The Charles University in Prague

Faculty of Physical Education and Sport

The Diploma Thesis

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Faculty of Physical Education and Sport

Model of focal brain ischemic lesion in neonatal rat

The diploma thesis

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Abstract

Title of work: Model of focal brain ischemic lesion in neonatal rats.

Work objectives: The aim of this diploma thesis was to determinate the parameter of the lesion after focal brain ischemia induced by photothrombosis, establish the changes of sensorimotor abilities and investigate the occurrence of post stroke epileptic seizures in this new model in P7 rat pups.

Materials and Methods: Intravenous application of bengal rose dye was followed by illumination of concentrated beam of high powered green laser over the sensorimotocortex for 5 minutes at 0,5s on/off cycles. To assess the motor deficit, rotarod walking test and bar holding test were performed in two months after stroke. The animals underwent implantation of EEG cortical and hippocampal electrodes followed by a week of recovery four months after stroke. EEG monitoring for five consecutive days have been done. Then animals were overdosed with urethane and transcardially perfused. The brains were cut into 50μm slice for histological staining. There were another five previous experiments, which were preliminary studies aiming at correct inducing of photothrombotic stroke and also EEG monitoring at all. Every study included a control groups that underwent the same surgical protocol. Present work includes only the last sixth experiment.

Results: Histological examination of the brain showed significant ischemic brain damage. The lesion size, localization and depth in the brain vary remarkably between animals. There is a statistically significant difference in ratio of the volume of the hemispheres between the control and experimental animals. Experimental animals were less skillful in almost all tests, unable to learn motor skills with repeated exposure in contrast to controls in the statistically mean. Our preliminary results show the occurrence of non-convulsive seizures in experimental animals.

Key words: photothrombosis, neonatal, epilepsy, EEG, seizures

Thesis Declaration

I hereby declare that this thesis is my own, unaided work and that recognition has been given to the references used. It has not been submitted for any degrees or examination at any other university.

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Anna Hráchová, April 2010, Prague

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Abbreviations

BBB - blood brain barrier

BRI –experimental animal

CA3 - region 3 Cornu Amonnis

CCAO – model of common carotid artery occlusion

CNS – central nervous system

contr. – control

CT – computer tomography

Diagr. - diagram

EEG – electroencephalogram

exp. - experimental

Fig. - figure

GABA - γ-Aminobutyric acid

i.v. – intravenous application

MCAO - model of middle cerebral artery occlusion

MRI – magnetic resonance

P7, P0, P56, P8, P28 – postnatal days of model, the age of 7, 0, 56, 8, 28

Sm. – statistically mean

Tab. – table

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THEORETICAL PART

1. Introduction

Stroke has been thought to be the third most common cause of death in adults in the developed world and primary concern among only the elderly people. However, recent evidence suggests that the occurrence of stroke in the neonatal brain is also very high. As recognised in the neonatal period, symptomatic perinatal stroke occurs in about one in 4,000 term neonates, ischemic stroke after the first month of life occurs with an annual incidence rate of about one in 30,000 children. (Nelson et. al., 2004) Neonatal stroke is associated with long-term morbidity and high social and medical cost to society. It is one of the most commonly recognized causes of severe neurologic deficits in neonates such as cerebral palsy, motor and learning disabilities, epilepsy, seizures and death. These findings have created a strong impulse to understand the causes and consequences of stroke in the developing brain, which, in turn, requires the development of appropriate animal models.

Many neonatal seizures in infants with arterial stroke are focal and may occur in the absence of other signs of neonatal encephalopathy such as abnormalities of tone or feeding, or depressed level of alertness. (Nelson et. al., 2004) Seizures are seen in infants with moderate or severe ischemic encephalopathy, who invariably have apparent neurological abnormalities. However, some studies was been found that the background EEG revealed remarkable abnormalities in infants, although they did not exhibit apparent neurological abnormalities. (Sato, 2003)

Although the importance of stroke as an etiologic factor for epilepsy is widely acknowledged, relatively few data are available regarding the mechanisms of post stroke epileptogenesis. The present study aimed to investigate the occurrence of post stroke epilepsy in neonatal P7 rats using the cortical photothrombosis with rose bengal dye. Brain development at P7 rats is comparable to human premature or full-term infants. (Sreenan et. al., 2000) Careful comparison suggests that the majority of structural and functional

milestones occurring during the first week of life in rat hippocampus take place during the third-trimester gestational period of the 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. (Bender et. al., 2007)

2. Basic Anatomy of the Human CNS

The human nervous system is composed of several hundreds of billions of neurons, the specialized cells designed to transmit information to other nerve cells, muscle, or gland cells. Neurons are the basic working units of the brain, each of which receive and give rise to thousands of connections. Some of these connections are located nearly a meter from the cell bodies of origin. The nervous system obtains sensory information from the environment, evaluates the significance of the information, and generates appropriate behavioural responses. Knowledge of neuronal structure and the pathways of information flow in the brain is important not only for understanding the normal function of the brain but also for identifying specific regions that are disturbed during neurological illness. (Kandel et. al, 2000; SNF, 2008)

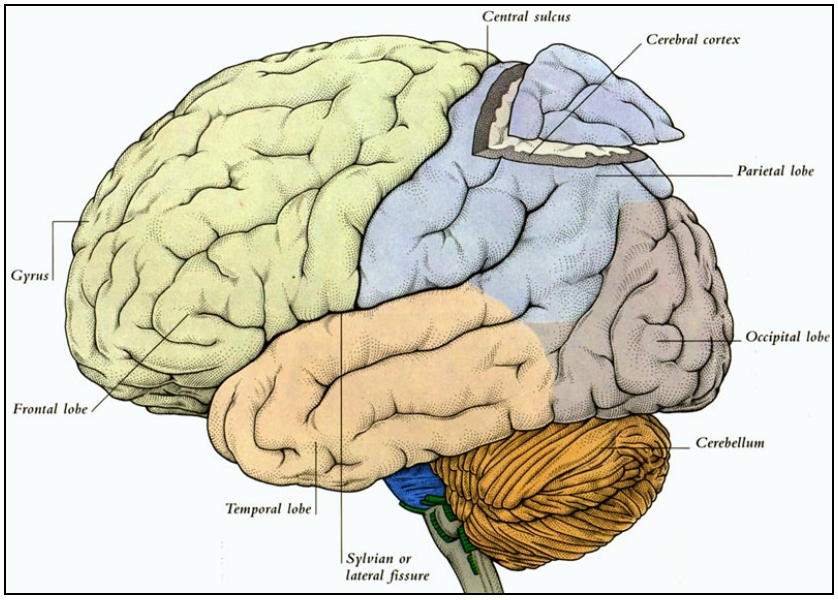


Figure 1: Central nervous system (Penney, 2010)

The nervous system has two anatomically distinct components: the central nervous system, consisting of the brain and the spinal cord, and the peripheral nervous system, composed of specialized clusters of neurons and peripheral nerves. The peripheral nervous system relays information to the central nervous system and executes motor commands generated in the brain and spinal cord. The simplest action involves the integrated activity of multiple sensory, motor, and motivational pathways in the central nervous system. Most neurons are precisely arranged into functional pathways that have the same anatomical arrangement in every individual. Many pathways cross from one side of the nervous system to the other. These basic principles govern the organization of the nervous system from the spinal cord through the brain stem to the highest levels of the cerebral cortex. (Kandel et. al, 2000; Crossman et. al., 2006)

2.1 Structure of the Central Nervous System

The brain is composed of six regions and each of them can be further subdivided into several anatomically and functionally distinct areas. The six major brain divisions are the medulla, pons, cerebellum, midbrain, diencephalon, and cerebral hemispheres (telencefalon). Each of these divisions is found in both hemispheres of the brain, but may differ in shape and size. (Kandel et. al, 2000)

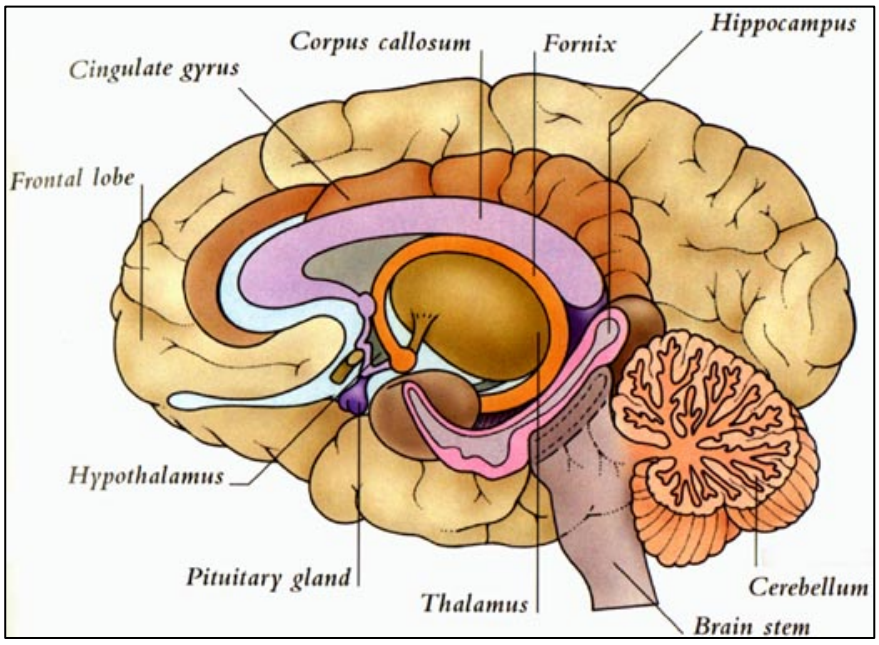


Figure 2: Central nervous system (Penney, 2010)

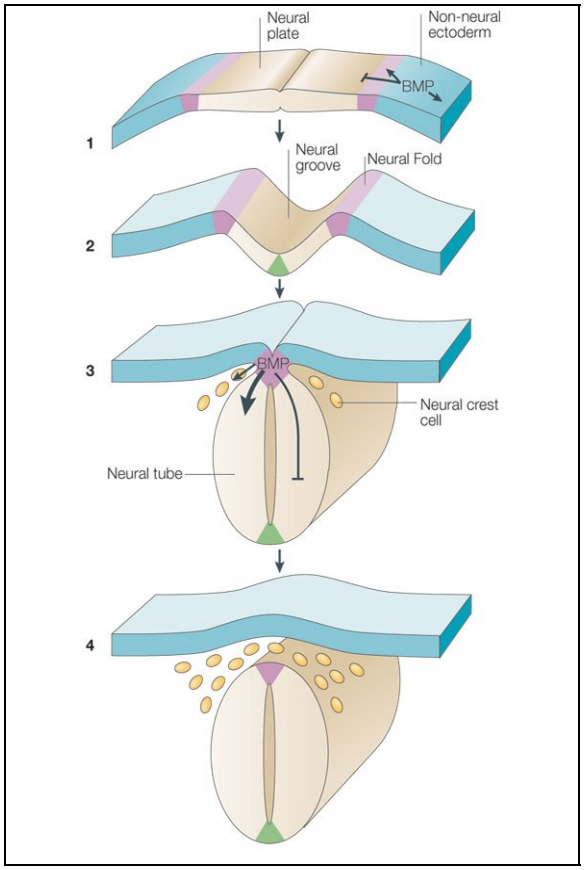
The cerebral hemispheres form the largest region of the human brain. They consist of the cerebral cortex, the underlying white matter, and three deep-lying structures: the basal ganglia, the amygdale, and the hippocampal formation. The cerebral hemispheres are concerned with perceptual, motor, and cognitive functions. The two hemispheres are interconnected by the corpus callosum, which connects symmetrical regions in both hemispheres. The corpus callosum, which is visible on the medial surface of the hemispheres, is the largest of the commissures, structures that contain fibres that mainly link similar regions of the left and right sides of the brain. The amygdale is concerned with social behaviour and the expression of emotion, the hippocampus with memory, and the basal ganglia with the control of movement. (Kandel et. al, 2000; Crossman et. al., 2006)

The cerebral cortex has a highly convoluted shape, formed by groove called sulci, that separate elevated regions. It arose during evolution to accommodate an increase in the number of neurons. The thickness of the cortex does not vary substantially in different species; it is always around 2 to 4 mm thick. The cerebral cortex is organized into cell layers. The number of layers and the details of their functional organization vary throughout the cortex. The most typical form of neocortex contains six layers, numbered from the outer surface pia mater of the cortex to the white matter. The cerebral cortex is divided into four major lobes named after the overlying cranial bones: frontal, parietal, temporal, and occipital. Each lobe includes many distinct functional domains. Many areas of the cerebral cortex are concerned primarily with processing sensory information or delivering motor commands. In addition, an area dedicated to a particular sensory modality or motor function includes several specialized areas that have different roles in processing information. These areas are known as primary, secondary, or tertiary sensory or motor areas, depending on their proximity to the peripheral sensory and motor pathways. (Kandel et. al, 2000; Crossman et. al, 2006; Gray, 2000)

3. Development of the Human CNS

The functions of the mature nervous system range from sensory perception and motor coordination to memory, emotion and motivation, depend on perfect connections formed between various types of nerve cells. These depend critically on the establishment of regionally distinct subdivisions of the neural tube. It is a complex process but can be analyzed in three major developmental steps. The generation of progenitor cells in the neural plate, the formation of the neural tube, and the generation of regional differences within the neural tube. (Kandel et. al, 2000)

The nervous system begins to develop at a relatively late stage in embryogenesis. Prior to its formation, three main cell layers have been generated. The endoderm, the innermost layer, gives rise to the bowels, liver and lungs; the mesoderm, the middle layer, gives rise to connective tissues, the vascular system and muscles; and the ectoderm, the outermost layer, gives rise to central and peripheral nervous systems.



1. Neural plate
2. Neural groove is triggered by the formation of a distinct hinge point in the ventral region- the floor plate
3. At the end of neurulation, the lateral edges of the neural plate fuse and segregate from the non-neural epithelium to form a neural tube
4. Roof plate and floor plate form at the dorsal and ventral midline of the neural tube

Figure 3: Formation of the neural tube (Liu et. al., 2005)

Initial stage of cell development starts when the zygote, the diploid cell resulting from union of a sperm and an ovum, implantates in the uterine wall and establishes the rudimentary placenta as the embryonic disk, which will give rise to the embryo. At about 14 days, the embryo is about 2 millimetres long. (Boon et. al., 2009) Neural and glial cells derive from a sheet of ectodermal cells located along the dorsal midline of the embryo at the gastrula stage. By the 17th - 20th day of gestation, this ectodermal sheet begins to acquire neural properties it forms the neural plate, a columnar epithelium, what will ultimately develop into the nervous system of the individual. Ectodermal cells that fail to follow the neural program of differentiation give rise instead to the epidermis of the skin. (Kandel et. al, 2000)

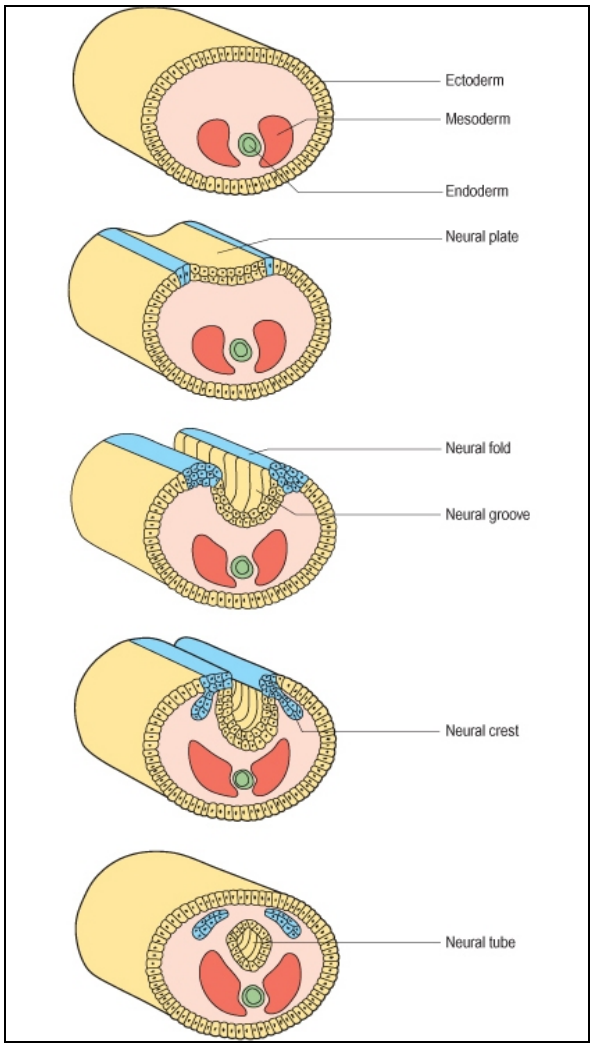


Figure 4: Formation of the neural tube (Liu et. al., 2005)

By the 23rd day, or the third week after the neural plate has formed it begins to fold into a tubular structure, called the neural tube, through a process called neurulation. First the neural groove (which is the embryonic brain structure) is visible. Two days later, the edges of this groove, which have continued to 'curl up' until now, start to join together to form the neural tube, which forms the basis of the entire nervous system. The closure of the neural tube is dependent upon protein bridges bound together by calcium. (Boon et. al., 2009) The caudal region of the neural tube gives rise to the spinal cord, and the rostral region becomes the brain. During these early stages of neural development, cells divide rapidly. (Kandel et. al, 2000) It is interesting to note that the neural tube, at this stage of development, contains around 125,000 cells. At birth, the human brain contains around 100 billion neurons - we can infer from this information that new neurons are being generated at the rate of about 250,000 per minute during the nine months of gestation. (Boon et. al., 2009) By the 27th day, the ends of the tube, the pores, close. [1]

Then the neural crest also begins to form. The crest is the source of neurons for the peripheral nervous system as well as for chromoform cells in the inner part of the adrenal gland. Chromoform cells are responsible for synthesising and secreting two important hormones instrumental in emotional arousal - epinepherine and norepinepherine. The neural crest is derived from neuroectodermal cells that originate in the dorsal aspect of the neural folds or neural tube; these cells leave the neural tube or folds and differentiate into various cell types including dorsal-root ganglion cells, autonomic ganglion cells, the chromaffin cells of the adrenal medulla, Schwann cells, sensory ganglia cells of cranial nerves, 5, 9, and 10, part of the meninges, or integumentary pigment cells. (Kandel et. al, 2000)

[1] Failure or ineffective closure of the neural tube, is the cause of *spina bifida* (embryologic failure of fusion of one or more vertebral arches; subtypes of spina bifida are based on degree and pattern of malformation associated with neuroectoderm involvement); *meningocele* (protrusion of the membranes of the brain or spinal cord through a defect in the skull or spinal column); *myelocele* (protrusion of the spinal cord in spina bifida), *anencephaly* (congenital defective development of the brain, with absence of the bones of the cranial vault and absent or rudimentary cerebral and cerebellar hemispheres, brainstem, and basal ganglia); and *pilonidal cyst* (a fistula or pit in the sacral region, communicating with the exterior, containing hair which may act as a foreign body producing chronic inflammation). (Boon et. al., 2009)

The process of cell proliferation is not uniform along the length of the neural tube. Individual regions of the neural epithelium expand at different rates and begin to form the specialized regions of the mature central nervous system. The proliferation of cells in the rostral part of the neural tube initially forms three brain vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). (Boon et. al., 2009; Kandel et. al, 2000)

At this early stage of development with these three-vesicles the brain flexes twice. First process called the cervical flexure is at the junction of the spinal cord and hindbrain, and the second one at the junction of the hindbrain and midbrain is called the cephalic flexure. A third flexure, the pontine flexure, forms at a later stage. Both the cervical and the pontine flexures eventually straighten out, but the cephalic flexure remains prominent throughout development. The persistence of this flexure is what causes the longitudinal axis of the forebrain to differ from that of the brain stem and spinal cord. (Kandel et. al, 2000) Later in development two of the three embryonic vesicles subdivide and together with the spinal cord, make up the six major regions of the mature central nervous system. The forebrain vesicle gives rise to the telencephalon and diencephalon, and the hindbrain vesicle gives rise to the metencephalon and myelencephalon. (Boon et. al., 2009; Kandel et. al, 2000)

14 days	Embryo is 2 millimetres long, has assumed a definite form and is more completely separated from the yolk sac, a cephalic and caudal extremities are easily distinguished. The neural folds are partly united.
17th -20th	Neural plate forms.
3 weeks	Neural groove forms to neural tube.
27th day	The ends of the tube close. The precursors of the eyes and ears are evident. A primary system for blood circulation is formed, and a rudimentary heart begins to beat. The alimentary canal, from mouth to anus is formed. The ends of the arms and legs are present. The tonic labyrinthine reflex is thought to develop. Three vesicles develop.
4th week	The development of the spinal cord. Marginal layer nerve fibres appear and begin to accept fibres of ganglion nerves.

5th week	The cerebral hemispheres differentiate.
7th week	The embryo is 20-25 millimetres long. Differentiation of the genitals and ossification of cartilage into bone occurs - at this stage most visible in the skull, ribs, scapulae, arms and legs, as well as the hard palate. The pineal gland forms.
8th week	All of the organ systems are established. Well defined finger and toe joints, ossification of the ulna, and radius, the illium, and of the fibula and tibia. First the frontal lobes form, then the parietal and concurrently, the temporal and occipital lobes. The limbic system is well developed by this time
3th month	The embryo is 75mm long and a growth rate of about 12 mm each week. At 11 weeks the palmar reflex emerges. Myelination of the nerve fibres (first the cranial nerves).
4th month	The embryo is 12-13 centimetres long. First bringing of its hands together and turning bodily within the uterus. All the spinal arches will close with cartilage which will later undergo ossification into bone. Corpus callosum is evident. Tonsils are present. At around 18 weeks, the asymmetrical tonic neck reflex emerges.
5th month	Intrauterine movements of the foetus. The germs of teeth both first and permanent are seen. Myelin formation in the spinal cord and dorsal root nerves begins.
6th month	255 millimetres. Rooting reflex emerges. Sebaceous glands are evident and lymph glands which will help to protect the foetus from noxious substances for all its life. The cerebral hemispheres now cover the whole top and sides of the brain including the cerebellum. Cerebellar development begins from this moment, but will not be complete until two years after birth. The body is covered by fine hairs (lanugo).
7th month	The communication lines between the brain and the periphery of the body (corticospinal tracts) are largely complete. The eyes open.
8th month	Body strengths and the nervous system will increase its connections and receive more sensory input, and gain more motor control.
9th month	The lanugo has largely disappeared from the trunk. The umbilicus is almost in the middle of the body and the testes are in the scrotum.

Table 1: The most important moments of development (Boon et. al., 2009; Gray, 2005; Kandel et. al, 2000)

The differentiation of cells in the nervous system, as in other organs, is the consequence of a complex program that directs the expression of specific genes within individual cells. Two major groups of factors determine which genes are expressed in a cell. The first, termed inducing factors, are signalling molecules provided by other cells. These factors can be freely diffusible and thus exert their actions over a long range, or they can be tethered to the cell surface and act locally. Because cells in different positions in the embryo are exposed to different inducing factors, the position that a cell occupies early in development is of critical importance in determining its fate. (Kandel et. al, 2000)

The second group of factors are the molecules that are activated or induced in cells upon exposure to an inducing factor from another cell. These molecules include surface receptors that mediate the effect of inducing factors. Activation of these receptors then modulates the activity of the transcription factors and regulates expression of genes that encode the proteins carrying out the specialized functions of the cell. The ability of the cell to respond to inductive signals, termed its competence, depends on the precise repertory of receptors, transduction molecules, and transcription factors expressed by the cell. (Boon et. al.; 2009, Gray, 2005; Kandel et. al, 2000)

The neural plate is induced by signals from non-neural cells – mesoderm. The fate of induced neural cells is controlled by two independent signalling systems. One system patterns the neural plate along its medial-to-lateral axis; after neurulation this axis becomes the dorsoventral axis of the neural tube. The second system controls the pattern of the neural plate along the anteroposterior axis. Signals along this axis divide the neural tube into its four major rostrocaudal subdivisions: the spinal cord, hindbrain, midbrain, and forebrain. The neural plate is patterned along its dorsoventral axis by signals from adjacent nonneural cells. Cell differentiation in both the dorsal and ventral halves of the neural tube is controlled by inductive signals. Ventral patterning is regulated by the activities of a single protein, sonic hedgehog, which generates different cell types at different concentrations. In contrast, dorsal patterning appears to involve several members of the bone morphologic protein family,

each of which may induce a particular set of cells. (Boon et. al., 2009; Kandel et. al, 2000)

The weight of the brain of the newborn is approximately 300 grams - around 10% of its body weight. In contrast to the adult brain which weighs approximately 1400 grams - only 2% of body weight. Brain weight increases with age and achieves 'adult' weight between six and fourteen years of life. We are born with our full complement of neurons. Postnatal growth of the brain is due to an increase in the size of neurons, and subsequent increase in number of supporting cells called glia, development of neural processes and synapses, and the laying down of the insulation of nerve processes (myelin sheaths). Synapses are formed at a very rapid rate during the early months of life, usually achieving maximum density between six and twelve months after birth. (Kandel et. al. 2000)

In the neonate, metabolic activity is most noticeable in the sensory-motor cortex and brain stem, areas necessary for reflex functions. At two to three months, metabolic activity is prominent in the visual and the adjacent parietal cortex which corresponds with the development of visual-spatial integrative function. Between six months and a year, metabolic activity is notably in the frontal cortex which corresponds to the development of higher cortical functions such as interactions with the immediate environment, stranger anxiety, etc. (Kandel et. al., 2000)

Although most of the neuronal cell death occurs in the embryo, the paring down of connections occurs in large part during critical periods in early postnatal life. These are windows of time during development when the nervous system must obtain certain critical experiences, such as sensory, movement, or emotional input, to develop properly. These periods are characterized by high learning rates. After a critical period, connections diminish in number and are less subject to change, but the ones that remain are stronger, more reliable, and more precise. Injury or deprivation, ether sensory or social, occurring at a certain stage of postnatal life may affect one aspect of development, whereas the same injury at a different period may affect another aspect. (Kandel et. al., 2000)

Interestingly, compared with adults, children have an increased incidence of certain disorders that involve excessive brain activity, such as epilepsy. Many epilepsy syndromes appear during childhood and fade away by adulthood. Brain development in people continues into the early 20s — even the brain of an adolescent is not completely mature. One of the later aspects of brain development is the completion of myelination of the axons connecting one brain area to another. This process starts around birth and moves from the back of the brain to the front: The frontal lobes are the last to become “connected” with fast conducting myelinated axons. Major functions of the frontal lobes are judgment, insight, and impulse control, and so the acquisition of these attributes becomes the last step in the creation of an adult human brain. (Kandel et. al., 2000)

4. The Development and the EEG Patterns

The EEG provides very good information of the development of the central nervous system. There is indeed an ontogenetic of the EEG, that is, a progressive maturation of the electrical activity of the brain, which is now well-known in its general lines. Whether EEG maturation relates to neuronal, biological or psychological maturation is of crucial importance for interpreting the EEG and delineating steps, phases or stages of evolution. A child is not, or not only, a small unachieved adult – so the EEG in children must not be judged in regard to the adult EEG, but in regard to specific child organization or successive organization. Also variability in children is much greater than in adults. The range of plasticity in reaction to external and internal factors, which may be of short or very long duration, is a fundamental component of the child organization to be taken into consideration. (Remond et. al., 1975)

EEG maturation must be related to „development“ as a whole, this term including different fields (somatic, psychological,…) Determining relationships between EEG and development is made difficult by two main factors: first, development is not a linear process. It proceeds by successive steps linked by phases of slowing, arrest or even progression of one or another field of evolution, second, there is no strict correspondence between physical,

biological and psychological development, each of which follows specific curves of evolution that are never superimposed or even parallel. (Remond et.al., 1975)

The bioelectrical activity of the central nervous system may be recorded through scalp electrodes. It is possible to record in incubator or during labour. It is important to record safely, without any risk for the baby.

We can find many studies, which describe EEG maturation in correlation with conceptional age or that the nerve conductions and its different values depend on gestational age. Limits of normality are difficult to establish. No clear limits of the normal range of variations of EEG in newborns have been described so far. (Bockhorst, 2008)

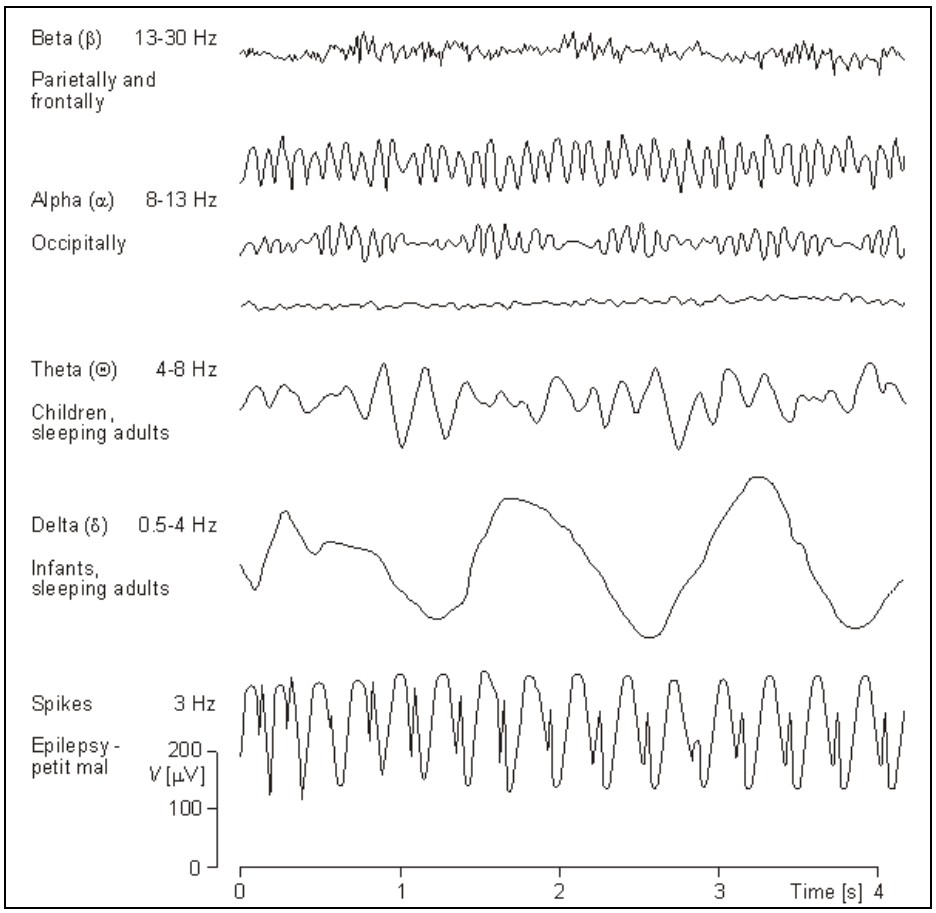


Figure 5: Normal EEG of the human adults (Eatpot, 2010)

The following table n.2 shows the EEG patterns during development from 24 weeks of conceptional age to the EEG of the full- term newborn. (Remond et. al., 1975)

AGE	MAIN PATTERNS	COMMENTS
Full-term new born	1/ Low voltage – activité moyenne (7c/sec, 50μV) 2/ Tracé alternant (1-3c/sec, 50-100 μV) 3/ Low voltage EEG patterns (2-4c/sec, 100 μV) 4/Continuous slow wave EEG patterns (0,5c/sec, 50 μV)	Activité moyenne in wakefulness, active and light sleep. Tracé alternant in quiet or deep sleep.
EEG of the premature infants between 36 and 41 weeks	Diffuse low voltage EEG patterns, continuous slow wave EEG patterns with superimposed rapid rhythms, and discontinuous patterns with bursts of variable shape.	Wakefulness and sleep are for the first time well correlated
EEG between 32 and 35 weeks of conceptional age	Slow waves 1-2c/sec with superimposed rapid rhythms in occipital, temporal and rolandic leads.	Patterns are not colerrated with wakefulness and sleep
EEG between 28 and 31 weeks of conceptional age	28 -29weeks: theta activity is synchronous (4-6c/sec, 25-100 μV) 30weeks: the typical patterns for 32-36weeks appear (1c/sec, 25-100 μV) and (10-14c/sec, 10-20 μV) – slow waves	At 30-31 weeks, wakefulness and sleep are still similar
The early premature infants (24-27weeks)	Slow waves 0,3-1c/sec, 300 μV), occipital predominance Alfa (8-14c/sec, 25-30 μV), rolandic Leeds	

Table 2: Development and the EEG patterns (Remond et. al., 1975)

The present study aimed to investigate the occurrence of post stroke epilepsy in neonatal P7 rats using the cortical photothrombosis with rose bengal dye. Brain development at P7 rats is comparable to that of premature or full-term infants. (Vannuci, 1999) Careful comparison suggests that the majority of structural and functional milestones occurring during the first week of life in rat hippocampus take place during the third-trimester gestational period of the 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. (Bender, 2007)

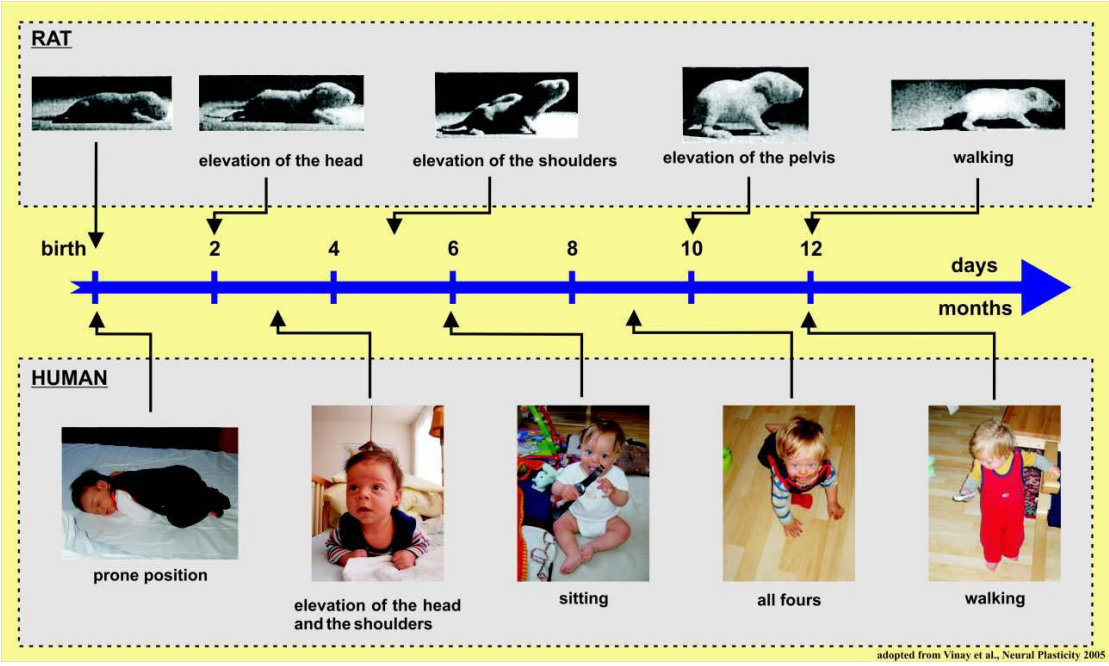


Table 3: Development of the human baby and rat pups (from poster in the Appendix)

5. Neonatal Brain Injury and Neurological Disorders

The brain can receive several different types of injuries and brain injury is unpredictable in its consequences. Neonatal brain can be affected by hypoxia-ischemia damages, strokes, traumatic injury, infections,... This thesis is focused on the focal ischemic damages. Previous works on neonatal brain injuries have been divided into studies with premature and full-term children, but most studies have combined both perinatal (from 28 weeks into the pregnancy to 7 days old) and neonatal (under 28 days old) events. (Nelson et. al., 2004) I will follow the frame line of premature and full-term children.

Clinical studies conducted with magnetic resonance imaging have supplemented post-mortem studies that demonstrate patterns of selective vulnerability in the developing brain at different ages. Developing white matter surrounding the ventricles is especially vulnerable to injury prior to 32 weeks gestation and injury to the white matter at this time causes the spastic diplegia form of cerebral palsy. In contrast, injuries later in the third trimester of gestation or shortly after term, are likely to damage the immature basal ganglia and motor cortex and spare the white matter. (Johnston, 1997)

Hypoxic-ischemic cerebral injury is one of the most commonly recognized causes of severe, long-term neurologic deficits in neonates such as cerebral palsy, epilepsy and seizures, motor and learning disabilities or death. These damages select regions of the immature brain at different ages and occur during the prenatal/neonatal stages of critical cellular or tissue differentiation process and have a serious impact on brain maturation. Insufficient supply of oxygen (hypoxia) and/or poor blood flow (ischemia) reaching particular area of the fetal or newborn brain lead to damage of the brain via activation of various cytotoxic agents and death pathways that induce neuronal injury and death. (Hossain, 2008) The etiology involved is multifactorial, complex and remain poorly understood.

It is generally accepted that hypoxic ischemic encephalopathy in term infants is usually associated with neurological abnormalities. Mild encephalopathy is characterized by hyperalertness, jitteriness and exaggerated primitive reflexes. Moderate encephalopathy is associated with lethargy, stupor,

hypotonia and suppressed tendon reflexes. Severe encephalopathy is associated invariably with coma, and brainstem and autonomic dysfunction. (Sato, 2003)

Arterial ischemic stroke is defined as cerebrovascular events that occur between 28 weeks of gestation and 28 days of postnatal age. Based on these results, neonatal stroke is recognized in approximately one of 4000 live births of full-term infants per year. Ischemic stroke after the first month of life occurs with an annual incidence rate of about one in 30 000 children. In other children, perinatal stroke is recognised only retrospectively, with emerging hemiparesis or seizures after the early months of life. (Nelson et. al., 2004) A stroke occurs when the blood supply to the brain is suddenly interrupted (ischemic stroke) or when a blood vessel in the brain bursts, pour blood out into the spaces surrounding the brain cells (hemorrhagic stroke). Arterial ischemic stroke can occur in infants born at any gestational age. Especially premature children with arterial ischemic stroke during early infancy often have complicated gestational and perinatal courses. They have additional neurological complications of prematurity in addition to their stroke, may have patterns of infarction that are rarely seen in term infants, have prolonged neonatal intensive care unit stays, and are frequently left with profound disability. Stroke appears to add to the impairments of prematurity and prematurity might add to the impairment due to very early stroke. (Golomb, 2007) Premature children are at high risk for neurologic disability even without arterial ischemic stroke, and the rate of disability increases dramatically as gestational age at birth drops. (Golomb, et. al., 2007) It is difficult to identify when these children clinically present with the first signs of stroke. Apnoea and seizures are common presenting signs of common complications of prematurity (respiratory distress syndrome, intraventricular haemorrhage, periventricular leucomalacia) and are also common presenting signs of arterial ischemic stroke. (Volpe, 2001)

There were presented the cases of 23 infants, born between 23 and 35 weeks gestational age, with focal arterial ischemic stroke occurring before 44 weeks gestational age in previous Golomb´s study. (Golomb et. al., 2007) The

most commonly affected territory was the middle cerebral artery territory. [2] Three children with extreme prematurity (<26 weeks) had cerebellar infarcts. Twelve children had unilateral or bilateral intraventricular haemorrhages. Twelve children had white matter injury (periventricular leukomalacia, hypoxic-ischemic encephalopathy, or both). All 22 children were left with different types of disabilities. (Golomb et. al., 2007)

The following table n.3 compares outcomes from three studies of neonatal stroke. In children <30 weeks gestational age at birth, 10% have cerebral palsy and 17% have severe cognitive impairment. (Marlow et. al., 2005) In children <26 weeks gestational age at birth, 46% have moderate severe disability. (Woodward, 2006) The rates of impairment in the Golomb´s study were even higher. For the group as a whole, no child was free of disability, >70% had cerebral palsy, >40% had moderate severe cognitive impairment and >80% had at least one moderate-severe disability. Children with an abnormal electroencephalogram during the first week after stroke typically develop hemiplegia. (Lynch et. al., 2002)

[2]There is a predominance of left hemisphere lesions, which may be caused by hemodynamic differences from a patent ductus arteriosus (Sreenan et. al., 2000) or a more direct route involving the left common carotid. The distribution of cerebral infarction differs somewhat with gestational age—preterm infants tend to have multifocal lesions involving the cortical or lenticulostriate branches of the middle cerebral artery, whereas full-term infants tend to have occlusions of the main branch. (De Vries, 1997)

Gestation age at birth	Cerebral palsy	Severe cognitive impairment	Moderate - severe disability	Epilepsy
<30 weeks (Marlow et. al., 2005; Woodward, 2006)	10%	17%	46%	-
Full term (Golomb et. al., 2007)	54%	22%	-	67%
Golomb´s study <30 weeks (Golomb et. al., 2007)	41%	18%	-	23%
Golomb´s study >30 weeks (Golomb et. al., 2007)	36%	23%	-	31%
Golomb´s study (All) (Golomb et. al., 2007)	77%	41%	>80%	54%

Table 4: Outcomes from three studies of neonatal stroke

The recurrence rate of neonatal stroke is much less than childhood stroke. In the Canadian Paediatric Ischemic Stroke Registry, the largest cohort of stroke in children, the recurrence rate of children with neonatal stroke was 3% to 5% and in survivors, outcome was normal in 33%. (Lynch et. al, 2002)

6. Epilepsy

Epilepsy is the collective name for a diverse set of disorders of the brain that have an abnormal predisposition to epileptic seizures. (Fisher, 2005) The definition of epilepsy includes the requirement of at least one seizure and the presence of an enduring alteration in the brain. (Fisher, 2005) Previous "classifications" of seizures and epilepsies were often treated as rigid doctrine. Advances in all areas of investigation (epidemiology, electrophysiology, imaging, developmental neurobiology, genetics, systems neurophysiology, and neurochemistry) have made it clear, however, that such a simple approach does not do justice to the complexity of the underlying developmental and physiological processes. (Berg et. al, 2009) Epilepsy syndromes or types of epilepsy can be classified into several groups. In the idiopathic epilepsy syndromes, the epilepsy exists without an underlying lesion or without any known brain disorder. (Fisher, 2005) Another classification of underlying causes will be grouped at genetic, structural/metabolic and unknown. (Berg et. al, 2009)

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

6.1 Seizures after Stroke

After an ischemic stroke, seizure occurrence exhibits a bimodal distribution. The first peak in seizure occurrence is within 2 weeks and the second peak is between 6 to 12 months after the stroke in human. About 40% of all epileptic seizures that are observed during the mean follow-up time of 9 months occur within the first day after the ischemic stroke and more than 20% of all seizures between 6 to 12 months. In contrast, after intracerebral haemorrhage, 60% of the seizures occur during the first 24 hours and after that time, the seizure occurrence declines. (Bladin et. al, 2005) The percentages of

patients suffering seizures and epilepsy have varied due to differences in data collection, patient inclusion criteria and follow-up time. To briefly summarize, seizures have been reported to occur from 3% to 15% and epilepsy from 3% to 14% of the patients with ischemic stroke in those studies with long follow-up times. (Karhunen, 2007; Karhunen et. al., 2007) After haemorrhage, 4% to 29% of the patients have been described as experiencing seizures and 3% to 15% develop epilepsy. (Karhunen, 2007; Karhunen et. al., 2007) The majority of patients with post stroke seizures have a lesion in the cerebral cortex with or without the involvement of the subcortical regions. (Bladin et. al, 2005)

6.2 Neonatal Seizures

The prevalence of neonatal seizures is approximately 1,5%. The overall **Chyba! Odkaz není platný.** is 3 per 1000 live births (58 to 132 per 1000 live births in pre-term infants). 18% occur in the first 1 to 2 days to the first week of life. The etiology of neonatal seizures is extensive and diverse, but hypoxic-ischemic encephalopathy is the most common cause (80% of all seizures in the first 2 days of life). (Panayitopoulos, 2007) Neonatal seizures are most commonly the clinical finding that triggers assessment, but in autopsy studies of infants who had ischemic cerebral infarcts, neonatal seizures were noted in 25–40% (Panayitopoulos, 2007), also despite of the high prevalence of neonatal seizures, epileptic syndromes in neonates are rare. (Nelson et. al., 2004) The incidence of epilepsy in premature infants with early stroke is not appreciably higher than in term children with perinatal stroke, but this may change as additional follow-up data are accumulated. (Golomb et. al., 2007) Four syndromes have been recognised by the The International League Against Epilepsy: 1. Benign familiar neonatal seizures, 2. Benign neonatal seizures (non-familiar), 3. Early myoclonic encephalopathy, 4. Ohtahara syndrome.

Despite high mortality (approximately 15%) and morbidity (approximately 30%), one half of neonates with seizures achieve a normal or near-normal state. Epilepsy has been developed in one third of the survivors. (Panayitopoulos, 2007) Epilepsy occurs in 15-60% of children with cerebral palsy. (Kwong, 2004) The retrospective study publicised by Kwong reviewed the prevalence, etiology and prognosis of epilepsy in cerebral palsy. Children with

cerebral palsy and epilepsy were compared with control epileptic children with normal neurodevelopment status during the same period. Children with cerebral palsy had a higher incidence of epilepsy with onset within the first year of age (47% vs. 10%), history of neonatal seizures (19% vs. 3%), status epilepticus (16% vs. 1.7%). They had a lower incidence of generalized seizures (28% vs. 59%) and remaining seizure free (37% vs. 90%). (Kwong, 2004)

Many neonatal seizures in infants with arterial stroke are focal and may occur in the absence of other signs of neonatal encephalopathy such as abnormalities of tone or feeding, or depressed level of alertness. (Nelson, 2004) Seizures are seen in infants with moderate or severe ischemic encephalopathy, who invariably have apparent neurological abnormalities. However, there were found the background EEG revealed remarkable abnormalities in infants, although they did not exhibit apparent neurological abnormalities. (Sato, 2003) In this study three term or near-term infants with seizures due to ischemic encephalopathy were without other neurological abnormalities. This report describes the clinical, laboratory, EEG and MRI features of these infants. The discrepancy between the EEG and clinical signs can be explained by the speculation that the participation of the cerebral cortex in movement or behaviour is relatively little in newborn infants. When the brainstem remains intact, the muscle tone can be normal and the primitive reflexes can be preserved even if cortical dysfunction occurs. (Sato, 2003)

Indicators of bad prognosis are the severe hypoxia–ischemia, the severe congenital malformations of cortical development and meningoencephalitis and the subtle and generalised tonic seizures. Intermediate prognosis is if moderately severe central nervous system infections or malformations, the most of the intracranial haemorrhages or infarctions, more serious metabolic CNS disturbances, frequent or prolonged clonic seizures and clonic status epilepticus and EEG persistence of immature patterns occur. (Kwong, 2004)

7. Epileptogenesis

Epileptogenesis is a transformation of a non-epileptic neural network to a seizure-generating network. It is also defined as the process of developing epilepsy—a disorder characterized by recurrent seizures following an initial insult. (Choi et. al., 2008; Rakhade et. al., 2009) In the case of acquired epilepsies, epileptogenesis has classically been divided into three phases: the inciting event (analogous to a mutation of an important gene or perhaps a cerebral malformation in the case of genetic epilepsies), the silent period when epileptogenesis takes place, and, finally, the onset of spontaneous recurrent seizures. (Bender, 2007)

Animal models of epilepsy and human tissue studies suggest that epileptogenesis involves a cascade of molecular, cellular and neuronal network alterations. Within minutes to days following the initial insult, there are acute early changes in neuronal networks, which include rapid alterations to ion channel kinetics as a result of membrane depolarization, post-translational modifications to existing functional proteins, and activation of immediate early genes. (Bender, 2007; Rakhade et. al., 2009). Subacute changes occur over hours to weeks, and include transcriptional events, neuronal death and activation of inflammatory cascades. The chronic changes that follow over weeks to months include anatomical changes, such as neurogenesis, mossy fibber sprouting, network reorganization, and gliosis. (Rakhade et. al., 2009) A variety of cerebral changes occur during epileptogenesis such as neuronal damage, gliosis, axonal and dendritic sprouting and alterations in the extracellular matrix and the induction of inflammation. (Karhunen, 2007; Karhunen et. al., 2007) These epileptogenic processes are developmentally regulated and might contribute to differences in epileptogenesis between adult and developing brains. (Rakhade et. al., 2009)

7.1 The Role of Inflammation in Epileptogenesis

Many scientists try to determine whether the degree of inflammation resulting from an initial neonatal seizure determines whether new blood vessels with a weakened blood-brain-barrier (BBB) function are formed and if these phenomena are important for chronic epilepsy to occur. Recent evidence indicates that inflammatory reactions are a common element in people with various types of epileptic disorders from different causes. (Bender, 2007; Rakhade et. al., 2009) Can brain inflammation be a common factor contributing or predisposing to development of seizure-generating neural networks?

Although the central nervous system used to be considered an immune-privileged system due to the presence of the BBB, graft acceptance, a lack of conventional lymphatic drainage, and relatively low levels of monocytes and lymphocytes, it is becoming clear that immune and inflammatory reactions do occur in the CNS, either intrinsically from the brain itself or acquired from systemic circulation through a damaged BBB. While the role of inflammation in the pathophysiology of human epilepsy remains hypothetical, inflammatory and immune reactions in the brains do occur in human epilepsy patients and in experimental models of epilepsy. (Choi et. al., 2008; Ravizza et. al., 2004)

The researchers hypothesize that conversion of brain cell circuits from normal to seizure-generating depends upon the extent of inflammatory reactions in the brain following an inciting event and on development of new blood vessels in the brain with a weakened BBB function, thus being more permeable to blood components usually excluded from the brain. (Choi et. al., 2008) There are the researches exploring and determining the first part of this hypothesis, whether brain tissue inflammation alters neuronal excitability, and promotes conversion of neural circuits to seizure-generating networks. Also the subsequent studies focus on determining whether an inflammation-related “cytokine” (called IL-1beta) triggers formation of new, more permeable blood vessels which weaken the BBB and promote brain cells’ excitability and vulnerability to damage. (Choi et. al., 2008; Ravizza et. al., 2004; Ballabh et. al., 2004)

7.2 Blood-brain-barrier (BBB)

The BBB consists of endothelial cells with interendothelial tight junctions, and its maintenance depends on normal functioning of pericytes, perivascular microglia, astrocytes, and the basal lamina. Under normal conditions, the BBB protects the CNS by regulating the entry of plasma-born substances and immune cells. Astrocytes are thought to act as important regulators of the balance between endothelial stability and permeability of the BBB. Transient changes have been demonstrated in the physiology and structures of the BBB in various CNS injuries such as status epilepticus, infections, and traumatic and ischemic events.http://www.ncbi.nlm.nih.gov/pubmed/15207256 (Ballabh et. al., 2004) An impaired BBB and inflammatory state are common features of neurological diseases associated with the late onset of epilepsy.

In many animal models of epilepsy, acute seizures cause glial activation and increased expression of transcription factors and cytokines that coordinate inflammatory responses. The activated glia and elevated cytokines contribute to seizure-related hippocampal pathology, such as neuronal death, neuronal birth, reactive gliosis, and mossy fibber sprouting. (McNamara, 1994) Accumulating experimental data also suggest that seizure-induced glial activation and up-regulation of pro-inflammatory cytokines can lead to neuronal excitability and neuronal injury either directly, by interacting with glutamatergic neurotransmission, or indirectly, by activating gene transcription. (Choi et. al., 2008)

Widespread microglial activation accompanied by neuronal injury occurs after acute seizures. As a rapid response, activation of microglia may be responsible for neurodegeneration rather than a consequence of neurodegeneration. Neuronal injury is detected 12 - 24 hours following status, many hours after cytokines are induced in the glia. (Ravizza et. al., 2005) Status epilepticus, prolonged seizures over 30 minutes, can cause neuronal death through glutamate-mediated excitotoxicity, necrosis, and activation of apoptosis. http://www.ncbi.nlm.nih.gov/pubmed/17371290 (Henshall, 2007) One to three days after status epilepticus, both neuronal and astrocytic death are observed in the dentate hilus within the hippocampus. (Henshall, 2007) Injured neurons and glia and their fragmented DNA are rapidly cleared by activated

microglia. These findings suggest that microglial activation associated with inflammation induces neuronal injury and suppresses neurogenesis. The occurrence of spontaneous seizures has been correlated with the extent of glial activation, as well as astrocyte and neuron degeneration in the hippocampus, and blockade of neuronal death failed to prevent epileptogenesis. (Choi et. al., 2008) Proinflammatory cytokines are elevated in experimental animal brains after ischemia and in the CSF from stroke and epilepsy patients. Astrocytes are a critical component of the BBB and have many important roles in glutamate and potassium uptake and the production of growth factors, cytokines, and extracellular matrix proteins. In response to immunologic challenges or brain injuries, astrocytes proliferate and become hypertrophic and fibrillary. (Henshall, 2007; Choi et. al., 2008)

Modulation of inflammatory reactions in the brain and targeting of inflammatory mediators may be effective therapeutic strategies to prevent or limit epileptogenesis in the vulnerable nervous system. (Choi et. al., 2008)

7.3 Inflammation and Brain Ischemia

Neuroinflammatory mediators play a crucial role in the pathophysiology of brain ischemia – including either deleterious effects on the progression of tissue damage or beneficial roles during repair and recovery. Within hours after the ischemic insult, increased levels of cytokines and chemokines enhance the expression of adhesion molecules on cerebral endothelial cells, facilitating the adhesion and transendothelial migration of circulating neutrophils and monocytes. These cells may accumulate in the capillaries, further impairing cerebral blood flow, or extravasate into the brain parenchyma. Infiltrating leukocytes, as well as resident brain cells, including neurons and glia, may release pro-inflammatory mediators, such as cytokines, chemokines and oxygen／nitrogen free radicals that contribute to the evolution of tissue damage. (Amantea et. al., 2009)

A few minutes after the onset of ischemia, tissue damage occurs in the centre of ischemic injury, where cerebral blood flow is reduced by more than 80%. In this core region, cell death rapidly develops as a consequence of the

acute energy failure and loss of ionic gradients associated with permanent and anoxic depolarization. (Hossmann, 1994) A few hours later, the infarct expands into the penumbra, an area of partially preserved energy metabolism, as a result of peri-infarct spreading depression and molecular injury pathways that are activated in the cellular and extracellular compartments. At this stage, cellular damage is mainly triggered by excitotoxicity, mitochondrial disturbances, reactive oxygen species production and programmed cell death. (Kriz, 2005) The evolution of tissue damage further perpetuates for days or even weeks as a result of secondary phenomena such as vasogenic edema and delayed inflammatory processes. (Balabh et. el., 2004)

There is increasing evidence demonstrating that neuroinflammatory processes play a pivotal role in the pathophysiology of brain ischemia. The inflammatory cascade is characterized by an immediate phase, which is initiated a few hours after stroke and may last for days and weeks as a delayed tissue reaction to injury. In addition to their deleterious contribution to ischemic tissue damage, inflammatory mediators may also exert beneficial effects on stroke recovery. (Amantea et. al., 2009)

8. The Diagnostic Methods

The assessment of neonatal patients with stroke includes a detailed history with questions regarding maternal disorders, pregnancy disorders (preeclampsia, history of fetal loss, placental abruption and haemorrhage), birth history, placental pathology, family histories of neurological disorders, premature vascular disease such as early myocardial infarction or stroke, deep venous thrombosis, and haematological disease. (Nelson et. al., 2004)

Cranial imaging procedures for neonatal stroke include MRI, CT and ultrasonography. By using the characteristic signal features for perinatal ischemic infarction from MRI diffusion-weighted imaging scanning, it is possible to diagnose prepartum, intrapartum, or postpartum ischemic stroke during the newborn period retrospectively and thus more precisely define the pre-, intra-, or postpartum factors that contribute to perinatal ischemic stroke. (Huppi et. al., 2006; Chun-Shan Wu et. al., 2008) Magnetic resonance angiography is also

useful for the detection of occlusion and hypoplastic vessels. If MRI is unavailable or not possible for a sick newborn, CT or ultrasonography should be considered. (Nelson et. al., 2004) The neonatal EEG is probably one of the best and most useful diagnostic method. EEG should be done in the first 24 hours, as some neonates with stroke will have seizures early in the postnatal period. (Nelson et. al., 2004) However, neonatal EEG recordings and interpretations require the special skills of well trained technologists and physicians. (Panayitopoulos, 2007)

In some infants not thought to be neurologically ill as neonates, perinatal stroke can be diagnosed in later months after with failure to reach developmental milestones such as rolling over, asymmetry and slow to reach and grasp, sitting up, crawling, walking and talking, or post neonatal seizures. There are also often the changes of muscle tone, lethargy or lack of alertness, irritability or fussiness, trembling of the arms and legs, poor feeding abilities secondary to problems sucking and swallowing, one side predilection, abnormal reflexes or eye fluttering. (Nelson et. al., 2004) A specialised motor examination may identify affected infants early. **Vojta's diagnostic method** is very sensitive in detecting injury of the central nervous system early in life, high correlation was found between cerebral palsy and asymmetry of the body, but not of the head. (Gajewska et. al, 2006) Method of Vojta is based on integrated system of early neurokinesiologic diagnostics and on the reflex locomotion. A complete assessment including maternal testing for coagulation abnormalities, assessment of the placenta, metabolic and coagulation tests, cardiac imaging, urine toxicology screening, lumbar puncture etc. can provide information useful in determining recurrence risk.

9. Previous Studies with Experimental Animals – Focal Brain Lesion and Epilepsy

An initial injury, such as a stroke, can trigger changes that alter the neural network and make it more prone to seizures. The process by which the normal brain is altered to a seizure prone state is called “epileptogenesis”, and it can continue progressively from several days to even years after the initial insult. A variety of cerebral changes occur during epileptogenesis such as neuronal damage, gliosis, axonal and dendritic sprouting, alterations in the extracellular matrix and the induction of inflammation. (Karhunen et. al., 2007)

In addition to neuronal damage, neurogenesis has been associated with the network plasticity following seizures in adult rodents. However, it was suggested recently that the connectivity of new neurons can develop in order to reduce the dysfunction in the epileptic brain. Both axonal and dendritic sprouting are also observed in association with seizures. (Karhunen, 2007; Karhunen et. al., 2007)

Focal cerebral ischemia results usually in dying neurons within an ischemic core that is surrounded by a penumbral zone in which neurons have the potential to survive. In addition, glial cells accumulate at the lesion border, prominent inflammation is present and neuronal reorganization and dendritic sprouting occurs. (Karhunen, 2007)

Present findings suggest that the lesion size and depth in the cortex can vary between epileptic animals, means that lesion extent and depth were similar in epileptic and non-epileptic rats with photothrombosis. Development of epilepsy was accompanied by light mossy fiber sprouting in the inner molecular layer of the dentate gyrus, indicating that not only the cortex but also the hippocampus might be affected by post-stroke epilepsy. (Lynch et. al., 2002) Scientists also observed that studies may produce unrealistically favourable results – for example if researchers typically use young, healthy rats with very specific focal damage to a particular brain structure producing comparatively mild motor impairments, for study stroke in human adults. (Kleim et. al., 2007)

9.1 Previous studies

Most of the previous studies of inducing epilepsy were done in adult rats. They have used various types of model of focal brain ischemia. Following text tries to review the results. A distinction is often made between early- and late-onset seizures. Early-onset seizures occur within the first or second week after stroke, but most of them occur within the first 24 hours. Late poststroke seizures occur after a latency period lasting from several months to years after the insult. (Epsztein et. al., 2008)

The occurrence of early and late seizures has been described after the intraluminal filament model of MCAO and after the combined occlusion of MCA and common carotid artery. Early seizures occur after transient and permanent MCAo with the intraluminal filament model in rats. After transient MCAO, about 80% of the rats demonstrate non-convulsive seizures, which occur approximately 25 min following the occlusion and last about 2 minutes. (Williams et. al., 2002) After the permanent MCAO, about 90% of the rats demonstrate non-convulsive seizures, which appear approximately 30 to 60 minutes following the occlusion and last, on average, for 50 to 70 seconds The majority of the early seizures occur during the first 2 hours after the MCA occlusion in rats. (Williams et. al., 2002) The occurrence of late seizures after large artery occlusions seems to depend on the age of the rats at the ischemic induction. In 2.5-months old Long-Evans rats, the combined occlusion of the MCA and CCA does not result in late epileptic seizures during the 6-month long follow-up period. (Kelly, 2007) In contrast, 25% of 4-month old rats and 100% of 20-month old F344 rats exhibit epileptic seizures. (Kelly, 2007)

Although the occurrence of early seizures has not been described in the cortical photothrombosis model, the occurrence of late seizures has been studied in more detail. In two month old rats, photothrombosis evoked late seizures in about 50% of the animals. (Kharmalov et. al., 2003) The duration of late seizures was reported to range from 2 to 319 seconds. (Kharmalov et. al., 2003) Late seizures were observed as early as the first recording day, which was 26 days after the cortical lesion had been made with photothrombosis. The latency time between photothrombosis and seizure, however, can also be

greatly delayed, these workers observed seizures occurring 181 days after the operation. (Kharmalov et. al., 2003) Seizures in other models of cerebral ischemia are described by Karhunen. (Karhunen, 2007; Karhunen et. al., 2007)

9.2 Studies with the Rat Pups

It was reported that injection of endothelin into the hippocampus results in the appearance of early seizures in 75 - 100% of the P12-P15 pups. (Mateffyova et. al., 2006) Three months after the operation, non-convulsive seizures are observed in 63-100% of the rats. (Mateffyova et. al., 2006) After Levine's model of hypoxia-ischemia, 40% of the seven days old rats develop spontaneous motor seizures. (Williams, 2004) The mean latency time from the operation to spontaneous motor seizures is 194 days and the animals exhibit one seizure every 4 to 5 days. (Williams, 2004) Thus in neonate rats a focal lesion in the hippocampus or global cerebral ischemia can induce epilepsy in later life.

Understanding the mechanisms by which early-life epilepsy develops would be very helpful. Then we would be able to design selective preventative or interventional strategies. Available possibilities such as global interruption of excitatory mechanisms can block seizure-evoked excitotoxic processes and this approach may not be suitable to the developing brain because it interferes with normal neuronal function. (Bender et al., 2007) If we understand these specific mechanisms for epileptogenesis we are able to design selective blockers, ideally without disruption of central nervous system maturation and function. (Bender et al., 2007)

PRACTICAL AND RESEARCH PART

The Aim of Work

The aim of this diploma thesis was to establish the model of focal brain ischemia induced by photothrombosis in P7 rat pups and to work on the following:

- determination of location and extension of the lesions in the brains
- determination of volumes of the lesions
- evaluation of sensorimotor tests made two and half month after inducing lesions
- description of the EEG made four month after inducing lesions

Hypothesis

I. With the defined parameters of laser duration, time of irradiation, location and placement the lesions in the brains will be identical

II. P7 animals after cortical photothrombotic ischemic stroke will be less skillful in sensorimotor tests made in their adultness

III. The focal ischemic brain injury in the P7 rat pups can lead to epileptogenesis

10. Experiments

10.1 Animals

The animal care and research procedures were carried out in accordance with the notice for working with experimental animals (265/19 Sb.) and the experimental protocol was approved by the Ethics Committee of Institute of Physiology AS CR. Animal were kept on a 12-hour light/12-hour dark cycle, temperature 22 ± 1°C and provided food and water ad libitum.

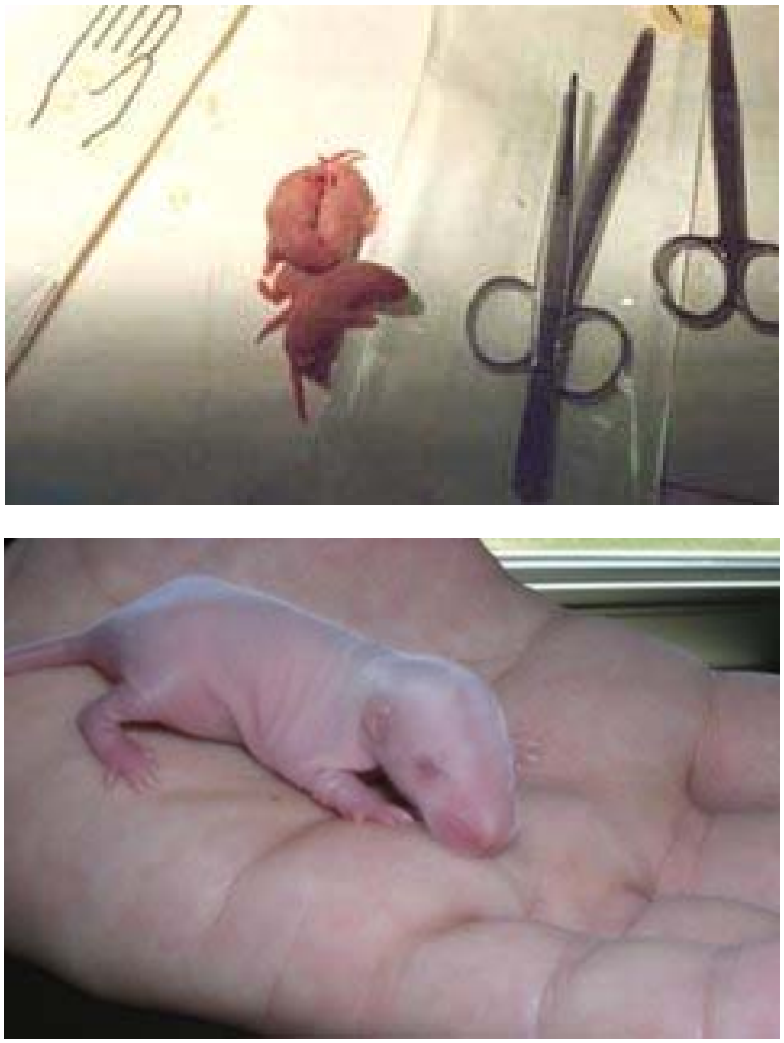


Figure 6: P7 Rat pups (photographies from experiment)

10.2 Inducing the Focal Brain Lesion

On postnatal day 7, rat pups (n=10) were systemically anesthetized using isoflurane (induction 2 - 4,5%) and were injected with fresh bengal rose dye (BRI 10mg/5ml, dosage 0,15ml) into right vena jugularis. An incision was sutured with one or two sutures and tissue glue was used. The scalp was incised sagitally in the middle and retracted to expose the skull. No craniotomy was done, because the skull of P7 rat pup is translucent to the green laser light. We used green laser light (50mW, Roithner Laser, Austria) over the sensorimotocortex for 5 minutes at 0,5s on/off cycles. The centre of the light beam was focused on rostrocaudal 0 mm, means bregma and 2 mm lateral to bregma corresponding to the posterior somatosensory and motor areas. The incision of the skull was sutured and glued immediately after irradiation. Animals were allowed to recover from anaesthesia on a thermic blanket and were returned to the home cage to the dam. Animal weight was regularly recorded then. To assess the motor deficit, rotarod walking test and bar holding test were performed in approximately two months later after stroke.

10.3 Testing of Sensorimotor Abilities

For evaluation of neuromuscular function in two and a half months old male rats after focal ischemic stroke at their age of seven days the following tests were used: the rotarod walking test and the bar holding test.

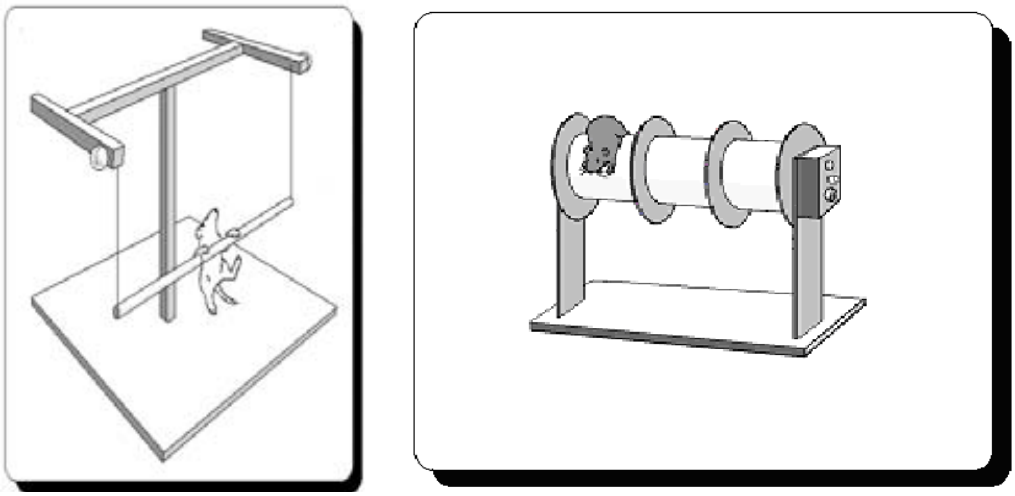


Figure 7: Bar holding and Rotarod tests (from poster in Appendix)

The rotarod test is used for testing of motor skills and motor learning of animals. Two relevant behavioural strategies, grasping and walking used by rats to maintain their equilibrium can be noted. Grasping behaviour consisted for the animals to grasp on the rotarod and to be passively rotated, the animals being motionless while the rod is rotated. Walking behaviour consisted for rats to walk asynchronously or synchronously upon the rotarod. Motor skills are pure motor abilities of animals; motor learning refers to a recalibration of fine turning of particular behaviour. The apparatus consisted of a horizontal rod (10 cm in diameter, 11 cm long) covered with sticking plaster in order to increase its roughness. The apparatus was placed 30 cm above a landing platform. It was programmed to rotate at five rounds per minute. Rats were placed on the rotating rod with their heads against the direction of rotation. The duration of their stay on the rod was measured for 120 s at maximum. The test was performed three times in a close succession. The mean of the three times was taken as a score. The rats where filmed from a frontal view, as they walked on the rotarod behaviour was analysed by frame-by-frame relay of the video records. In order to evaluate the differences in walking patterns between control and experimental rats the video records was examined by two independent observers blind to the experimental conditions. (Whishaw, 2005)

For the bar holding test a wooden bar 25 cm long, 1 cm in diameter was suspended 25 cm above a padded soft surface. An animal is to grip a horizontal bar with its forepaws only and released suspended. It is considered to have passed the test if it succeeds in lifting its body to touch the bar with both hind-paws within a pre-set period. Both the time during which the animal kept hanging without falling and the number of paws used for the purpose are recorded. Is important to write down, if the animal holds itself with both forepaws or only one forepaw or pulls up and supports with one or two hind-paws or whether it is unable to grasp the bar. Time of fore- and hind-limb grasping within the 120 s was recorded. (Whishaw, 2005)

10.4 EEG Monitoring

Photothrombosis was induced in P7 rat pups (n=10, seven experimental and three controls) of which eight rats underwent EEG monitoring (two groups of three experimental and one control animal). The animals were underwent implantation of the silver EEG cortical and hippocampal electrodes to record electrical activity of the brain followed by a week of recovery. Electrodes were implanted four months after photothrombosis. Animals were anesthetized using isoflurane (1 - 2,5%) The scalp was incised sagitally in the middle line and all soft tissues were retracted around and cleaned to expose the skull. Then six holes were drilled for epidural placement of electrodes. The electrodes were placed over the motor cortex into the epidural spaces (0 mm rostrocaudal and 2mm lateral). To monitor hippocampal activity insulated silver wire was stereotactically introduced into the CA3 region of the hippocampus (4 mm rostrocaudal, 4 mm lateral, 4 mm ventral). Reference and ground electrodes were implanted into the occipital bone over the cerebellum. The electrodes and connector were fixed to the skull by means of dental acrylic duracrol together with anchoring screws mounted into the nasal and occipital bone to secure the headset in place. EEG monitoring for 4-5 consecutive days have been done after a week of recovery (Fig. 8, Fig. 9).

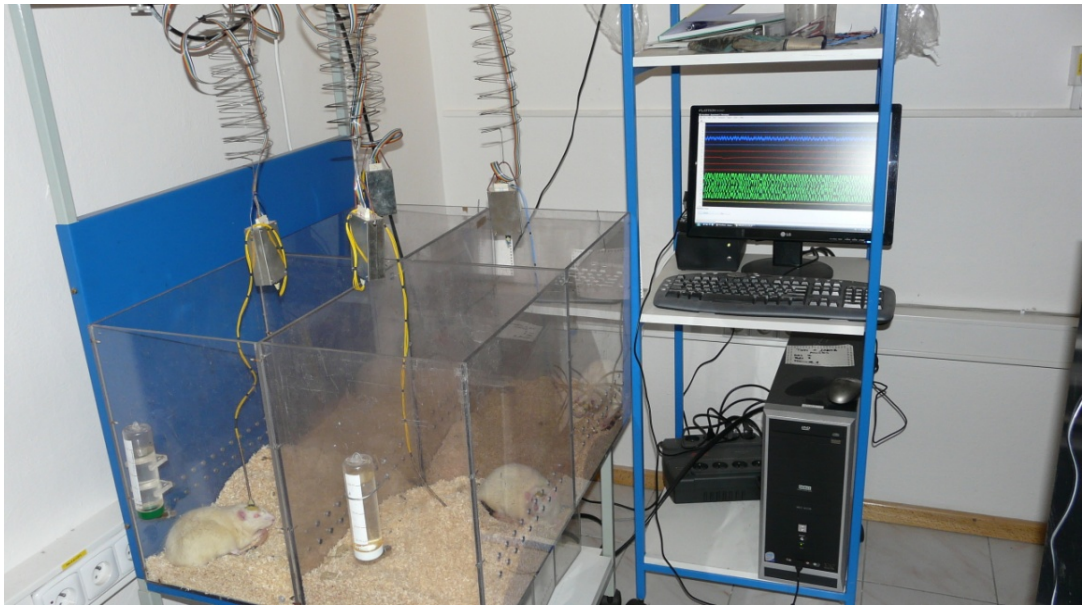


Figure 8: EEE monitoring (photography from experiment)

10.5 EEG Monitoring Protocol

The video-EEG was recorded continuously for 4-5 day. EEG was collected using the two recording systems with two groups of animals. The first group (three experimental and one control animal) were monitored with the older EEG Pentusa system (TDT, USA), the second group (with another three experimental and one control animal) were monitored using newer VisionBrain (FGU AVCR, Czech Republic). The behaviour of the animals was recorded using a video camera (MSI StarcamRacer) that was positioned above the cages. An infrared light was used at night to allow visibility continuously for 24 hours/day.

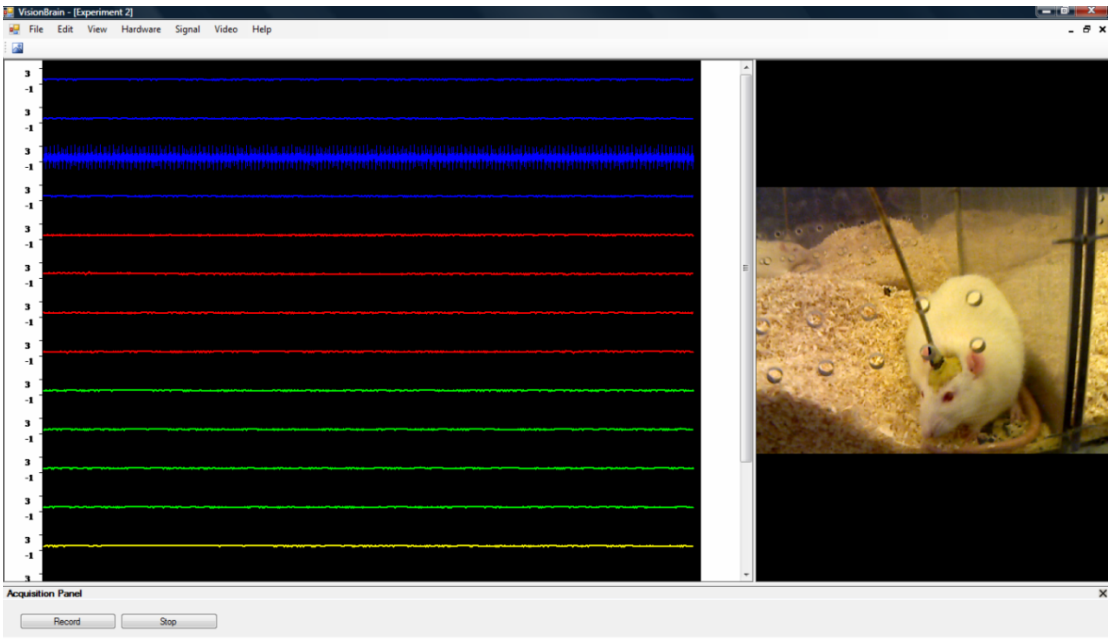


Figure 9: EEG monitoring and analysis (from poster in Appendix)

10.6 Histology

The fixation of the tissues is needed for the histology. Perfusion technique is used to aid extraction of the brain and spinal cord from a dead animal prior to histological analysis. The aim of the fixation is a quick and regardful of protein denaturation of the cell protoplasm and of structures of the tissues. The standard procedure is to inject the animal with a lethal dose of urethane (2,5 g/kg, i.v.). Immediately after death, the heard is exposed and a cannula inserted into the left ventricle and the right auricle is cut. Fluid passed through the cannula circulates around the body via the open right auricle. A neutral fluid is initially perfused: physiological saline or some other appropriate fluid. This removes the blood from the body. After this, fixative fluid appropriate to the histological procedures is perfused (paraformaldehyde in 0,1 M phosphate powder, pH 7.4 (1 ml/ g), $+4^0$C, for 10 minutes). This procedure has the effect of fixing the brain in place: brain can be easily removed from the skull. Then the brain is fixated for next three hours in paraformaldehyde solution.

Then the prepared brains were put in the cryprotective solution of saccharose (10, 20 a 30%) in +4°C and then frozen in dry ice at -70^0C. The brains were cut into 50μm slice with Cryocut Leika 1600 and put in 30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate powder) for following histological staining.

10.7 Nissle Staining

Nissl is a term used by classical cytologists for the endoplasmic reticulum. Since all cells contain the endoplasmic reticulum, a Nissl stain (cresyl violet) will stain all cell bodies in the brain. The staining procedure consists of sequentially dipping the mounted brain slices in different solutions for specified amounts of time. An initial alcohol baths remove the lipids and fixative from the tissue, the sections are stained, followed by dehydrating the tissue by a series of alcohol baths. The Nissl stain is used widely in many research labs to examine the overall morphology of the brain. It was used to verify the location of a lesion, to determine the infarct size and depth and to assess the severity of neuronal damage. (Goble, 2010)

Protocol: Place staining dishes in fume hood and label them in order 95% ethanol, 70% ethanol, 50% ethanol, distilled water, distilled water, cresyl violet stain, 50% ethanol, 70% acid ethanol, 95% ethanol, 100% ethanol. Turn the fume hood and put on slides. Insert dishes into slide racks. Pass the slide racks through the following sequence of bath for the appropriate time indicated:

1. 95% ethanol 15min
2. 70% ethanol 1min
3. 50% ethanol 1min
4. distilled water 2min
5. distilled water 1min
6. cresyl violet stain 5min
7. distilled water 1min
8. 50% ethanol 1min
9. 70% acid ethanol 2min
10. 95% ethanol 2min
11. 95% ethanol a few dips
12. 100% ethanol 1min

10.8 Measurement of the Lesions´Volume

The characterization of lesion location in the brain is an important step in studying brain function. Putative brain centres or networks that control motor, sensation and cognition can be assessed by correlating deficits with lesion location across a group of subjects. So after adjustment of contrast, the brain lesions were described with the help of neuroanatomy atlas with the microscope, then the pictures of the slices were taken (microscope Olympus AX70 with epifluorescence and cooled CCD camera Olympus DP-70). The contours of the hemispheres were traced manually on each slice (Fig.10) in the program Ellipse. The position of the midline was determined using neuroanatomic landmarks. Volumes of the ipsilateral and contralateral hemispheres and of the lesion were calculated by summing up the volumes from all slices (program SigmaStat s SigmaPlot).

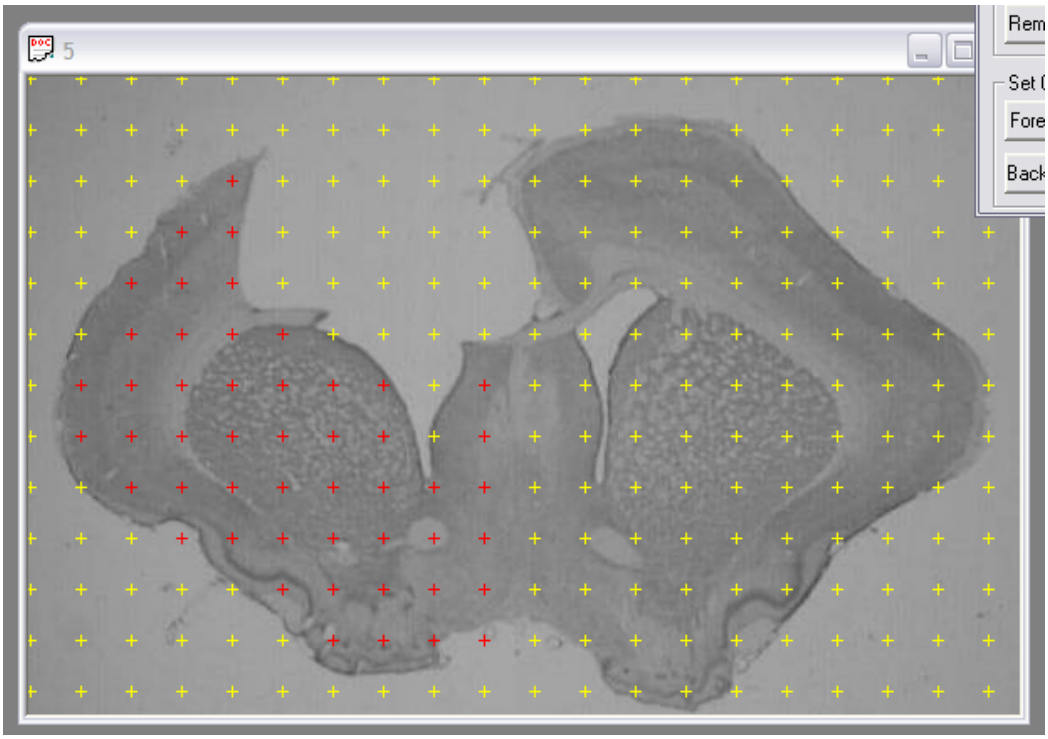


Figure 10: Tracing the contours of the hemispheres on each slice

10.9 EEG Analysis

To detect spontaneous seizures, the EEG was able to be visually analyzed on a computer screen (EEG Viewer for Matlab, Jakub Otáhal 2005-10). An electrographic seizure was defined as a paroxysmal discharge with rhythmic repetitive waveforms that lasted for at least 5 s, had a clear beginning and ending, and temporal evolution in amplitude and frequency. Number, duration, and frequency of seizures (number of seizures/number of video-EEG monitoring days) will be calculated. If electrical seizure activity is observed on the EEG, the behaviour of the rat is assessed from video recordings. The control rats underwent similar video-EEG follow-up as the animals with cortical photothrombosis.

The classification proposed by the International League Against Epilepsy and the International is use to define whether the rat was epileptic or not. Epilepsy can be considered if there is an enduring alteration in the brain and at least one seizure.

11. Results

Prior to photothrombosis laser duration, time of irradiation, location and placement of the lesion had to be specified. Therefore there were fifth other previous experiments. The *first experiment* for determination the size lesion, duration of laser beam and the time of irradiation was on the 18th November 2008. In this first experiment, the focal lesions were induced to P7 rats (n=5), with the time of laser irradiation at 5, 10, 20, 30min (one rat pup died during operation) using green laser light (50mW, Roithner Laser, Austria) over the sensorimotocortex for 5 minutes at 0,5s on/off cycles. On the25th November 2008, the second identical experiment has been done. Due to results of the final size of lesions after these experiments, laser irradiation time was determined for 5min. There were also several problems: experiment from the 5th December 2008 was not use for this work, because of faulty with numbering of animals during time. On the 16th of December 2008 animals died due to heat-over rating from the heating pad, only two of them were used for perfusion and histology after 6 hours (acute ischemic histology). The next experiment on the 13th of January 2009 was a preliminary study aiming at correct inducing of photothrombotic stroke and also EEG monitoring at all.

The last two experiments included a *control groups* that underwent the same protocol as the rats with cortical photothrombotic stroke. Surgical operations were similar as the induction of photothrombosis performed (including injection of saline solution dye, laser lightening and electrode implantation). Because of the saline solution instead of the bengal rose was used on photoactivation leading to ischemia did not occur. This work includes only the last experiment (operation for inducing the lesions was on the 23th April 2009).

11.1 Determination of location and expansion of the lesion in the brain

Histological examination of the brains showed significant ischemic brain damages. The lesion size, localization and depth in the brain vary remarkably between animals. The lesions of experimental animals (n=7) were located in the left hemisphere, vary from small lesions in somatosensory cortex (n=2), to lesions in almost whole left hemisphere (n=3). The rest of lesions (n=2) had been always effected corpus callosum, parts of hippocampus and the underlying white structures of the brain. After 5 minutes surface blood vessels irradiation the focal ischemic lesion is often large (n=5) and extends to the subcortical white matter damaging the corpus callosum and encroaching on the lateral ventricles. The next picture examples demonstrate the brain injury after photothrombosis.

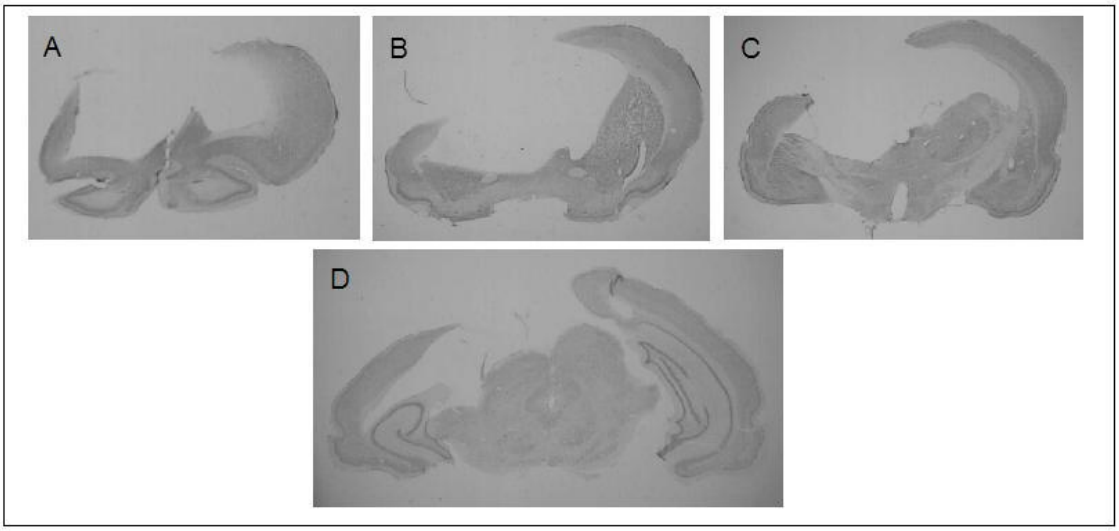


Figure 11: BRI 39: The large lesion

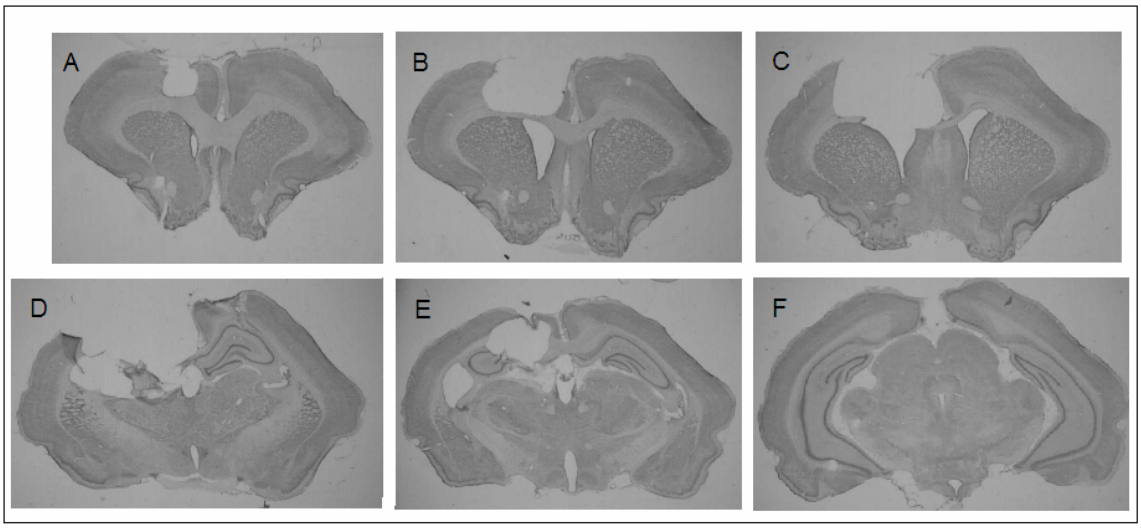


Figure 12: BRI 35: The medium lesion

The last picture demonstrates the control animal without lesion. Control animals underwent the same protocol as the experimental, surgical operations were similar as the induction of photothrombosis was performed; including injection of saline solution dye, laser lightening and electrode implantation. Because of the saline solution instead of the bengal rose was used on photoactivation leading to ischemia did not occur. The lesion occurred in the right hemisphere is caused by EEG wires using for EEG monitoring.

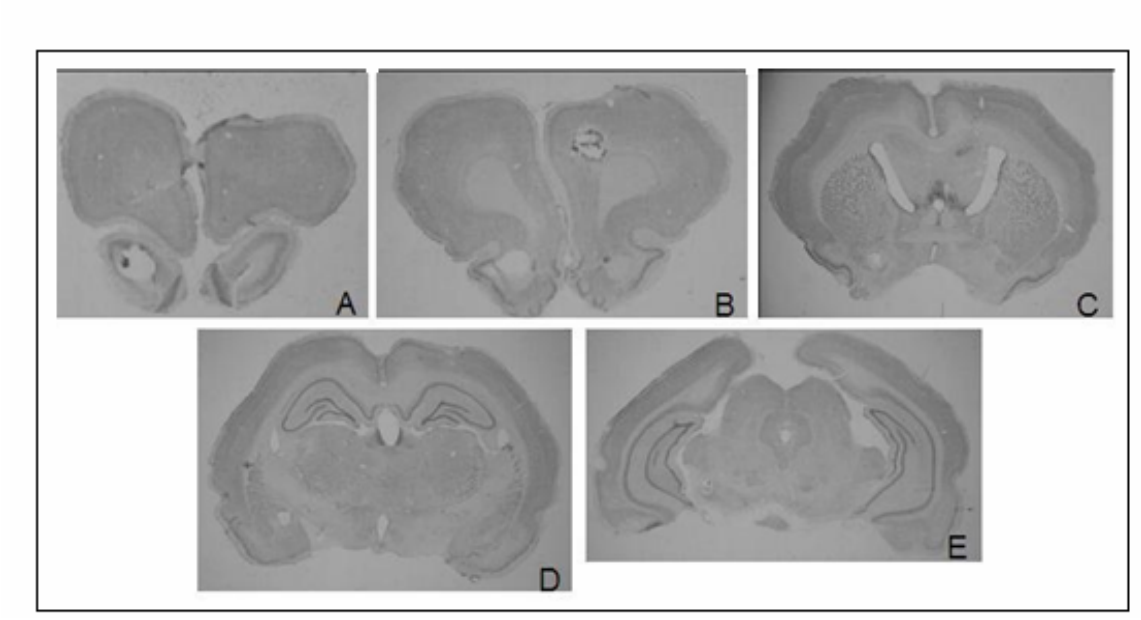


Figure 13: Control animal

There are picture example selections and description of the lesions of all the experimental animals (n=7). Pictures were chosen to demonstrate the range of lesion and approximate location.

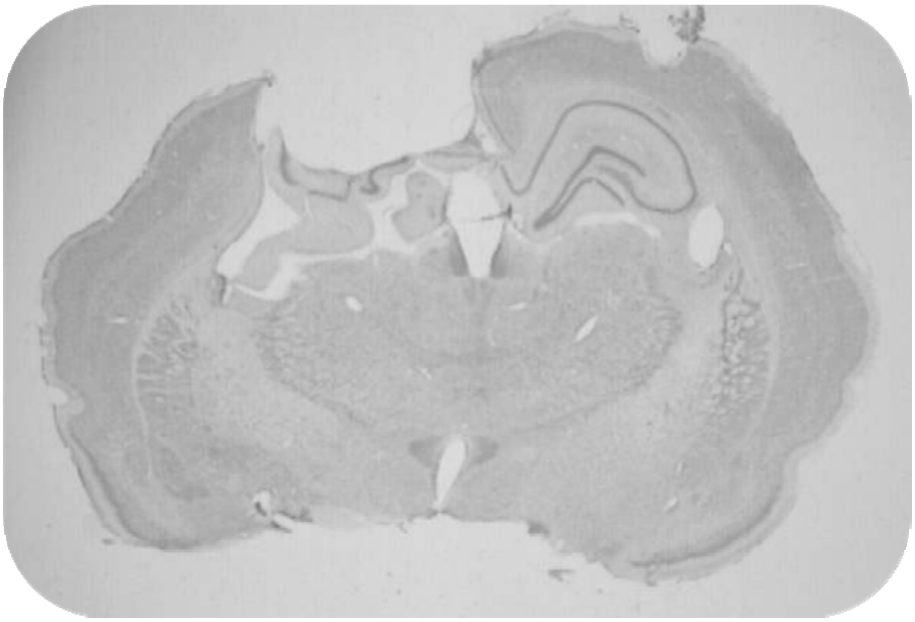


Figure 14: BRI 33: The lesion is in the left side, starts in primary and secondary somatosensory cortex, caudate putamen (striatum), primary motor cortex, lateral ventricle, then internal capsule cingulate cortex, prelimbic cortex, corpus calosum. In the sagitally view lesion spreads from the frontal brain and ends in from of the cerebellum.

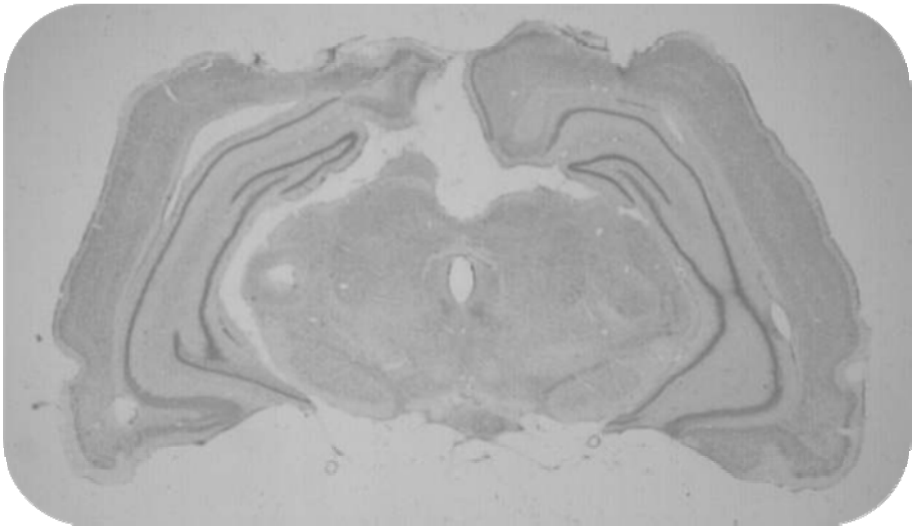


Figure 15: BRI 34: Small lesion in the left hemisphere above thalamus and midbrain, lesion is not in somatosensory cortex, affects primary and secondary visual cortex, and parietal associal cortex.

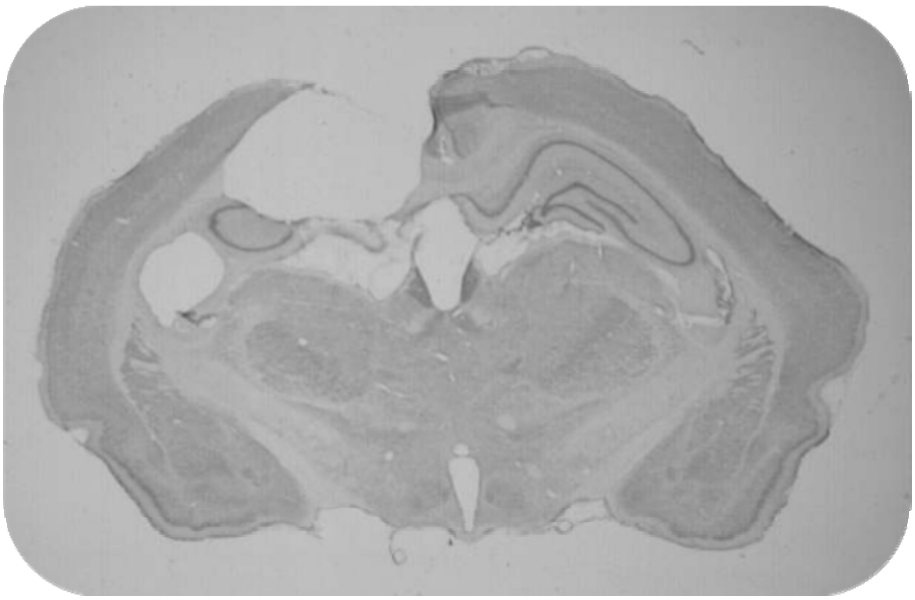


Figure 16: BRI 35: Lesion is in the left side, it starts at secondary motor cortex, spreads deeper and widen, affects genu of the corpus callosum, caudate putamen (striatum), corpus calosum is broken. Then it affects field CA3 of hippocampus, primary somatosensory cortex, parietal association cortex forceps major of the corpus callosum, hippocampal fissure, cingulum, ending in the line with the fourth ventricle.

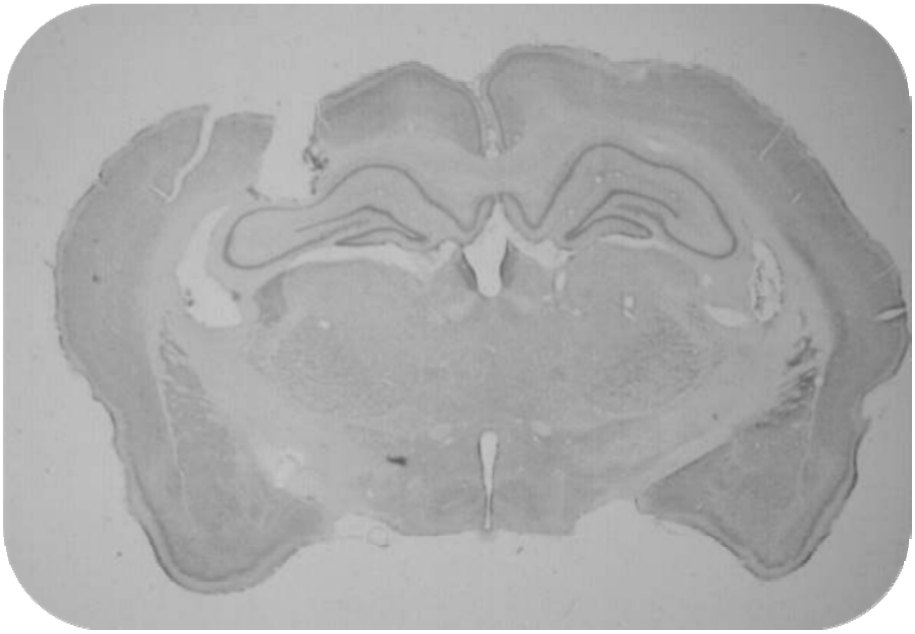


Figure 17: BRI 36: Lesion start in primary somatosensory cortex in the left side, spreads deeper to the corpus callosum, pariental cortex, field CA1 of hippocampus.

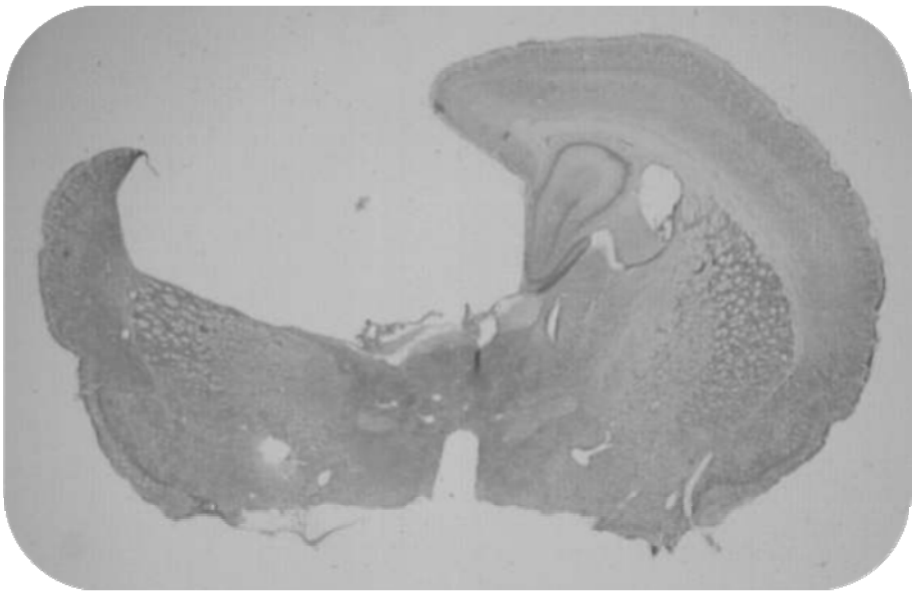


Figure 18: BRI 37

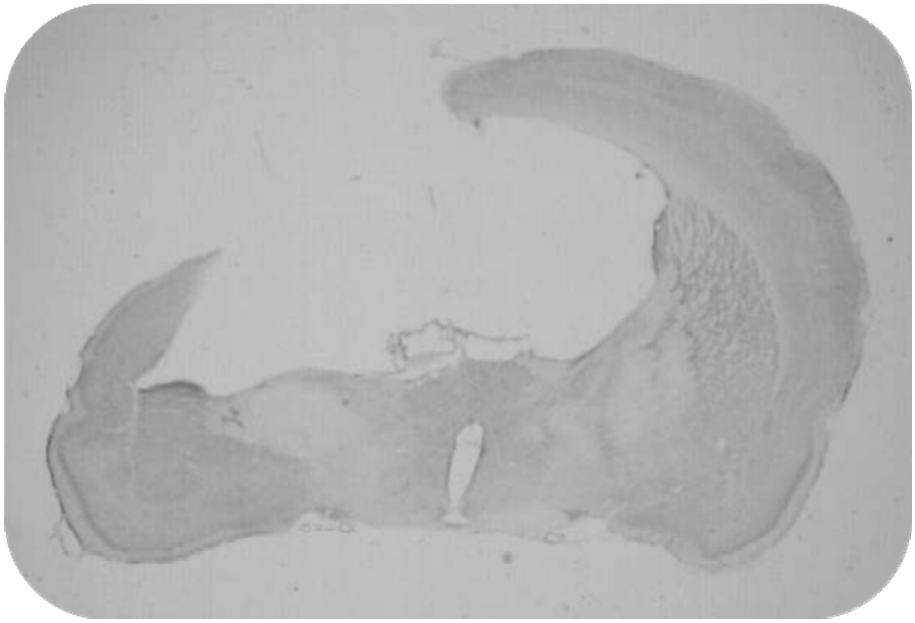


Figure 19: BRI 38

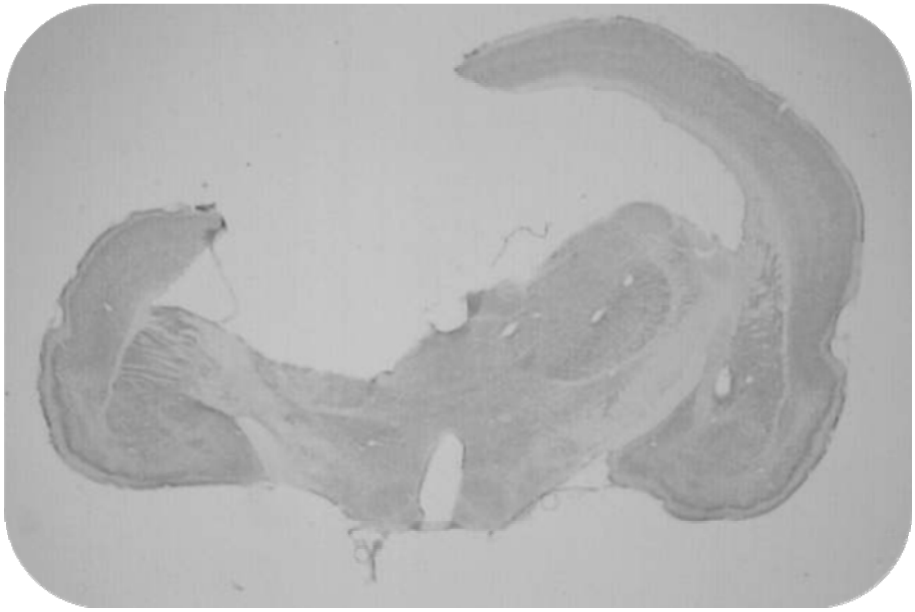


Figure 20: BRI 39

BRI 37, 38, 39 : Lesions are large, expand to almost through whole the left hemisphere, encroaching also the right parts of the brain, white matter and deep grey structures.

11.2 Ratio of the volume of the hemispheres

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,050). There is 95 percent confidence interval for difference of means :-0,514 to -0,000289. Power of performed test with alpha was 0,050: 0,442. It means there is significant decrease of the volume of hemisphere in the affected brain with the lesion. Difference is -0,257. t = -2,309 with 8 degrees of freedom (P = 0,050).

Group Name	Number	Missing	Mean	STD	SEM
ischemic	7	0	0,786	0,184	0,0695
control	3	0	1,043	0,0526	0,0304

Table 5. : Mann-Whitney Rank Sum Test

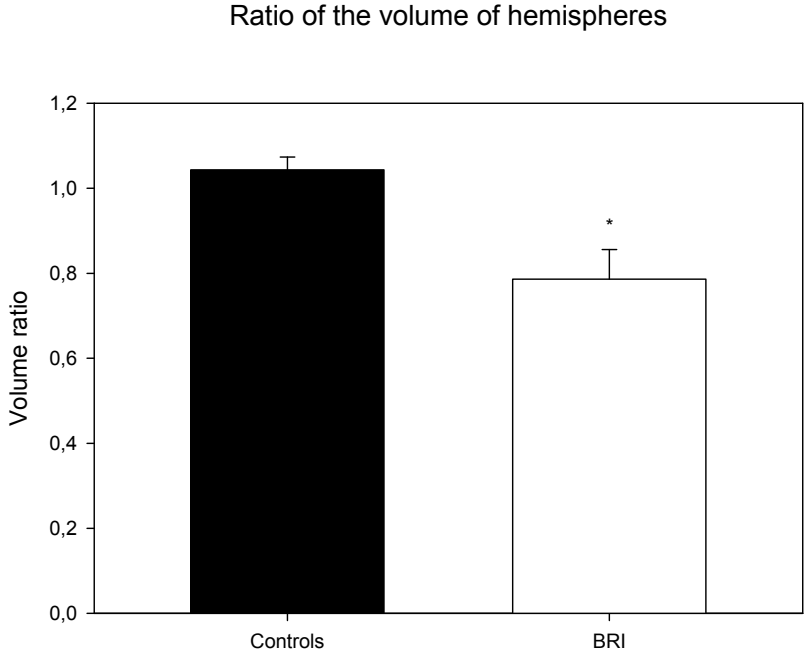


Diagram 1: Diagram of the ratio of the volume of hemispheres

11.3 Testing of Sensorimotor Skills

Numbering: For testing of sensorimotor skills we used another numbering of animals. The next table coordinates the numbers of animals of new and previous experiments.

E-1	BRI 39	E-7	BRI 37
E-2	BRI 34	E-8	BRI 38
E-4	BRI 35	C-3	BRI 40
E-5	BRI 36	C-9	BRI 41
E-6	BRI 33	C-10	BRI 42

Table 6: Numbering of animals

Bar Holding Test: In controls, improved performance with repeated trails was observed. The latency in which they kept hanging on the rod without falling increased with repeated trails in contrast to the experimental animals (Diagr. 2).

Rotarod Test: When comparing the experimental animals to the controls, we observed no differences in their initial performance but with repeated trials the control animals presented the ability to learn and maintain equilibrium on the rotarod. Hence improving their grip and increasing their walking latency. (Diagr.3)

All these results represent statistically mean of seven experimental and three control animals. (Diagrams 2, 3) Individual results (Table 7, 8) show that experimental animal achieved comparable abilities to control animal. In addition, although the lesions were very large (BRI 37, BRI 38, BRI 39), these animals improved their achievement and acquired good results essentially. For example animal BRI 37 achieved in bar holding 2s, 2s, 4s, in rotarod testing 3s, 3s, 5s, animal BRI 38 in bar holding 2s, 4s, 6s, in rotarod 4s, 55s, 63s and animal BRI 39 in bar holding 4s, 2s, 1s, in rotarod 36s, 120s, 82s.

Bar holding test

Name	Trail 1	Trail 2	Trail 3
	t [s]		
CONTROLES			
C-3	8	6	3
C -9	2	1	4
C-10	3	5	17
Sm	4.3	4.0	8.0
EXPERIMENTAL			
E-1	2	2	8
E-2	2	4	6
E-4	4	10	6
E-5	4	7	2
E-6	3	5	3
E-7	2	4	6
E-8	4	2	1
Sm	3.0	4.9	4.6

Table 7: Bar Holding Test

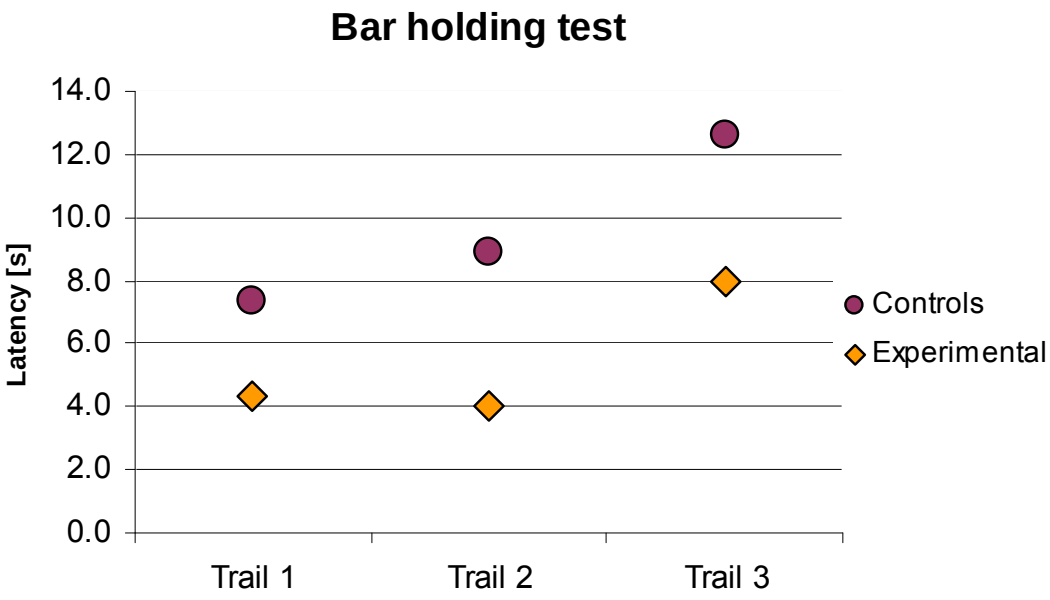


Diagram 2: Bar Holding Test

Rotarod test

Name	Trail 1	Trail 2	Trail 3
	t [s]		
CONTROLES			
C-3	2	4	120
C -9	28	29	22
C-10	28	13	26
Sm	19.3	15.3	56.0
EXPERIMENTAL			
E-1	3	3	5
E-2	4	17	18
E-4	12	31	29
E-5	4	44	0
E-6	60	9	26
E-7	4	55	63
E-8	36	120	82
Sm	17.6	39.9	31.9

Table 8: Rotarod walking test

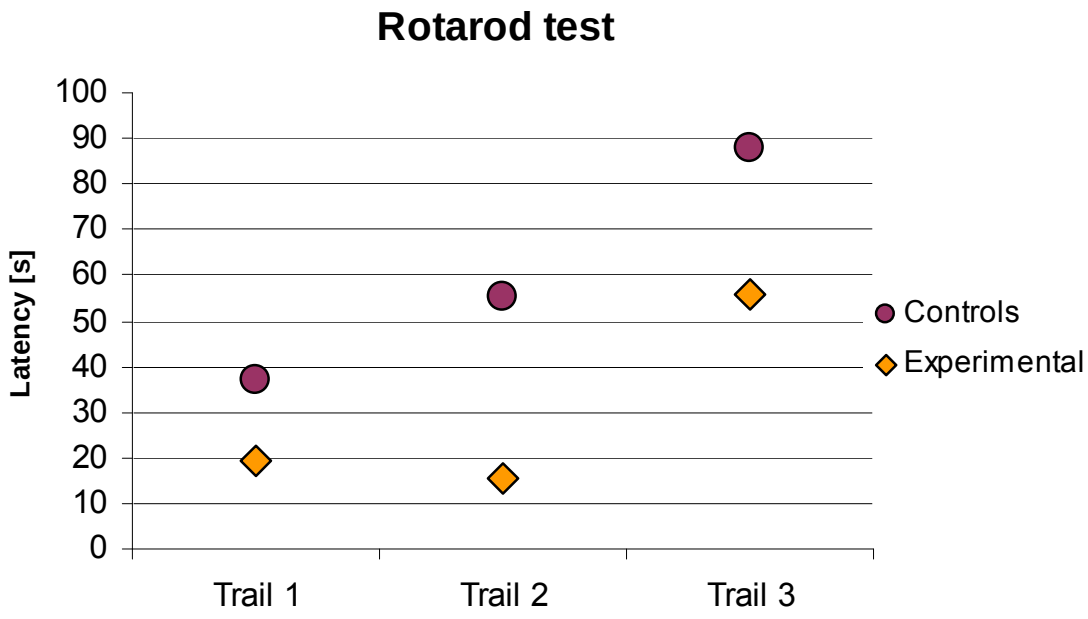


Diagram 3: Rotarod walking test

11.4 EEG Analysis

The EEG was recorded continuously for 4-5 day. All of animals were more active during the night and all of them showed sleep EEG signals. We have found overall increased activity of EEG in the experimental animals during the EEG monitoring. Our preliminary results have shown non-convulsive electrographic seizures observed 4 month after surgery, an example is shown in Fig. 21. Convulsive epileptic seizures were not detected during the EEG recordings. Electrographic seizures were not detected in rats in the control group. The mean seizure frequency and mean seizure duration will be defined in the following studies.

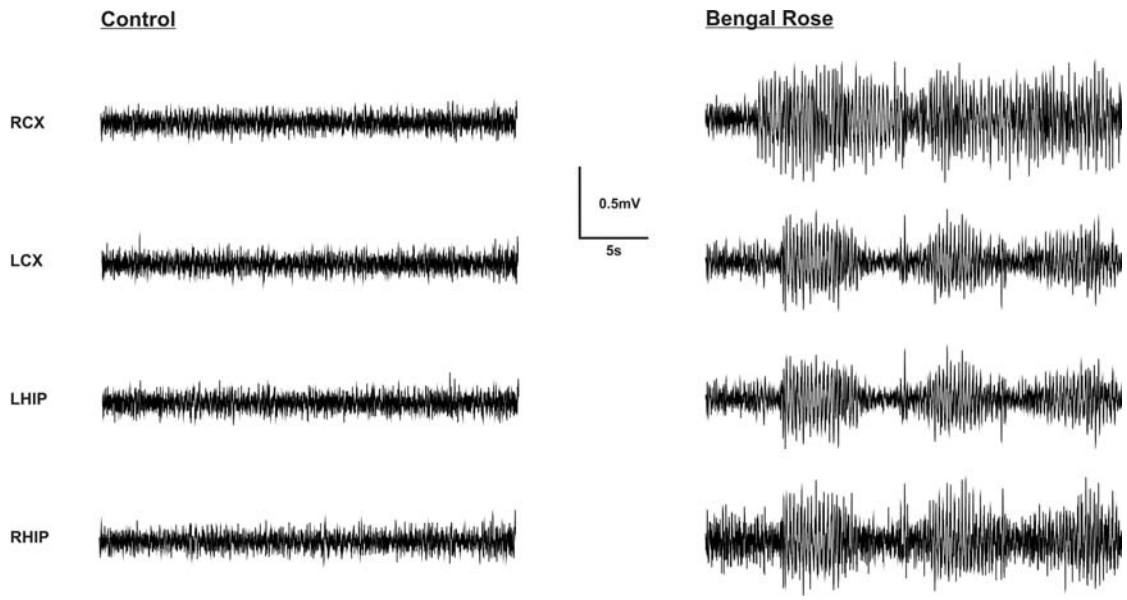


Figure 21: Example of EEG of control and experimental animals

12. Discussion

Our study demonstrates that unilateral photothrombosis of the senzorimotor cortex in P7 rat leads to lesion ipsilaterally to the infarct. The aim of this study was also to investigate the occurrence of post stroke epilepsy in the new model of P7 rat pups. There were almost no literature, articles and reviews with the neonatal models of focal brain ischemia induced by photothrombosis. It was necessary to develop the new model for P7 rats. Therefore there were fifth another previous experiments described in the beginning of the chapter Results. In the discussion, I would like to write about the results, conclusions of my hypothesis and assess the aim of this work.

In this study the depth, size and localization of the lesions were more variable than expected based on previous studies. (Karhunen, 2007; Karhunen et. al., 2007; Kelly, 2007; Kharmalov, 2005) We expected the lesions should have the same parameters and will be smaller directly in the cortical layers. In result the lesions vary remarkably between animals and extend to the subcortical white matter damaging the corpus callosum and encroaching on the lateral ventricles. The variation in the lesion size can provide an opportunity to investigate the association of lesion size with the EEG and development of epilepsy. Simultaneously it would be interesting to differentiate the place of epileptogenesis and rising seizures. That is why it would be more appropriate to be able make lesions using different parameters of photothrombosis, for creating smaller lesions exactly in the cortex layers.

Factors which may have contributed to variability of the lesion parameters in the present study include intensity of the light beam, time of irradiation, the placement of the light, parameters of the bengal rose dye and maybe using some neuroprotective dyes. Previous studies varying in the time of irradiation, type of laser and parameters of the light beam (in Appendix 3). There was only one study, in that time, working with neonatal animals, P7 mice. (Maxwell, 2005) In Maxwell´s study, they have adapted the photothrombotic model for use in neonatal mice and describe modifications that allow discrete and reliable definitions of the position, shape and size of the infarct. By placing a mask having an aperture of defined shape and size on the skull surface, they

were able to reliably and reproducibly induce infarcts in discretely defined cortical regions. Further, they demonstrate explicit control of infarct volume by modifying the duration of laser exposure. (Maxwell, 2005). In the following studies it will be necessary to create the lesion only in the cortical layers.

Human neonatal brain can be affected by hypoxia-ischemia damages, strokes, traumatic injury, infections... This thesis is focused on the focal ischemic brain damages. Injury or deprivation occurring at a certain stage of human postnatal life may affect one aspect of development, whereas the same injury at a different period may affect another aspect. Developing white matter surrounding the ventricles is especially vulnerable to injury prior to 32 weeks gestation and injury to the white matter at this time causes the spastic diplegia form of cerebral palsy. In contrast, injuries later in the third trimester of gestation or shortly after term are likely to damage the immature basal ganglia and motor cortex and spare the white matter. (Johnston, 2007) In some human infants not thought to be neurologically ill as neonates, perinatal stroke can be diagnosed in later months after with failure to reach developmental milestones such as rolling over, asymmetry and slow to reach and grasp, sitting up, crawling, walking and talking, or post-neonatal seizures. (Nelson, 2004) A specialised motor examination by Vojta identifies affected infants early.

Brain development at P7 rats is comparable to human premature or full-term infants (Vannucci, 1999). 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. (Bender, 2007) Careful comparison can be used for experimental studies with rat pups as simulating the human neonatal injuries and brain damages for researching, understanding, defining and treating of them.

Testing of motoric skills of animals with different brain damage is very useful for explaining the plasticity, regeneration and degeneration of the brain tissues. We can test animals almost immediately after the injury at the early age and periodically test them until their adultness, compare the results, improvement or aggravation of their senzomotoric skills. These tests are good for comparing outcomes after infarcts, testing and researching the effects of

different drugs. Testing is quantitative, objective, and reproducible measures of functional impairment.

In our study, the experimental animals were less skillful in almost all tests in statistically mean, unable to learn motor skills with repeated exposure in contrast to controls. But in comparison with individual results, the conclusion was different. It was interesting to compare the extension of the lesion with the motoric skills. Although the lesions were very large, animals improved their achievement almost in all three testing essentially. These animals, with the lesion in almost whole the left hemisphere and in some cases also the part of the right hemisphere, were able to manage tests in the same level such as the control animals.

It is known, that in animals, unilateral inhibition of the sensorimotor cortex during development results in a sparse contralateral projection from this cortex and retention of a greater number of ipsilateral projections from the more active cortex. The final pattern of the origin and termination of the corticospinal tract is shaped during development by the balance between projection and withdrawal of axons. (Eyre, 2007). There are also studies with human babies after neonatal or prenatal brain damage. Interestingly, no site or size-of-lesion effects, common to adult stroke, were again identified. These findings identify poor long-term outcome with early brain insults at stages far removed from the onset of injury. (Chapman et. al., 2002)

Developmental plasticity is the ability of the brain to adapt itself to changes in the environment, how the brain is able to develop, to function and to recover from central nervous system damage. The immature brain grows and creates its own neuronal network as the brain is in contact with new input from the outside world. Neuronal connections and cortical maps are continuously remodelled and activity plays a key role in the formation and maturation of synapses.

Brain plasticity in children can be divided into several types, such as adaptive plasticity that enhances skill development or recovery from brain injury. Impaired plasticity associated with cognitive impairment and excessive plasticity leading to maladaptive brain circuits. (Johnston, 2003)

I expected the occurrence of late seizures after focal brain injury in P7 rats in my third hypothesis. Previous studies have demonstrated that adult rats can develop epilepsy after cortical photothrombotic lesions (Karhunen et al., 2007; Kelly et al., 2007; Kharmalov et al., 2003). The long-term studies of epileptogenesis after focal cerebral ischemia in rats by Karhunen (Karhunen et al., 2007) aimed at exploring experimental post stroke epilepsy in the adult rats in further detail, using three models: the transient intraluminal filament model of MCAo, ET-induced MCAo and the cortical photothrombosis with rose bengal dye. Our preliminary results have shown non-convulsive electrographic seizures observed four month after inducing focal brain lesions in P7 rat pups. All experimental animals had overall increased EEG activity. The following studies are needed to explore the development of epilepsy in this type of experiment. The mean seizure frequency and mean seizure duration will be also defined in the following studies.

13. Conclussion

The aim of this diploma thesis was to determinate the parameter of lesion after focal brain ischemia induced by photothrombosis in P7 rat pups, establish the changes of sensorimotor abilities and investigate the occurrence of post stroke epileptic seizures in their adultness. All these aims were realized in the Academy of science of the Czech Republic, Institute of Physiology. Histological examination of the brain showed significant ischemic brain damage, there was the statistically significant difference in ratio of the volume of the hemispheres between the control and experimental animals. The lesion size, localization and depth in the brain vary remarkably between experimental animals. Our preliminary results show the occurrence of non-convulsive seizure in experimental animals. Although the statistically mean shows that experimental animal were less skilful in motoric tests, individual comparison the size of lesion to achieved motoric abilities shows improvement and good skills almost completely comparable to control group animals.

14. Resources

1. AMANTEA, NAPPI, BERNARDI. Post-ischemic brain damage: pathophysiology and role of inflammatory mediators. FEBS Journal, 2009; Vol. 276, p. 13-26
2. BALLABH, BRAUN, NEDERGAARD. *The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiology Dis.* 2004; Vol. 16, p.1–13.
3. BENDER, BARAM. Epileptogenesis in the Developing Brain: What Can We Learn from Animal Models? Epilepsia. 2007; Vol. 48, p.2–6.
4. BERG ET. AL. Comments on the Report: Revised terminology and concepts for organization of the epilepsies: Report of the Commission on Classification and Terminology. Internmetional League Against Epilepsy: 2009.
5. BLADIN ET. AL. Seizures after stroke: a prospective multicenter study. Archives of Neurology 2000; Vol. 57, p. 1617−1622.
6. BOCKHORST ET.AL. Early Postnatal Development of Rat Brain: In Vivo Diffusion Tensor Imaging. Journal of Neuroscience Research; 2008. Vol. 86, p. 1520–1528.
7. BOON, MONTFORT. Stages of Brain Development. Retrieved November 29, 2009 from <http://www.learningdiscoveries.org/StagesofBrainDevelopment.htm>
8. CHAPMAN et.al. Discourse plasticity in children after stroke: age at injury and lesion effects. Pediatric Neurology, 2003; Vol. 29, Issue 1 2003, p. 34-41
9. CHEN. Brain development. Retrieved February 13, 2010 from <http://bio1152.nicerweb.com/Locked/media/ch48/
10. CHOI, KOH. Role of Brain Inflammation in Epileptogenesis. Yonsei Medicine Journal 2008; Vol. 49, p. 1–18.

11. CHUN-SHAN WU ET. AL. Prenatal Ischemic Stroke as a cause of Neonatal Seizure Diagnosed by Magnetic Resonance Diffusion-Weighted Imaging. Journal of Medicine and Science, 2008; Vol. 28, p. 135-140.

12. CROSSMAN, NEARY. Neuroanatomy, 3rd Edition. Churchill Livingstone, 2006. ISBN: 978-0-443-10036-9.

13. DE VRIES ET. AL. Infarcts in the vascular distribution of the middle cerebral artery in preterm and fullterm infants. Neuropediatrics 1997; Vol. 28, p. 88–96.

14. EATPOT. EEG specific stages. Retrieved February 13, 2010 from <http://members.arstechnica.com/x/albino_eatpod/specific-eeg-states.gif>

15. EPSZTEIN ET. AL. Late-Onset Epileptogenesis and Seizure Genesis: Lessons From Models of Cerebral Ischemia. The Neuroscientist 2008; Vol. 14, No. 1, p. 78-90

16. EYRE. Corticospinal tract development and its plasticity after perinatal injury. Neuroscience & Biobehavioral Reviews, 2007. Vol. 31, Issue 8, p. 1136-1149

17. FISHER. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), Epilepsia 2005; Vol. 46, p. 470−472.

18. GAJEWSKA, SAMBORSKI. Application of Vojta's method for early detection of developmental disturbances in very low birthweight infants with regard to Apgar score and asymmetric body positions. Annals Acadademy of Medicine Stetin; 2006. Vol. 52, p.101

19. GOBLE. Protocol for Nissl *Staining*. Retrieved April 3, 2010 from <http://www.scientistsolutions.com/a543-protocol-protocol+for+nissl+staining.aspx>

20. GOLOMB, SAHA, GARG, AZZOUZ, WILLIAMS. Association of cerebral palsy with other disabilities in children with perinatal arterial ischemic stroke. Pediatric Neuroly 2007; Vol. 37, p. 245-9.

21. GOLOMB. Very Early Arterial Ischemic Stroke in Premature Infants. Pediatric neurology, 2007; Vol. 38, No. 5, p. 329-334.

22. GRAY. Anatomy of the Human Body, Twentieth edition. New York: Bartleby.com, 2000.

23. GUPTA ET.AL. Diffusion tensor imaging of the developing human cerebrum. Journal Neuroscience Research; 2005. Vol. 81, p.72–178.

24. HENSHALL. *Apoptosis signalling pathways in seizure-induced neuronal death and epilepsy.* Biochem Soc Trans. 2007; Vol. 35, p. 421–423.

25. HOSSAIN. Hypoxic-ischemic injury in neonatal brain: involvement of a novel neuronal molecule in neuronal cell dech and potential target for neuroprotection. International Journal of Developmental Neuroscience, 2008; Vol 26, p. 93–101.

26. HOSSMANN. Viability thresholds and the penumbra of focal ischemia. Annual Neurology, 1994; Vol. 36, p. 557–565.

27. HUPPI, DUBOIS. Diffusion tensor imaging of brain development. Seminary Fetal Neonatal Medicine; 2006. Vol. 11, p.489–497.

28. JOHNSTON. Clinical disorders of brain plasticity. Brain and Development, 2004; Vol. 26, Issue 2, p. 73-80

29. JOHNSTON. Hypoxic and ischemic disorders of infants and children. Brain & Development 1997; Vol. 19, p. 235-239.

30. JOLKKONEN. Subacute hemorrhage and resolution of edema in Rose Bengal stroke model in rats coincides with improved sensorimotor functions. Neuroscience Letters; 2007. Vol. 428, p. 99–102.

31. KANDEL, SCHWARZ, JESSELL. Principles of Neural Science *, 4/e*. New York: McGraw Hill, 2000. ISBN: 0-8385-7701-6.

32. KARHUNEN ET. AL. Epileptogenesis after cortical phototrombosic brain lesion in rats. Neuroscience 2007; Vol.148, p. 314–324

33. KARHUNEN. Long-term studies of epileptogenesis after focal cerebral ischemia in rats. Kuopio, Finland 2007. ISBN ISBN 978-951-27-0821-5

34. KELLY ET. AL. Long-term video-EEG recordings following transient unilateral middle cerebral and common carotid artery occlusion in Long-Evans rats, Experimental Neurology 2006; Vol. 201, p. 495−506.

35. KELLY. Animal Modeling of Poststroke Seizures and Epilepsy: 5-Year Update. Epilepsy Curr; 2007. Vol. 7, p. 159–162.

36. KHARLAMOV ET. AL. Electrobehavioral characteristics of epileptic rats following photothrombotic brain infarction. Epilepsy Research 2003; Vol. 56, p. 185−203.

37. KLEIM, BOYCHUK, ADKINS. Rat Models of Upper Extremity Impairment in Stroke. ILAR 2007; Vol. 48, No. 4, p. 374-84

38. KRIZ. Inflammation in ischemic brain injury: timing is important. Critical Review of Neurobiology, 2006; Vol. 18, p. 145–157.

39. KUROIWA ET. AL. Development of a Rat Model of Photothrombotic Ischemia and Infarction Within the Caudoputamen. Stroke, 2009; Vol. 40, p. 248.

40. KWONG. Epilepsy in Children With Cerebral Palsy. Pediatric Neurology, 2004; Volume 19, Issue 1, p. 31-36.

41. LEE, PARK, KIM. Photochemically induced cerebral ischemia in a mouse model. Surgical Neurology 2007; Vol. 67, p. 620– 625

42. LIU, NISWANDER. Bone morphogenetic protein signalling and vertebrate nervous system development. Nature Reviews Neuroscience, 2005; Vol. 6, p. 945-954.

43. LYNCH, HIRTZ, DEVEBER, NELSON. Report of the National Institute of Neurological Disorders and Stroke Workshop John on Perinatal and Childhood Stroke. Journal of pediatrics; 2002; Vol. 109 No. 1.

44. MARLOW, WOLKE, BRACEWELL. Neurologic and developmental disability at six years of age after extremely preterm birth. The New England Journal of Medicine 2005; Vol. 352, p. 9-19.

45. MATEFFYOVA, OTAHAL, TSENOV, MARES, KUBOVA. Intrahippocampal injection of endothelin-1 in immature rats results in neuronal death, development of epilepsy and behavioral abnormalities later in life. The European journal of Neuroscience 2006; Vol. 24, p. 351−360.

46. MAXWELL, DYCK. Induction of Reproducible Focal Ischemic Lesions in Neonatal Mice by Photothrombosis. Developmental Neuroscience; 2005, Vol. 27, p. 121-126.

47. MCNAMARA. *Cellular and molecular basis of epilepsy. Journal Neuroscience* 1994; Vol. 14, p. 3413–3425.

48. NELSON, LYNCH. Stroke in newborn infants. Lancet Neurology, 2004; Vol. 3, p. 150-158.

49. PANAYITOPOULOS. A clinical guide to epileptic syndromes and their treatment. Second edition. London: Springer; 2007

50. PENNEY. Outer Brain Surface. Retrieved February 13, 2010 from <http://www.coheadquarters.com/coOuterBrain1.htm>

51. RAKHADE, JENSEN. Epileptogenesis in the immature brain: emerging mechanisms. Nature Reviews Neurology 2009; Vol. 5, p. 380-39.

52. RAVIZZA ET AL. *Inflammatory response and glia activation in developing rat hippocampus after status epilepticus*. Epilepsia. 2005; Vol. 46, p. 113–117.

53. REMOND, LAIRY. Handbook of EEG and clinical neurophysiology. Vol. 6, The normal EEG Thoughout Life. Part B: The Evolution of the EEG from birth to adulthood. Paris: 1975. ISBN 0-444-41390-1

54. SATO et al. Hypoxic ischemic encephalopathy associated with neonatal seizures without other neurological abnormalities. Brain & Development, 2003; Vol. 25, p. 215–219.

55. SNF organization - SOCIETY FOR NEUROSCIENCE. *Brain facts. A Primer on the Brain and Nervous System*. Washington: Society for neuroscience, 2008. Retrieved November 29, 2009 from <http://www.sfn.org/skins/main/pdf/brainfacts/2008/brain_development.pdf

56. SREENAN, BHARGAVA, ROBERTSON. Cerebral infarction in the term newborn: clinical presentation and long-term outcome. Journal Pediatric, 2000; Vol. 137, p. 351–55.

57. SULEJCZAK ET. AL. Focal Photothrombotic Lesion of the Rat Motor Cortex Increases BDNF Levels in Motor-Sensory Cortical Areas Not Accompanied by Recovery of Forelimb Motor Skills. Journal of neurotrauma 2007; Vol. 24, p. 1362–1377

58. VANNUCCI. Rat model of perinatal hypoxic-ischemic brain damage. Journal of Neuroscience 1999; Vol. 55, p. 158–163.

59. VOLPE. Neurology of the newborn. 4th ed. Philadelphia: W.B. Saunders, 2001.

60. WHISHAW. The behavior of the laboratory rat: a handbook with tests. Oxford university press 2005, ISBN 0195162854

61. WILLIAMS, TORTELLA. Neuroprotective effects of the sodium channel blocker RS100642 and attenuation of ischemia-induced brain seizures in the rat, Brain Research 2002; Vol. 932, p. 45−55.

62. WILLIAMS. Antiepileptic drug treatment of nonconvulsive seizures induced by experimental focal brain ischemia, The Journal of Pharmacology and Experimental Therapeutics 2004; Vol. 311, p. 220−227.

63. WOODWARD. Neonatal MRI to predict neurodevelopmental outcomes in preterm infants. The New England Journal of Medicine 2006; Vol. 355, p. 685-94.

15. Appendix

Appendix 1

This poster was used for presentation

in occasion of conference of Česká Společnost pro Neurovědy 2009.

Appendix 2

This poster was used for presentation in occasion of congress of American Epilepsy Society in Boston Massachusetts

Appendix 3

Localization of the lesion, type of the laser and its duration, parameters of bengal rose dye and animals in the previous studies

Author	Localization	Laser and Time of irradiation	Bengal Rose	Animals	Controls
Kuroiwa et al.,2009	Caudate light exposure	1700 and 2500 lux for optic fibers with 0.5- and 0.75-mm diameters, 10 minutes	20 mg/mL saline, 1 mL/kg, disodium 4,5,6,7-tetraiodofluorescein; Sigma. Femoral vein for 10 seconds	Male Sprague-Dawley rats, weighing 270 to 320 grams (n=143)	caudate light exposure without Rose Bengal dye infusion
Karhunen 2007	1.8 mm posterior and 2.2 mm lateral to bregma corresponding to the posterior somatosensory and motor areas	4 mm in diameter; (Olympus, Glostrup, Denmark,10 minutes	20 mg/kg; 20 mg/ml; (Sigma-Aldrich, Munich, Germany) Femoral (exp 1) or saphenous vein (expt 2) at a rate of 150 l/min.	51 male Sprague–Dawley rats, weighing 310–380 g (age 9–12 weeks)	In ctrl (*n*=6) the light was not turned . Ctrl (*n*=5) electrodes implanted into the hippocampus and the skull.
Sulejczak 2007	3.5 mm anterior to bregma and 3 mm lateral to the midline	cold, white light beam (150 W, E 2.75), 20 minutes	During the first minute of illumination, rose bengal (Sigma Aldrich) dye (10 mg/mL saline solution, 0.133 mL/kg body wt) was injected intravenously.	Adult male Wistar rats (n=28), weighing 260–340g	infusion of rose bengal without illminationu
Jolkkonn, 2007	0.5mm anterior to the bregma and 3.7mm lateral to the midline over the right motor cortex	cold white light (Olympus, Denmark) with a 4mm, 10 minutes	(20 mg/kg), femoral vein via a microinjection pump within 2 min	Male Wistar rats (*n* = 16), age 3–4 months	Sham operated animals were treated similarly but light was not in use.
Maxwell, 2005	2 mm lateral to the midline and 0.5 mm posterior to bregma. , custom-made mask for kontrol the shape of lesion	diode-pumped laser (20 mW; 532 nm; B&WTek, Del., USA)	rose bengal (50 mg/kg i.p.; Sigma-Aldrich) 15 min for dye absorption		
Karhunen , long term study, 2007	1.8 mm posterior and 2.2 mm lateral to the bregma. Additional group of animals with coordinates 0.5 mm posterior and 3.7 mm lateral.	(4 mm in diameter; Olympus, Denmark), 10 minutes	Rose Bengal (20 mg/kg; 20 mg/ml; Sigma Aldrich Chemie, Germany) femoral (Exp 1) or saphenous vein (Exp 2) at a speed of 150 µl/min.	Male Sprague-Dawley rats weighed 275-315 g (I, II) or 310-380 g (III), n= 51	light was not turned on and thus,The second control group underwent only the electrode implantation.