin et al. 2003; Neidlinger-Wilke et al. 2002). The mechanical environment of endothelial cells influences migration, proliferation and tube formation, protease and other secretory activities (Sumpio et al. 1990), cellular and cytoskeletal organization (Iba et al. 1991), expression of genes likewise the surface molecules and cell signalling (Azuma et al. 2000).

Tissue injury such as cerebral ischemic lesions and tumors, induce inflammatory processes and the deposition of fibrin matrix containing fibroblast, secreted by the surrounding tissues. Neutrophills and monocyte-macropheage lineage cells invade and remove necrotic tissue as well as infectious agents. In about 2 – 3 days activated and proliferating fibroblasts (protomyofibroblasts) produce tensial stress contracting wound matrix. At about 4 – 5 days

formation and growth of granulation tissue restores tissue integrity. Tensial stress, ED-A fibronectin and macrophage-derived growth factors leads to the differentiation of granulation tissue into a myofibroblasts expressing α- smooth muscle actin (Kilarski et al. 2009). Myofibroblasts then secretes extracellular components contracting the wound and hence transmitting tension across intercellular actin stress fibers connected to the extracellular matrix, showing that granulation tissue can be formed without immediate angiogenesis (Kilarski et al. 2009).

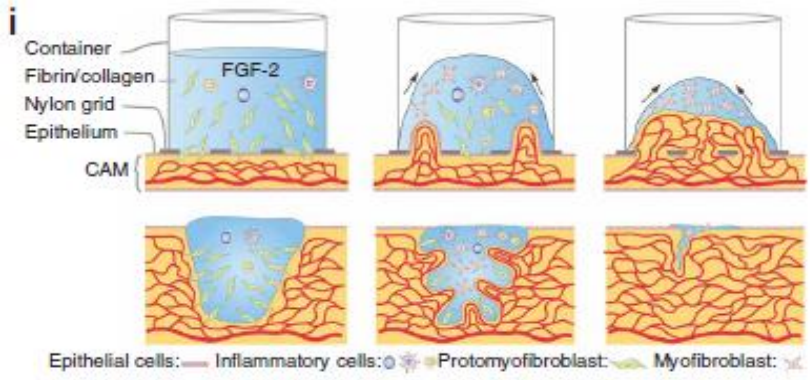

Figure 4: Fibroblast Growth Factor-2(FGF-2); taken from (Kilarski et al. 2009)

Activated fibroblasts and myofibroblasts contracts the provincial matrix and wound margin producing tension that mediates and directs expansion of vascularized tissue. Functional pre-existing vessels translocate in to the wound after which angiogenesis takes control, fine tuning vascular tissue by remodulation and regression. (Kilarski et al. 2009) demonstrated the above by blocking Vascular Endothelial Growth Factor Receptor – 2 which still resulted in vascular growth confirming the hypothesis that biomechanical forces are sufficient and required to mediate initial vascular growth without endothelial sprouting or proliferation characterized by angiogenesis (Kilarski et al. 2009).

Extracellular matrix (ECM) belongs to one of the most important structures that regulate angiogenesis. Matrix molecules, such as proto-myo-fibriblasts, are responsible for inducing tissue alignment and providing architectural clues for the neovessels (Kilarski et al. 2009). In addition ECM acts as a biochemical regulator of endothelial cell (EC) phenotypes and serves as a structural framework to support sprout and neovessel structure and function. It also provides a foundation from which intracellular stresses, critical to EC function, are generated. Matrix flexibility or stiffness is a significant modulator of metalloproteinase activity, an essential aspect of angiogenesis, in ECs (Seandel et al. 2001; Vernon & Sage 1996).

CHAPTER 2

AIMS OF RESEARCH

The purpose of the present dissertation is to investigate the effect of photothromosis on motor and cognitive functions of adult rats after focal cerebral ischemia induced in the early postnatal periods in rats. In addition, seizure susceptibility in a PTZ elicited model of epileptic seizures, as a consequence of stroke was also analysed. The specific aims were:

1. Assess the effect of stroke using simple motor and cognitive functional responses, such as gross motor coordination in adulthood.
2. To investigate the relationship between morphological and behavioural change after stroke.
3. To analyze changes of post stroke susceptibility to PTZ–induced seizures in immature rats as early as five and eighteen days after photothrombosis.

EXPERIMENT 1

THE EFFECT OF EARLY POSTNATAL STROKE ON MOTOR PERFORMANCE IN ADULT RATS AND IT CORRELATION TO THE ACCOMPANYING MORPHOLOGICAL CHANGES

Materials And Methods

Animals

Experiments were performed in male albino Wistar rats (n = 139), provided by the Institute of Physiology of the Academy of Sciences of Czech Republic. At postnatal day (P) 7 the animals were brought to the experimental room and divided into four groups: animals (n=30) exposed to laser light for 5 min. (BR_5 min); animals (n=38) exposed to laser light for 30 sec. (BR_30 s); sham-operated controls (Sham) treated with saline (n=31) and intact control group (C, n=40). Behavioral tests were performed at P 67. A group of seventy-eight rats (BR_5 min = 20, BR-30 s = 19, Sham = 19 and C = 20) were submitted to rotarod, bar holding and inclined grid tests. A second group of sixty-one rats (BR_5 min = 10, BR_30 s = 19, Sham = 12 and C = 20), were submitted to ladder rung walking tests and open field (OF). A subset of twenty-one animals, n = 7 from each experimental group, were used to confirm the ischemic injury. Rats were housed in standard plastic cages in a temperature-controlled environment of 22±1˚C, humidity 50-60% with a 12-h light/dark cycle (lights on at 6 a.m.) with free access to food and water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The protocol of the experiments was approved by the animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic (v.v.i) to be in agreement with the Animal Protection Law of the Czech Republic, which is fully compatible with the guidelines of the European Community Council directives 86/609/EEC. The Institute

possesses The Statement of Compliance with Standards of Human Care and Use of Laboratory Animals #A5228-01 from NIH.

SURGICAL PROCEDURES

Induction of Photothrombosis

Cortical brain lesions were induced by photothrombosis according to (Brant D et al. 2009); with slight modifications. Before the procedures started rats where weighted and marked. They were anesthetized with isoflurane (1.5 – 5%). A midline scalp incision was made and the scalp was retracted laterally to expose the skull. Freshly prepared Bengal Rose solution (Sigma®, BR 10 mg/1 ml, 0.15 ml per animal) was injected into the exposed right jugular vein, rats were then transferred within 1 minute to a stereotaxic frame and their brains were exposed (through an intact skull) to Green laser light (Roithner Laser, Austria 50 mW output) for 60 sec at 0.5s on/off cycles. The center of the light beam was focused on the left sensorimotor cortex, 1.8 mm from Bregma anterior posteriorly (AP) and 2.8 mm laterally (L) extending to the midline medially (AP = 1.8mm, L = 2.8mm) (George Paxinos & Charles Watson 1998). The incisions were immediately sutured and collodium was applied on the suture. Animals were allowed to recover from anesthesia for about 45 minutes before returning to their mother. Sham-operated controls followed the same protocol, except that saline was used instead of Bengal Rose injection before laser light illumination. Two animals were removed from experiment due to procedural problems during surgery. Body temperatures were monitored and maintained at 37^{o} C throughout the surgery. The health condition of animals was checked regularly.

MOTOR FUNCTION ANALYSIS

Behavioral testing was performed in adult animals, 60 days after photothrombosis. The deficits of weight supported stepping, paws coordination, endurance, muscle strength, skilled walking, were assessed with a battery of simple tests adopted from (Mikulecká & Mareš 2002; Ticha et al. 2011). Before behavioral testing, all animals were weighted and left to adapt to the experimental room for one hour.

Bar holding

An animal was held by the nape and its forepaws were allowed to touch a wooden bar (25 cm long, 1 cm in diameter, suspended 25 cm above a padded soft surface). Time of fore- and hind-limb grasping was recorded with a limit of 60 sec. The mean of the three grasping latencies was taken as the score.

Rotarod test

Motor coordination and equilibrium was observed using an automatic rotarod treadmill unit, set to rotate at a constant 5rpms and placed 30 cm above a landing platform. The unit consisted of a plywood horizontal rod (10cm in diameter 11cm long) covered with thick plaster to increase its roughness. The rats were placed individually on the rotating rod with their heads directed against the direction of rotation. Three trials were performed in a close succession. Maximal score in maintaining equilibrium was arbitrarily fixed at 60 seconds. The mean of three trials was taken as a score. A video camera was placed in front of the rotarod, to observe the behavioral strategies used by an animal to maintain equilibrium. Two strategies were observed passive rotation and walking. The passive rotation consisted in

hanging on the rotarod and being passively rotated. During the walking behavior the rat walked asynchronously (the movement of the paws was not perfectly coordinated) and synchronously (the rat walked evenly, the paws being perfectly coordinated).

Inclined grid

Turning on the inclined grid was assessed by a modification of the procedure described by Marshall (1982). A grid (75 cm with x 100 cm height) made of stainless was held in a horizontal position. A rat was placed on it, approximately in the center. Then, the grid was lowered until it attained a negative inclination of 30 with respect to the horizontal plane. The latency to turn on the grid to 180^0 was measured for a maximum of 60 sec. If the rat failed to turn on the grid, the latency was set to 60 sec. The mean of three turning latencies was taken as a score.

Ladder rung walking

A horizontal ladder rung walking test apparatus, consisted of side walls made of clear Plexiglas and metal rungs (3 mm diameter), which could be inserted to create a floor with a minimum distance of 1cm between rungs. The ladder was elevated 30 cm above the ground with a empty starting cage and a refuge (home cage) at the end. The width of the alley was adjusted to the size of the animal, to prevent the animal from turning around. The time to cross the runway in one trial with regular gaps and another trial with irregular gaps was assessed. The rats were tested with a limit of 60 sec.

Open field

The open field (OF) test was performed in a square arena (45x45x30 cm), with a camera installed above the center. Immediately after a rat was placed in the center of the arena; locomotor behavior was recorded automatically by a computerised system (Etho Vision Noldus Information Technology) for 5 min. Locomotor activity expressed as distance moved (cm) was calculated.

To reduce any lingering olfactory cues all devices were cleaned after each rat was tested.

HISTOLOGY

Tissue preparation

One week after behavioral testing, animals were overdosed with urethane (2g/kg i.p.), and perfused transcardially with ice cold 0.01 M phosphate-buffered saline (PBS; pH 7.5) followed by 4% formaldehyde. The brains were removed and post fixed in 4% formaldehyde at 4^0C and then sequentially soaked in 10%, 20% and 30% sucrose. The brains were then frozen in dry ice and stored at -70^0C. Coronal sections (50μm thick; Cryocut Leika 1600) were cut through the entire brain and every fifth section was collected and placed in 4% formaldehyde. To assess cell morphology, brain sections were derived from all investigated rats, mounted onto gelatin-coated slides and stained with cresyl violet or Nissl staining (Otahal et al. 2005). Slides were cover-slipped and assessed using a light microscope (Olympus® AX 70 microscope with bright field optics).

Evaluation

To evaluate the magnitude of lesions; ratios between hemispheres (affected/contralateral) were calculated. Volumes of these hemispheres were stereologically estimated by Cavalieri principle and pointgrid probe in the Ellipse software (ViDiTo, Slovakia). Briefly, every fifth slice (50µm thick) was obtained from a cutting series of slices throughout the brain. These slices were stained with Nissl and placed under a stereomicroscope, to captur their images, with a digital camera. A set of slices from one brain and a measuring scale bar were captured using the same magnification and camera setting. The obtained images included both hemispheres, this allowed us to use the same settings in the pointgrid for both hemispheres, affected and no-affected contralateral hemispheres. Spacing of the pointgrid probe was set to 0.2mm.

STATISTICS

Statistical analysis was performed with a program (Sigma Stat3.5®SPSS). To assess the difference in tissue volume non parametrical Mann-Whitney test was used. The sensorimotor performance data were analysed using one-way ANOVA with post hoc Student-Newman-Keuls Method. The differences in the ratio of correct response (animals crossed the ladder rung walking within the 60 sec.) were evaluated by means of Chi^2 -test. All data was expressed as mean ± SEM and statistical significance $p<0.05$ was accepted.

RESULTS

Morphology of the photothrombotic lesions

Two different exposition times to green laser light (30 s and 5 min) focused on sensorimotor cortex, responsible for sensorimotor limb function (Castro-Alamancos & Borrel 1995), were chosen to induce focal cerebral ischemia. The shorter exposition time (30 s - BR_30 s) induced lesions of diameters ranging from 0.3 – 2.5, penetrating through all cortical layers leaving subcortical white matter intact (Fig.5A). The longer exposition time (5 min - BR_5 min) induced larger lesions of 2.4 -3.7 mm in diameter involving all cortical layers including the underlying white matter and subcortical structures (e.g. striatum) of affected hemisphere (Fig.5B). The animals with large lesion presented a mean volume ratio of (0.79±0.07) ischemic hemisphere versus the contralateral hemisphere, while those with small lesion had a mean volume ratio of (0.94±0.02). Mann-Whitney test revealed a significant difference in volume ratio between intact controls (C), sham-operated controls (Sham) and rats with large lesion ($p< 0.05$), and between both controls (C and Sham) and rats with small lesions ($p<0.05$) (Fig.1C). All control animals, intact controls and sham-operated controls, presented no structural evidence of cerebral damage.

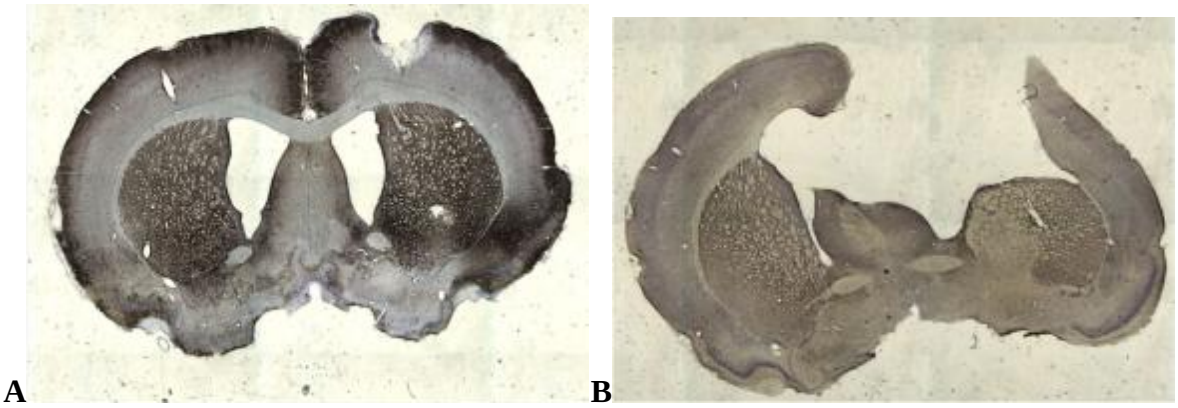

Figure. 5 (I): Representative, Nissl-stained coronal brain section of photothrombotic infarct in adult rats showing, the involvement of all cortical layers in BR_30 (A) and BR_5(B) including the underling subcortical white matter.

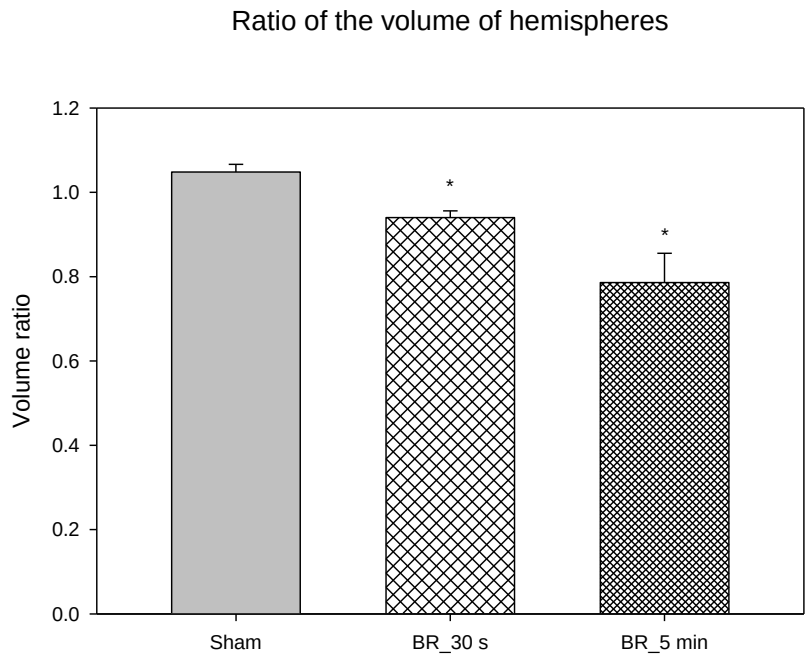

Figure. 5 (II): Sections obtained at 50µm intervals throughout the lesion were used to calculate lesion volumes ratio of sham-operated controls, BR_5 and BR_30.

MOTOR FUNCTION ANALYSIS

Bar holding

A significant main effect of photothrombotic lesion on the time spent on the bar was demonstrated F (3, 74) =10.79, p<0.001. The time spent holding the bar was significantly shorter in both groups of rats subjected to stroke; rats with small lesion (16.3±2.2) and rats with large lesion (6.2 ± 0.65) compared to both sham-operated (19.5±2.5) and intact controls (22.95± 1.89). Moreover, the animals with large lesions spent, a significantly shorter time holding the bar compared to animals with small lesions.

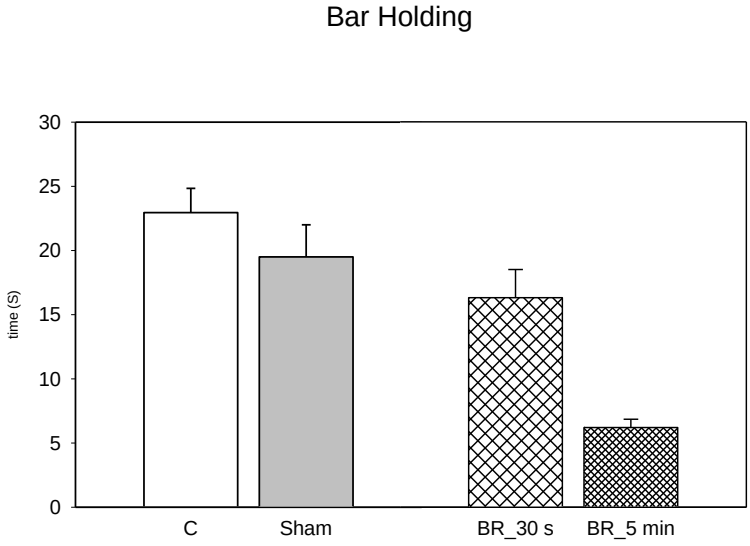

Figure. 6.

Rotarod test

The overall analysis of the time spent on the rotarod revealed significant main effects of experimental condition F (3, 74) =24.62, p<0.001. Both groups of rats with either small lesions or large lesions walked for a significantly shorter time on the rotarod (29.16±3.79 and 20.23± 2.5, respectively) compared to controls (51.57± 2.22) or Sham-operated rats (45.6±3.09). In addition, lesion dependent performance in motor performance was observed; the animals with large lesions spent less time on the rotarod compared to animals with small lesions.

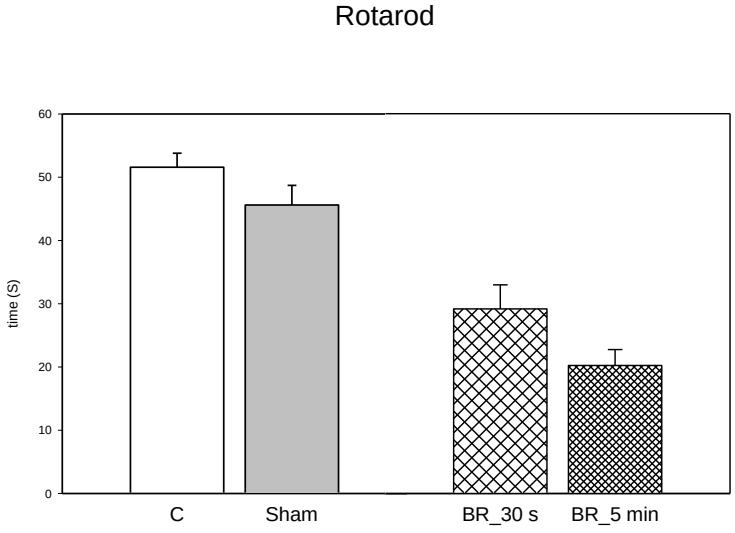

Figure 7.

Inclined grid

The latency to turn on the inclined grid significantly increased in both groups subjected to stroke, $F(3,74)=10.78$, $p<0.001$; with values of (29.16±3.79) in rats with small lesions and (20.23 ±2.5) in those with large lesion compared to both intact (9.4±1.4) and (6.9±0.8) sham-operated controls. In addition, animals with the large lesion had an increased latency to turning on the grid compared to the animals with small lesion.

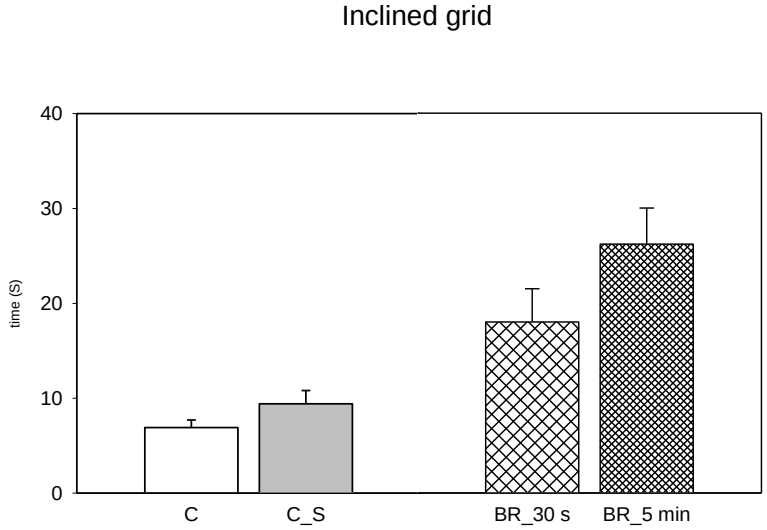

Figure. 8.

Ladder rung walking

In the ladder rung test, 95% of controls, 83% of sham-operated animals, 68% of BR_30 s and 50% of BR_5 min animals, mastered the regular gaps trial within 60 s (Chi^2 =9.4, p= 0.02). In the trial using irregular gaps, 70% controls, 75% sham-operated animals, 37% BR_30 s and 41% BR_5 min group mastered the task within 60 s (Chi^2 =10.3, p=0.06). The ANOVA showed that the latency to cross the ladder with regular gaps was affected F(3,57)=3.67, p =0.017. Surprisingly, there were no statistically significant differences in the latency to cross

the ladder in both trials with regullar or irregular gaps F93,57)= 3.67=0.017 ;$F(3,57)=1.74$, p =0.017 respectively.

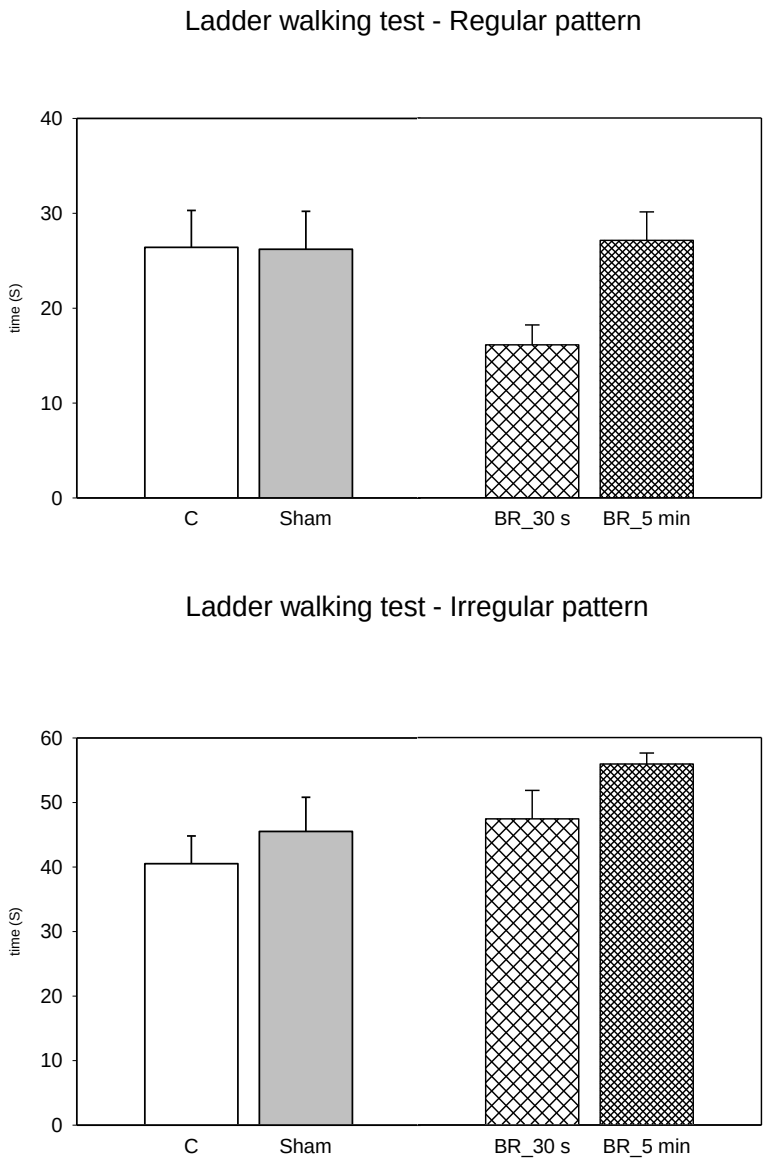

Figure. 9.

Figures. 6-9: Effects of photothrombosis induced at P7 on the sensorimotor behavior of adult rats; Abscissa: C (controls); Sham (sham operated controls); BR_30s (group exposed to laser light for 30s); and BR_5 min (group exposed to laser light for 5min). Ordinate: mean + S.E.M. for time (s) it took to perform in rotarod, bar holding, inclined grid (P<0.05) and ladder rank walking test (P<0.06):*****Compared to both C and Sham, #Compared to Sham, +Compared to BR-30 s.

Open field

The distance moved in the OF revealed significant main effect of lesion size on the loco motion in the animals subjected to stroke F (3, 57) =13.69, p<0.001. The post hoc test showed that only the animals with large lesion walked a shorter distance (791.4± 77.5) compared to all other groups. There were no significant differences between the animals with the small lesions (2097.8±182.1) and both control groups (sham-operated - 2015. ± 185.3 and intact controls - 2378.0± 144.4).

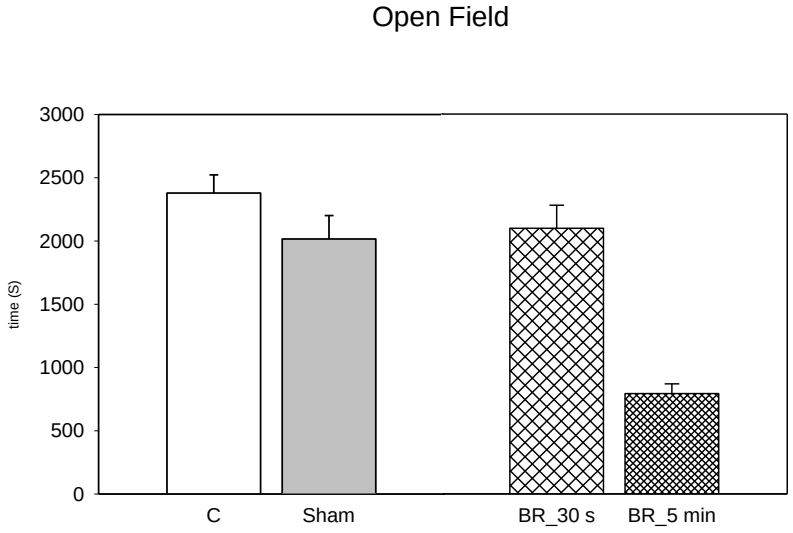

Figure. 10: The effect of stroke, on the locomotor activity of adult rats in the open field test. Abscissa: C (controls); Sham (sham operated controls); BR_30s (group exposed to laser light for 30s); and BR_5 min (group exposed to laser light for 5min). Ordinate: mean + S.E.M. for distance moved. P<0.05: *Compared to both C and Sham, +Compared to BR_30 seconds.

EXPERIMENT 2

INCREASED SUSCEPTIBILITY TO PENTETRAZOL-INDUCED SEIZURES IN DEVELOPING RATS AFTER CORTICAL PHOTOTHROMBOTIC ISCHEMIC STROKE AT P7

MATERIALS AND METHODS

Animals

A total of eighty-nine rats were used in this study. The photothrombotic stroke was induced in immature male albino Wistar rats at P7 (the day of birth was defined as day 0). Sham-operated animals of the same age served as controls. Two experiments were performed: experiment 1 for the induction of motor seizures by PTZ in a set of rat at P 12 and P 25 while in experiment 2, rats underwent video EEG monitoring at P25. The animals (n=65) in experiment 1, were divided into two basic groups–rats with photothrombotic lesion (n=33) and surgical controls (n=32). These groups were sub divided; half of these were administered with the 60 mg/kg dose of PTZ and the other half was injected with a dose of 100 mg/kg both at P 12 and P 25 i.e. 5 or 18 days after ischemic insult respectively. In experiment 2, rats (n=24) were divided into two groups - surgical controls (n=15) and rats with photothrombotic lesion (n=9), all of them were injected with two successive doses (20 mg/kg and 40 mg/kg) of PTZ to elicit EEG spike-and-wave rhythm.

All rats were housed in standard plastic cages, nests of ten pups per mother, in a temperature-controlled environment of 22±1˚C, humidity 50-60% with a 12-h light/dark cycle (lights on at 6 am) with free access to food and water.

INDUCTION OF PHOTOTHROMBOSIS

Cortical brain lesions were induced in half the set of the animals (n=32) at P7, using photothrombosis according to (Watson and Prado, 2009) with minor modifications. The animals were anesthetized with isoflurane (3.5–5%); a midline scalp incision was made and the scalp was retracted laterally to expose the skull. Bengal Rose (Sigma-Aldrich, Czech Republic, BRI 10 mg/1 ml, 0.15 ml per animal) was injected into the exposed right jugular vein and within one minute the rats were transferred to the stereotaxic frame, where the brains were illuminated through an intact skull with green laser light (Roithner Laser, Austria 50 mW output) for 60 seconds at 0.5s on/off cycles. The center of the light beam was focused on the left somatosensory cortex (AP=1.8mm, L=2.8mm). The incision was immediately sutured and collodium was applied on the suture. The animals were allowed to recover from anesthesia for about 45 minutes before returning to their mother in the home cage. All surgical controls (n=32) followed the same protocol but treated with saline instead of Bengal Rose before laser light illumination. Body temperature was monitored and maintained throughout the entire experiment.

ANALYSIS OF PTZ-INDUCED SEIZURES

Rats were placed individually in plastic chambers (15x15cm 30cm high), body temperature of P12 rats was maintained by means of a pad heated at 34˚C. Pentylentetrazol (Sigma, St. Louis, Mo., USA) solution was freshly prepared in two different concentrations, always injecting a volume of 2 ml/kg b.w. Half the set of animals in both age groups P12 and P25 received 60 mg/kg s.c. to assess possible proconvulsant action of lesions; the other half received 100 mg/kg s.c. to analyze its interaction in fully developed seizures. Occurrences of two types of seizures were recorded and seizure severity was scored using a five-point scale

(Pohl and Mares, 1987). The latencies of seizures were also registered; all data from lesioned rats were compared to those from surgical controls.

EEG ANALYSIS

Implantation of EEG electrodes

A different set of rats n=24 (9 rats after photothrombosis, 15 surgical controls) underwent cortical EEG electrode implantation at P25. Four epidural flat silver electrodes were implanted under ether anesthesia, over the sensorimotor and occipital cortices, while indifferent and ground electrodes were inserted into the occipital bone. All electrodes were fixed to the skull with fast-curing dental acrylic. After recovery period of two hours, the animals were connected to video EEG monitoring system and EEG recording was initiated.

Video-EEG monitoring

Animals were placed individually in plastic chambers (15x15cm. 30cm high) and electrodes were connected to EEG monitoring system (Vision-Brain Video-EEG system, FGU ASCR, Czech Republic). EEG signals were pass-band filtered at a range of 1.6 – 500 Hz and digitalized at a rate of 2 kHz and control EEG was recorded continuously for at least 10 minutes before the first PTZ injections. Registration continued after intraperitoneal PTZ injections (20 mg/kg as the first dose and 40 mg/kg 20 minutes later) up to 30 minutes after the second dose of PTZ. Latency of the first RMA episode was measured and the number and total duration of all RMA episodes were counted within the whole 20 minutes after the first PTZ injection. Both the number of animals exhibiting seizures and the number of seizures after the second dose of PTZ were counted. Qualitative EEG evaluation was performed off-

line with EEG Viewer software (EEG Viewer for Matlab, FGU ASCR, Czech Republic). Fast Fourier transform was used for calculation of EEG power in 30-s windows of EEG before and after the first PTZ injection. Results were expressed as a total power and power of individual frequency bands (delta=1-4 Hz, theta=4-8, alpha=8-12, beta 1=12-16, beta 2=16-32, gamma=32-50 Hz).

HISTOLOGY

Tissue preparation

The brains were histologically examined after the end of experiments to confirm the presents and extent of lesions. Rats were decapitated under an overdose of anesthesia, brains removed and frozen in dry ice. The brains were then sectioned in a coronal plane (50 μm) with a cryocut Leica CM 1900, mounted onto gelatin-coated slides and stained using Nissl staining (Otahal et al. 2005). Slides were then cover-slipped and assessed using a light microscope (Olympus® AX 70 microscope with bright field optics).

STATISTICS

All data were expressed as a mean ± standard error of the mean. One Way ANOVA (Sigma Stat3.5® SPSS) was used to assess and compare results in both age groups. Statistics of data from EEG recordings was performed by means of t-test and/or Mann-Whitney Rank sum test (SigmaStat started with a testing of distribution), numbers of animals exhibiting seizures were compared by means of Fisher exact test. Statistical significance was accepted when $p<0.05$.

RESULTS

Analysis of PTZ-induced seizures

The two age groups studied, differed in patterns of motor seizures elicited by the 100 mg/kg dose of PTZ. The younger group (P12) exhibited only generalized tonic-clonic seizures with a loss of righting abilities whereas the 25-day-old rats generated at first minimal clonic seizures with preserved righting reflexes and after a longer latency generalized tonic-clonic seizures. Data from sham-operated controls from the present experiments did not significantly differ from those of naïve rats obtained in other experiments performed approximately at the same time (Mikulecka et al. 2012).

The 60 mg/kg dose of PTZ

Only one 12-day-old surgical control and two lesioned rats (out of eight) exhibited generalized seizures – one control and one lesioned rat exhibited complete generalized tonic-clonic seizures, the remaining lesioned animal exhibited generalized clonic seizures. Seizure severity reflected the presence of seizures, which was 1.5±0.5 in controls and 1.88±0.58 in lesioned rats. The dose of 60 mg/kg PTZ administered to 25-day-old rats elicited seizures in two out of nine surgical controls but failed to elicit seizures in lesioned animals. Seizure severity in this age group was 1.44±0.29 in surgical controls and 1.0±0 in lesioned rats. No significant difference was found between the control and lesioned groups at either age. Latencies of seizures could not be statistically evaluated due to low incidence of seizures.

The 100 mg/kg dose of PTZ

All eight 12-day-old controls exhibited generalized seizures; seven complete GTCS and one generalized clonic seizures. In the group of rats with photothrombotic lesions of the same age

8 rats exhibited complete generalized tonic-clonic seizures and one did not exhibit seizures at all. Seizure severity did not differ between these two groups (4.88±0.12 in controls, 4.56±0.44 in lesioned animals, respectively). Latencies of seizures were significantly shorter in lesioned rats than in controls (196.3±16.5 vs. 286.9±27.1 s, respectively).

The high dose of PTZ induced seizures in all 25-day-old controls. Six of them exhibited GTCS (five the common sequence of mS and GTCS, one rat started directly with GTCS), the remaining two exhibited only mS. Seven out of 8 rats with photothrombotic lesion exhibited GTCS, six of them with mS as the first seizure. The patterns and latencies of both minimal and generalized seizures did not differ between the two groups. An outlined difference in latencies of GTCS in 25-day-old rats (opposite to that in the younger group) did not reach the level of statistical significance due to high variability of data.

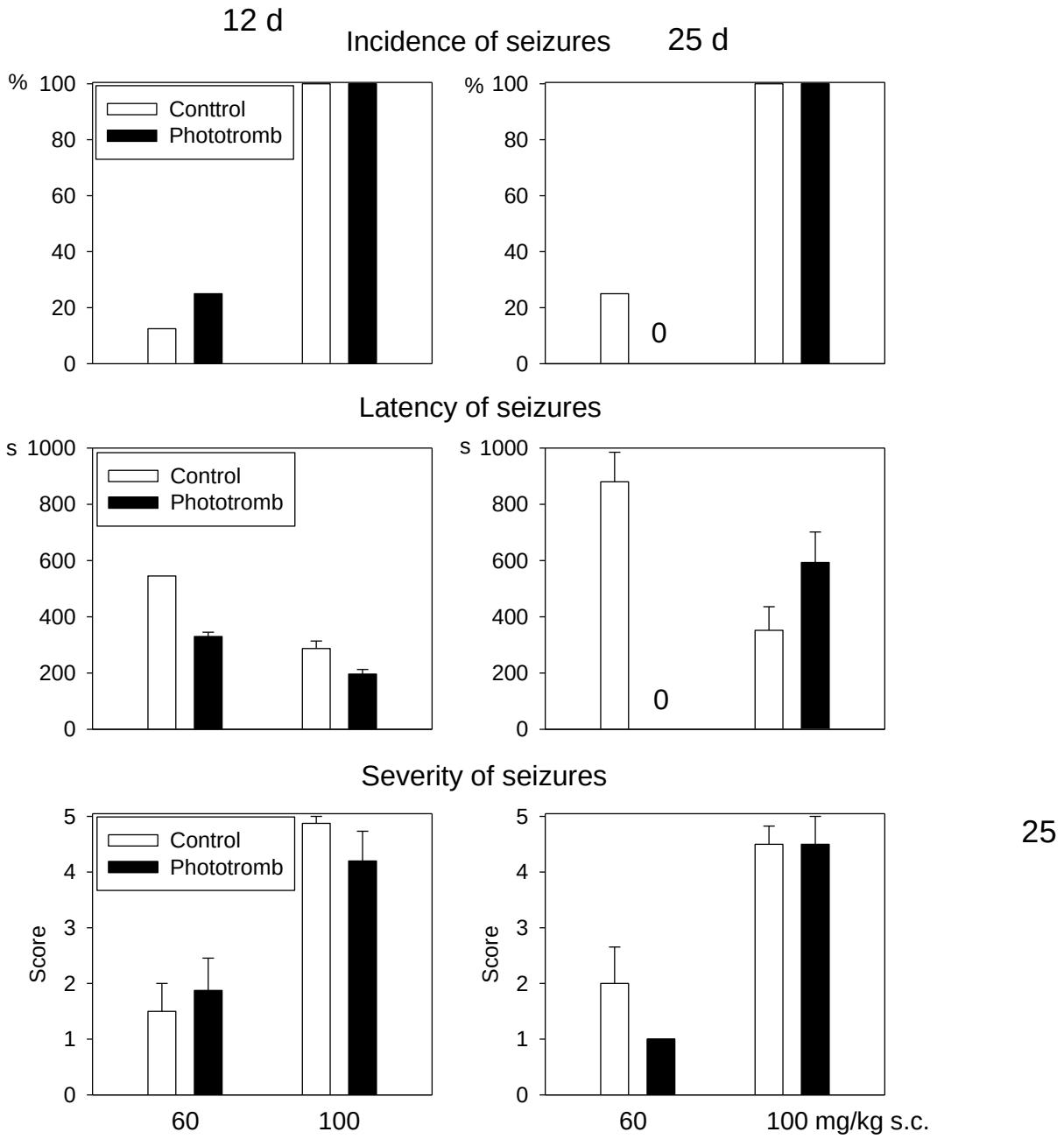

Figure 11:. PTZ-induced seizures in 12- (left) and 25-day-old rats (right). From top to bottom: Incidence of seizures, i.e. percentage of rats exhibiting seizures; latency of seizures (mean+S.E.M.); severity of seizures (mean+S.E.M.) evaluated by a five-point scale (Pohl and Mareš 1987). White columns – sham-operated controls with Bengal rose administration; black columns – rats with a phototrombotic lesion. Abscissae: two doses of PTZ (60 and 100 mg/kg s.c.), ordinates from top to bottom: percents of rats; latency in seconds; five-point scale.

EEG ANALYSIS

Rhythmic metrazol activity (RMA) induced by the 20 mg/kg dose of PTZ

EEG recordings prior to PTZ injection failed to demonstrate noticeable changes in the cortex contralateral to the lesion. The first dose (20 mg/kg i.p.) elicited episodes of spike-and-wave rhythm (rhythmic metrazol activity, RMA) with a frequency of 4–5 Hz (Figure.14). Statistical comparison revealed a significant effect of cortical lesions on the latency of the first RMA (Figure. 12); lesioned rats exhibited significantly longer latencies as compared to surgical controls (944±240 vs. 120±120 s, respectively). A marked tendency of higher number of RMA episodes in the lesioned animals, after the first dose of PTZ (Figure.12) stayed just below the level of significance (p=0.066). There was a significantly longer total duration of RMA episodes in lesioned rats in comparison to the controls (Figure.12).

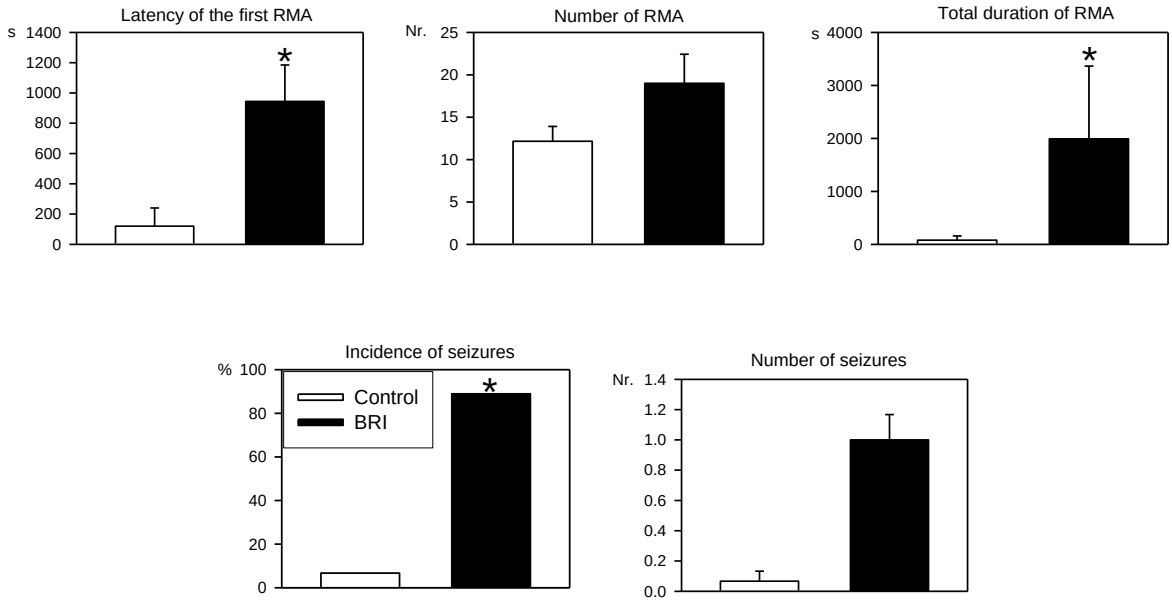

Figure 12: Graphs from left to right: latency of the first rhythmic activity (RMA); number of RMA; total duration of RMA; incidence of minimal clonic seizures; average number of seizures. Asterisks denote significant difference between controls (white columns) and phototrombotic animals (black columns).

Analysis of EEG demonstrated a marked increase in power from native activity to recordings after PTZ administration. It did not show any difference between rats with photothrombotic lesions and surgical controls. Therefore analysis of individual frequency bands was done and it demonstrated that the increase of power after PTZ administration is mainly in low frequency bands (delta, theta and alpha). Comparison of lesioned and control animals did not reveal any significant difference, there was only a tendency of a higher increase in theta frequency band in lesioned rats than in the controls.

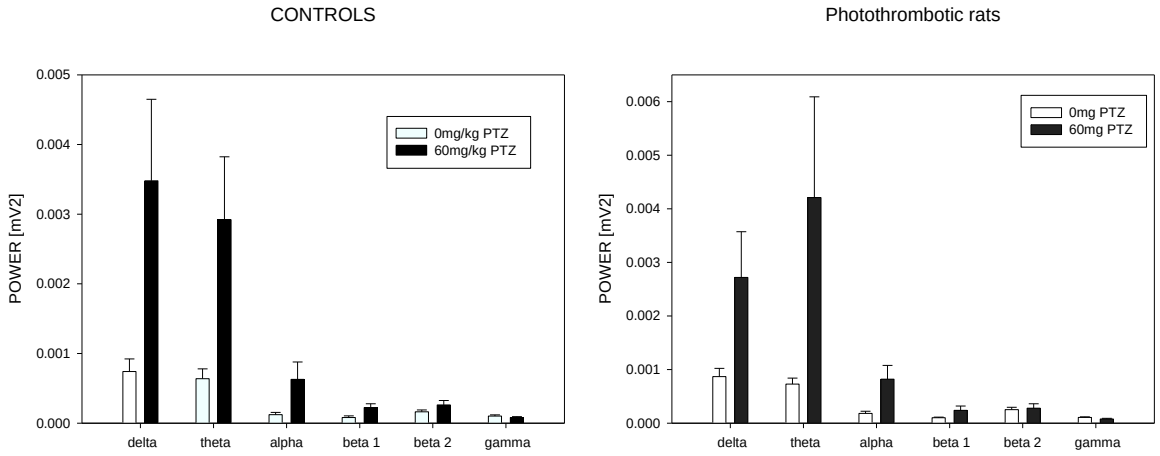

Figure. 13: Spectral analysis of EEG of 25-day-old rats. Left – surgical controls, Right – rats with photothrombotic lesion. Abscissae – individual frequency bands (see Methods); ordinates – power in mV2. White columns – activity before PTZ administration, black columns – activity 10 min after the second PTZ injection.

Minimal motor seizures following the second dose of PTZ

The second dose of PTZ (40 mg/kg i.p.) elicited minimal clonic seizures in seven out of eight lesioned rats; it is a significantly higher incidence than in the control group, where only one out of eight rats demonstrated ictal activity accompanied by convulsive seizures (Figure. 12). Minimal clonic seizures involved mainly muscles of the head and forelimbs; the hind limbs

were widely abducted, the tail erected and righting ability was preserved. EEG pattern of minimal seizures was characterized by a spike-and-wave rhythm (Figure.14)

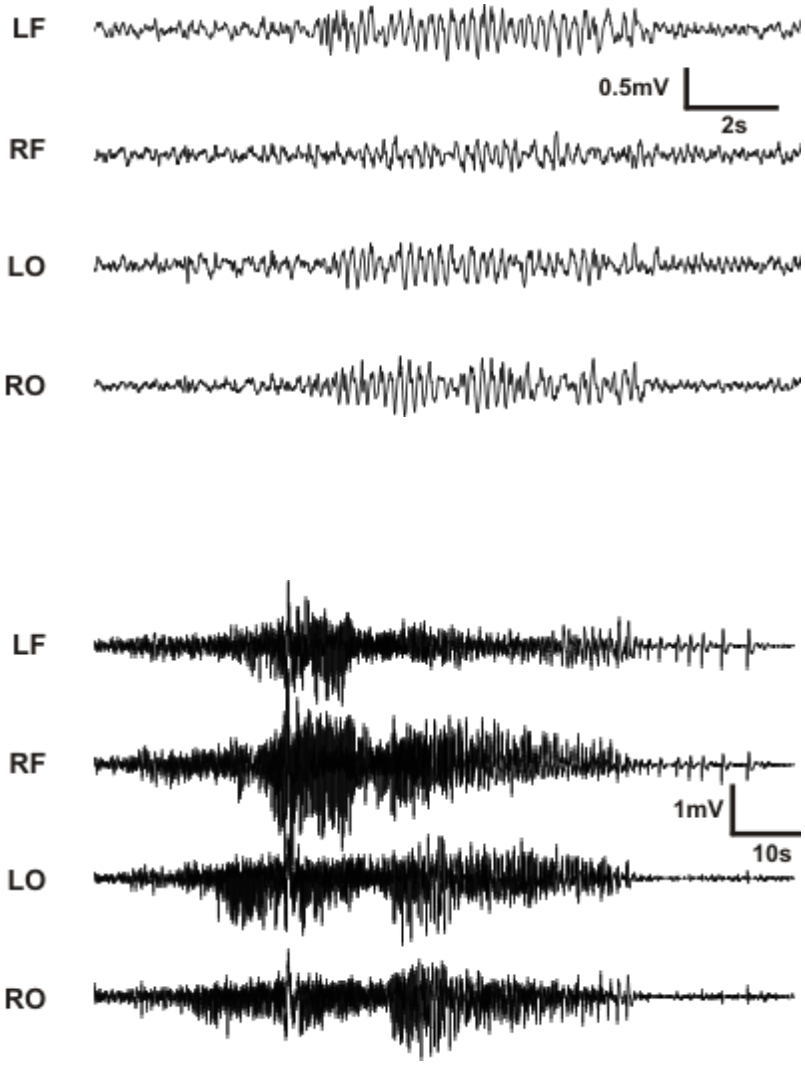

Figure. 14: Original EEG recordings: demonstrating rhythmic metrazol activity (upper recording) and clonic seizure (bottom recording). Individual leads from top to bottom: RF – right frontal cortex; LF – left frontal cortex; LO – left occipital cortex; RO – right occipital cortex. Upper part - time mark 2 s, amplitude calibration 0.5 mV; lower part – time mark 10 s, amplitude calibration 1 mV.

HISTOLOGY

Morphology

The lesions were localized in the left somatosensory cortex extending from 2.2 to -2.3mm rostro-caudally and 0.5 to 3mm lateral to bregma according to Paxinos and Watson (1998) (Figure.14). A difference in the involvement of white matter was observed; some animals had a pure gray matter lesion, while others exhibited deeper lesions involving white matter. The involvement of white matter had no influence on PTZ-induced phenomena nor did it affect the convulsions or EEG phenomena.

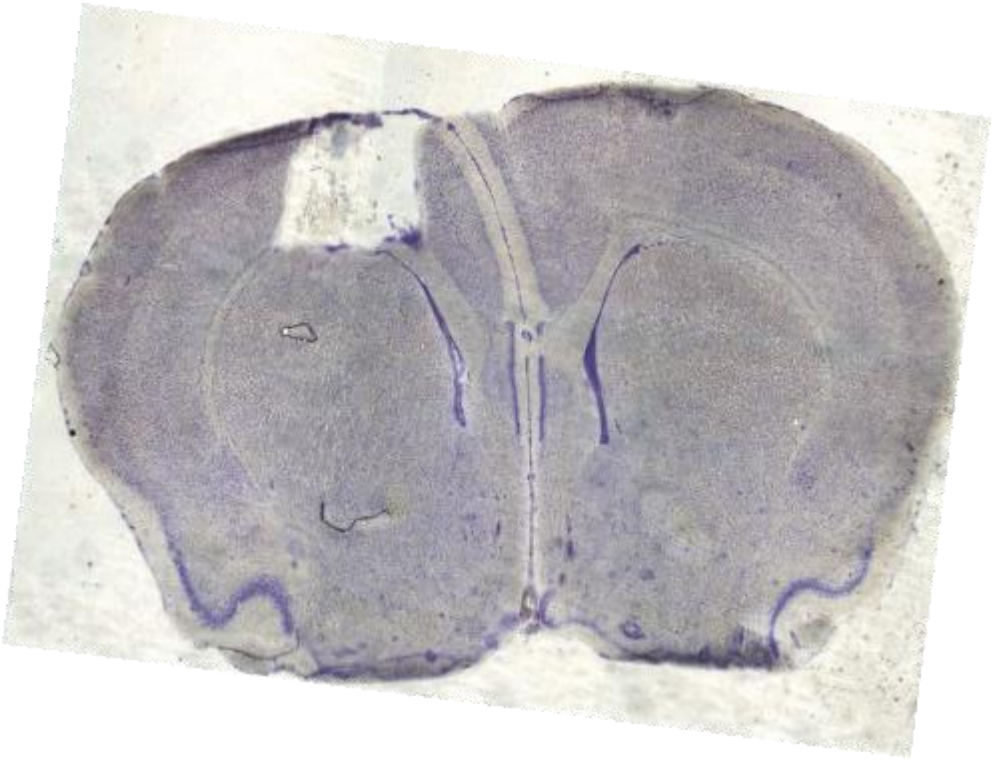

Figure.15. Nissl stained frontal section of the brain of a 25-day-old rat from the Experiment 2. This animal exhibited transition from episodes of rhythmic EEG activity to convulsive seizures. Lesion only touched the white matter.

DISCUSSION

EXPERIMENT 1

THE EFFECT OF EARLY POSTNATAL STROKE ON MOTOR PERFORMANCE IN ADULT RATS AND ITS CORRELATION TO THE ACCOMPANYING MORPHOLOGICAL CHANGES

The effect of cerebral ischemia has been analysed by many authors, mostly assessing acute and chronic outcomes of lesions induced in adult animals but rarely the impact of stroke induced in immature animals and assessed in adulthood. Several reports showed that the immature brain reacts differently to ischemia than does the mature brain, for a review see (Yager & Thornhill 1997; Chen et al. 1999; Aden et al. 2002; Ditelberg et al. 1996; Saucier et al. 2007). Therefore, it is critical to use an experimental model of an appropriate age (Aden et al. 2002). Furthermore, to improve the validity of animal models of stroke, recommendations from experts stress the importance of long-term functional outcomes rather than relying exclusively on acute consequences (Lindner et al. 2003).

The present study investigated sensorimotor performances and locomotor activity of adult animals subjected to focal photothrombotic stroke at an early (P7) age. We adopted a technique developed initially for use in neonatal mice (Maxwell & Dyck 2005) to induce photothrombotic stroke in immature rats. Firstly, we focused on assessing whether neonatal photothrombotic stroke of two infract sizes has different effect on sensorimotor performance in adulthood. Secondly, whether there is a correlation between infarct size and degree of behavioral impairments.

In our study, all animals survived until adulthood except two animals that died during the surgical procedure. Moreover, no differences in animal body weight that might affect motor performances were observed between the controls and animals subjected to stroke (data not

shown). In contrast to previous studies (Chou et al. 2001; Felt et al. 2002; Hoane et al. 2000), after the induction of photothrombotic ischemic stroke our animals were not intermittently submitted to any form of stimulation such as rehabilitative training in order to enhance sensorimotor recovery.

Our data depicted two kinds of lesions with different shapes and sizes relative to laser illumination. Exposure to laser light for 30 sec resulted in small lesion restricted to the sensorimotor cortex leaving the subcortical structures intact. While exposure for 5 min resulted in large lesions that involved all cortical layers including the underlying white matter and striatum of affected hemisphere. The large ischemic lesions had significantly less remaining tissue relative to the group with the small lesions. Motor ability and coordination, includes all processes that affect the brain's ability to synchronize the function of interrelated muscles. Synchronization of muscle movement occurs primarily in the cerebellum. The cerebral cortex is important in controlling the forelimbs and less important in control of the predominantly ambulatory hind-limb. Rats with destroyed cerebellum or inferior olivary complex are unable to perform complex sensorimotor tasks (Rondi-Reig et al. 1997). In this study, motor coordination was measured as the total time each rat was able to remain on a rotating cylinder or the time it was able to remain on an inclined platform as the angle of inclination was progressively increased. Our results revealed that the performance of rats submitted to stroke was slower with a greater magnitude in animals with large lesion. In the bar holding test, one of the most demanding tests of motor coordination, impairments of motor performance were more expressed. Specifically, the animals with larger lesions were only able to grasp the bar for a very short time. In both groups of rats with small or large lesions, no statistical differences in percentage to complete the task within 60 s were found in ladder walking test with regular pattern. Similar results were found in the task with irregular pattern but the percentage of successful animals was decreased to a large extent. We

observed during testing, that the animals alternated a few steps with a resting period; therefore, they were not able to fulfil the task within 60 s. These results indicate a decreased motivation of these animals to rejoin the cage mate. The animals with larger lesion walked a shorter distance suggesting a marked inhibition of locomotion. Surprisingly, in our animals, no signs of ataxia or loss in balance where observed. Our results indicate a clear relationship between sensorimotor impairments and lesion extend. Contrary to the previous observation, (Chou et al. 2001) indicting no relationship between OF behavior and lesion severity. We observed a correlation firstly between the time of exposure and lesion size and secondly between lesion size and sensorimotor performances and locomotion. Our results are in agreement with previous findings demonstrating that the animals exposed to laser for a longer time had the largest lesions and demonstrated the greatest sensorimotor and locomotion impairments (Sulejczak et al. 2007). Also in a combined analysis of controls and HI-subjected rats, a strong correlation between the degree of brain damage and long-term sensorimotor deficits was demonstrated (Bona et al. 1997). In their study, neurological functions were evaluated in a set of four sensorimotor tests performed between five and six weeks after hypoxia-ischemia; asymmetries of limb placing, foot-faults and abnormality in the postural reflex tests were detected (Bona et al. 1997). Other studies noted particular differences between behavioral outcomes and lesion size. Jones and colleagues, observed for example, large unilateral lesions in HI rats accompanied by reduction in forelimb grasping strength, increased number of foot faults in grid-walking test and forelimb asymmetry in cylinder test compared with sham-operated controls (Jones et al. 2008). On the other hand, Shanina demonstrated that small lesions with intact subcortical layers resulted in significant sensorimotor impairments detectable only during the first two weeks after photothrombotic ischemic stroke (Shanina et al. 2006). Similarly, Biernaskie and colleagues demonstrated that, the degree of behavioral recovery in animals treated with lidocaine after endotelin-1

induced stroke was dependent on the infract size (Biernaskie et al. 2005). In their study, animals with large lesions were unable to retrieve food pellets; whereas animals with small lesions returned to pre-ischemia performance levels. Our results generated from a photothrombotic model are in accordance with the data described above.

To date, the most frequently used neonatal stroke model of long-term neurobehavioral and functional outcomes is hypoxia-ischemia (HI) and middle carotid artery occlusion (MAC-O) (Almli et al. 2000). According to Brant D. Waston and Prado, models such as these produce cerebral ischemia, but not stroke *per se.* These models were reported to have high mortality rates (Maxwell & Dyck 2005) and affect both animals body weight and behavior (F.Josef van der Staay 1998). For instance in a study by (Renolleau et al. 1998) all 7-day-old pups subjected to left MCA electrocoagulation with transient carotid occlusion lasting for more than 1 h died during procedure; if occlusion lasted an hour of less, only 10 % of pups died during the first 2 h. Unlike these models, photothrombosis in its ischemic thromboembolic form is a more realistic model of human stroke because it simulates more accurately the characteristics common to human stroke. Among these characteristics are the occlusion initiating events in human stroke, formation of permanent occlusion, and penumbral region (Hao et al. 1992). Photothrombotic lesion location and size can be controlled by adjusting the intensity and duration of laser exposure. This makes it possible to mimic lesion size and location as observed in most human stroke to be small in size ranging from 4.5 to 14% of the ipsilateral hemisphere (Carmichael 2005) with predominance of left hemisphere lesions in perinatal periods (Nelson & Lynch 2004); providing the opportunity for long-term evaluation of behavioral and neuropathological endpoints.

There are great variety of techniques developed for the study of cerebral stroke initially developed for the use in adult rats and modified for the use in immature rats (Northington

2006) that require sophisticated microsurgical skills due to the size and tiny anatomy of immature rats. Although these techniques are continuously being refined and modified, so far no appropriate models based on photothrombosis in immature rats and focused purely on the assessment of long-term sensorimotor abilities are available. Future considerations may therefore be aim to explore and refine basic experimental issues such as age of photothrombotic stroke induction in conjunction to different developmental periods to assess functional and behavioral outcomes.

EXPERIMENT 2

INCREASED SUSCEPTIBILITY TO PENTETRAZOL-INDUCED SEIZURES IN DEVELOPING RATS AFTER CORTICAL PHOTOTHROMBOTIC ISCHEMIC STROKE AT P7

Cortical photothrombotic lesions induced in immature rats in this study, affected seizures elicited by pentetrazol, later during postnatal development. Major changes were found in a model of human absences induced by a low dose of PTZ and an easy transition from EEG spike-and-wave rhythm into minimal clonic seizures; while convulsive seizures remained almost unaffected. These changes were observed in 25-day-old rats, i.e. 18 days after lesion induction, indicating permanent or at least long lasting changes in selective seizure susceptibility.

We used all three types of seizures elicited by PTZ: episodes of spike-and-wave rhythm, minimal clonic seizures and generalized tonic-clonic seizures. These seizures have different semiology, different ontogenetic development and also different sites of origin. Spike-and-wave episodes are accompanied by minor motor phenomena (jerks of vibrissae), generated in cortico-thalamo-cortical circuits (Avanzini et al. 1992; Lourenco et al. 2010; Browning & Nelson 1985) and accepted as a human model of absences (Snead, III et al. 2000). Minimal clonic seizures generated in the basal forebrain (Browning & Nelson 1985) are characterized by clonic seizures of forepaw and head muscles and by spike-and-wave rhythm in the EEG (Mares 1998) accepted as a human model of myoclonic seizures (Loscher & Schmidt 1988). These two types of seizures could be elicited in rats as from the third postnatal week (Velisek et al. 1992). Generalized tonic-clonic seizures are formed by a short initial phase of wild running, followed by a sequence of short tonic (up to a maximum of 15-20 s) and long clonic phase (up to tens of minutes). Righting ability is lost at the beginning of the tonic phase.

These seizures are generated in the brainstem (Browning & Nelson 1985) and isolated spinal cord is also able to generate them (Mareš 2006). These types of seizures are not age specific and can be induced at any developmental stage (Velisek et al. 1992).

Cortical lesions were found to potentiate spike-and-wave activity more than two weeks after photothrombosis. It is in agreement with increase in excitability of homo- as well as contralateral cortex 7 days after photothrombotic cortical lesion in adult rats demonstrated in vitro by (Buchkremer-Ratzmann et al. 1996) as well as with hyperexcitability of an undercut cortex due to increased AMPA/kainate excitatory postsynaptic potentials and a decrease of inhibitory postsynaptic potentials (Li & Prince 2002). The increase in sensitivity to PTZ-induced phenomena may be partly explained by an absence of colossal afferents. A part of this augmentation may be also due to changes in thalamic nuclei (Paz et al. 2010). An easier transition of nonconvulsive seizures (rhythmic EEG episodes) into convulsive (minimal clonic) seizures may be also due to an increased cortical excitability. A part of this augmentation may be also an increased transition into convulsive seizures – minimal clonic ones. With cortical photothrombotic lesions there is a loss of part of the cerebral cortex and a formation of transitory zone with partially damaged neurons between necrotic and healthy cortex. A similar situation as in cortical cobalt foci, partially damaged neurons may be a source of pathologic activity (Fischer 1969). Failure of cortical lesions to affect brainstem seizures demonstrated a restricted effect of neocortical lesions. We can conclude that photothrombotic cortical lesions induced at early stage of development is not sufficient to result in epilepsy at least in a nearly three-week interval, but this insult may lead to hyperexcitability - possible background of later epilepsy (Jensen & Baram 2000).

Brain injuries including stroke are commonly known to trigger epileptogenesis, a process leading to the occurrence of spontaneous seizures (Friedman et al. 2009). During the acute

phase, minutes after focal cerebral ischemia, exitotoxic mechanisms were shown to trigger neuronal and glial damage; causing acute and delayed cell death while triggering the initiation of post-ischemic inflammation gene expression. This leads to a production of inflammatory mediators and cytokines such as platelet-activation factor, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1α) (Dirnagl et al. 1999) and interleukin-6 predominantly present in the central nervous system after injury (de Vries et al. 1997). These inflammatory mediators are produced and secreted by damaged brain cells, endothelial cells, circulating immune cells as well as by activated brain parenchymal cells: microphages, microglial cells, astrocytes and neurons (Vezzani 2005). The presence of these inflammatory mediators and cytokines enhance neuronal excitability, impair cell survival and were shown to be involved in the pathogenesis of epilepsy, for example IL-1α has proconvulsant action, facilitating seizures. Moreover, activated immunocompetent cells and neurons produce a high amount of nitric oxide, which we recently found to be an important molecule in the initiation of seizures in vitro (Kovacs et al., 2009). Acute inflammation and its general proconvulsive action might explain the increased sensitivity to PTZ observed in this study 5-18 days post photothrombotic ischemic stroke. Cerebral brain injuries at any stage of life, including during perinatal periods, is accompanied by BBB breakdown, usually triggered by perivascular astrocytes or perivascular microglia activated by ischemia. Induced cortical photothrombosis likewise, leads to massive focal disruption of BBB revealed by T2, DW MRI and Evans Blue extravasations. This causes vascular injury contributing to increased BBB permeability and extravasations of serum components into the brain parenchyma. Ultra-structural studies carried out in animals and human epileptic tissue revealed multiple forms of epilepsy common after brain injury and local diminished BBB integrity (Cacheaux et al. 2009). This implies that compromised BBB integrity may be a trigger event leading to epilepsy as shown by (Seiffert et al. 2004). Exposure of brain tissue to blood constituents via diminished BBB integrity after

stroke has devastating effects. Serum proteins were shown to lower the threshold for generating epileptic seizures producing long-lasting neuronal reactivity enhancement to afferent stimulation (Maggio et al. 2008). Among these proteins are thrombin and albumin, proven to have sufficient ability to recapitulate epileptiform activity induced by BBB disruption (Lee et al. 1997; Cacheaux et al. 2009). Cacheaux et al. (2009), for example, showed albumin to be critical in the generation of epilepsy after BBB compromise while TGF-1β pathway activation by TGF- β1 proved to result in epileptiform activity. This phenomenon might be the reason why the animals post photothrombosis, in our study presented a lowered PTZ threshold; PTZ at 20+40 mg/kg was able to elicit minimal clonic seizures, not normally observed at such doses. Whatever the mechanism, the presented results clearly indicate that cortical photothrombotic lesions induced in immature rats produce long lasting changes in seizure susceptibility.

CONCLUSION

Closing remarks

Results from the studies that make up the back bone of the present thesis, demonstrated that photothrombotic cerebral ischemic stroke induced in the early postnatal period and tested in adult rats, indeed influenced functional task performance governed by the affected cortical area. A further analysis of one of the major consequences of stroke, epilepsy, revealed that these cortical lesions had an effect on the sensitivity to PTZ-induced seizures, shortly after stroke. Despite the fact that all animals subjected to stroke performed poorly as compared to the controls, they were all able to fulfil the given tasks. No signs of ataxia or loss in balance where observed. The only exception was that at least half the animals were not able to cross the ladder with irregular pattern arrangement within 60 sec. This might be brought about by the brain's ability to compensate for the loss functions; possibly due to plasticity of the immature brain.

Lourenco et al. (2010) states that motor performance variables has a potential to better identify motor control deficits than clinical outcome measures alone. Future considerations may therefore be aim to explore motor patterns used for task performance post stroke. Kinematic variables were shown to provide detailed measures of movement quality, such as reaching movements - joint ranges of motion, grasping and supination, postural adjustments – change of body angle or shoulder and trunk movement. It will be of great interest to extend this study to analyze kinematic changes and task performance such as lime placing, foot faults and postural motor function. Task performance analysis of animals submitted to stroke during adulthood compared to those submitted to stroke during perinatal periods might shed light to our current results.

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