**Charles University in Prague**

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Postgraduate study program of Biomedical Science
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# Modulation of consequences of ischemia and hypoxia

# Možnost ovlivnění důsledků ischémie a hypoxie

# (role volných radikálů)

## Thesis

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## Author’s disclosure

I, Kateryna Nohejlová, MD, maiden Deykun, certify that all personal, professional and financial relationships with other people and organizations that pose a conflict of interest, that could personably be perceived as posing a conflict of interests, or that could potentially influence or bias my work described in the thesis have been fully and truthfully disclosed in the Acknowledgements and Reference sections of the thesis.

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## Summary

Various brain insults can lead to the development of functional changes. The pathogenesis of those includes different mechanisms. Literature suggests that many types of insults are accompanied by the increase of free radicals level. The other widely described fact is that free radicals could lead to tissue damage itself. A raise of free radicals, therefore, could participate on functional changes following different types of cerebral insult in a more general manner. Even though, fluctuations of free radicals' levels in certain extension are regular in a living tissue, those levels do not reach threshold that is critical for tissue damage. This tissue homeostasis is provided by the systems that liquidate free radicals overload. Never the less, free radicals also play an important role in physiological processes, as intra and extracellular mediators and signal transducers.

The models of three pathological states, which are known to be related to the changes in free radicals production, were used for the purposes of the work. (1) The model of focal photothrombotic ischemia of sensorimotor cortex of laboratory rat; and two global insults models: (2) generalized epileptic seizure elicited by flurothyl and (3) intermittent normobaric hypoxia. The used paradigms served to the assessment of the hypothesis that the role of free radicals in pathogenesis of CNS disturbances has more general trend.

The next approach of models preference was the intensity of action that borderlines the threshold of mainly functional CNS disturbances. With the respect to the used behavioral methods of assessment was need for the animals to survive the interventions. For the estimation of the degree of disturbances were used tests of postural motor function and motor learning, as well as the tests of evaluation of cognitive function in terms of spatial orientation.

The core aim of the work was the estimation of possible influences of a raise of free radicals. It was conducted in such a way that when compared to the outcomes of plain insults it would be possible to deduce the extension of free radicals participation in pathogenesis of functional state of an animal. In order to do so free radicals scavengers and antioxidants were used at the dosed that were expected to block their pathological increase. Two free radical scavengers and antioxidants were

chosen, melatonin and tempol, which act extracellularly as well as intracellularly. From the experimental perspective the preventive application, before an insult, was self-suggesting. However, in the respect of the potential clinical application, it was needed to use them as a treatment, meaning to apply them after an exposition to pathogenic factors.

The first set of experiments targeted on the introduction of the model of minor ischemic lesion in the sensorimotor area of the cortex of laboratory rat. The young adult albino Wistar male rats (190 to 250 g) were used. The classical photothrombic model of ischemia was used. Anesthetized animals received photosensitive dye, Rose Bengal, *i.v.* and were transcranially irradiated by green laser in the area of sensorimotor cortex. The superficial ischemic lesion was induced in that area (according to the length of irradiation) as a result of thrombotic occlusions of the vessels.

From 24 hours after an induction of ischemia the animals were subjected to behavioral tests.

In the test of beam balance animals with the ischemic lesion initially did not show any significant differences from naïve control and sham operated animals. However, 48 hours after an induction of ischemia the animals' quality of performance decreased.

Next, animals were tested on the rotarod and reverse rotarod. The rotarod test results of affected and control animals did not differ. In the case of more complicated task of reverse rotarod test the animals with ischemic lesion performed worse then control animals.

The same day animals were also tested for motor function in the Morris water maze. In this test animals did not have to search for a platform that was located above the water level, therefore only quality of swimming was evaluated. Paradoxically, swimming velocity was increased in animals with ischemic lesion comparing to control animals.

The following day animals proceeded to the 6-day testing of spatial learning, i.e. hidden platform acquisition. The affected animals showed decreased learning

ability, mainly in terms of searching strategy. There was also increased swimming velocity throughout the trials that suggests to be related to hyperactivity.

Two other groups were tested in the same manner but received melatonin or tempol 10 minutes after an induction of ischemia. The animals, which received tempol, did not show any significant improvement. An application of melatonin enhanced animals' performance in the most of the tests.

The morphological evaluation of the ischemic lesions with use of 2,3,5-triphenyltetrazolium chloride reduction staining of surviving mitochondria showed minor but distinguishable changes in each animal. The lesions were maximally transcortical. The animals treated with melatonin and tempol had lesions reaching maximally into the IV-V cortical layers.

These findings support the hypothesis that application of free radicals scavengers and antioxidants as treatment after an induction of ischemia improve its outcome.

Second set of experiments was aimed on the possibility of limitation of epileptic seizure consequences. The seizures were elicited by flurothyl and the consequences were evaluated by the changes of Morris water maze performance.

Previously was describe that one generalized epileptic seizure evoked by flurothyl vapors caused worsening of spatial learning in the Morris water maze. This outcome was improved by exposition of animals to hypobaric hypoxia (1 hour; 8000 m) three days prior to induction of seizure. It is assumed that this effect is closely related to the phenomenon of preconditioning and activation of antioxidant systems.

The model of one generalized epileptic seizure evoked by flurothyl was used for testing the hypothesis that high dosage of melatonin applied before and after seizure induction would lead to improvement of spatial learning.

The experiment was conducted on naïve freely moving laboratory rats (Wistar, 190-240 g). Animals were randomly divided into following groups: naïve control, animals with induced seizure by flurothyl, and three combinations of seizure and melatonin application – 1hour before induction of seizure, and 150 seconds and 6 hours after the induction of seizure.

In all experimental groups the tonic-clonic seizure was elicited by vapors of flurothyl. The vapors were immediately evacuated after the seizure development. The duration of the seizure was three minutes maximally. 24 hours later was initiated the testing of spatial learning in the Morris water maze. Animals' goal was to learn the position of the submerged platform during 7-days period and latency of searching was measured.

The control animals showed better learning ability than the animals after the seizure, and this difference was more prominent on the last trial days.

Preventive application of melatonin significantly improved animals' performance and again it was more prominent on the last days of learning trial.

An application of melatonin 150 seconds after an induction of epileptic seizure had similar effect, although in lesser degree.

An application of melatonin 6 hours after the seizure did not have significant influence on performance in comparison to the animals with plain seizure.

On the basis of experimental sets' and previous results it could be concluded that the demonstrated effects are related to the interaction of melatonin with the raise of free radicals. The second conclusion is that free radicals participate in pathogenesis of decrease learning ability after seizure. Certainly, it has to be taken into account and the other possible mechanisms of melatonin action, e.g., involvement of neuronal melatonin receptors. Despite the well-known fact that melatonin in general attenuates seizure-related worsening of cognition it should not have an effect when applied after seizure. That is why the combination of mechanisms has to be suggested.

The third experiment was aimed on evaluation of hypoxic preconditioning to cognitive function worsening by ischemic lesion.

The model of intermittent normobaric hypoxia was applied. Fraction of oxygen in inhaled air cycled from 21 to 8% and back to 21% every one minute during one hour. The normobaric pressure was achieved by admixture of nitrogen to ambient air. Concerning the ischemic lesion, the duration of laser irradiation was slightly increased in comparison to the settings of first experiment to make the cognitive

changes of the lesion more prominent for evaluation.

Experimental groups were following: (1) animals exposed to intermittent hypoxia four days before testing in Morris water maze, (2) animals with photothrombic ischemic lesion induced 24 hours before the testing and (3) combination of both – exposition to hypoxia three days prior to ischemia induction with following testing.

All of the animals showed the ability of spatial learning. The naïve control group differed significantly only from animals that were preconditioned by hypoxia before ischemia. The preconditioned animals performed worse.

The results of the last experimental set did not show an expected development of protective hypoxic preconditioning induced by intermittent hypoxia. More over, there was worsening of spatial learning ability. Two factors could be involved: by prolonged laser irradiation and its combination with more severe type of hypoxia. To facilitate development of hypoxic preconditioning induced by intermittent normobaric hypoxia the new experimental design should be proposed. The results suggest certain interaction of ischemic lesions with repetitive hypoxia, which cause disorder of cognition. This negative result could be beneficial for future implementation of animal model of obstructive sleep apnea syndrome.

The obtained experimental results support the hypothesis that free radicals could play important role in the development of negative outcome of focal cortical ischemia and seizures induced by flurothyl. The aims were achieved. Certainly, melatonin application has positive effect on cognitive and sensorimotor function of affected animals. Although, the hypoxic preconditioning effect of intermittent normobaric hypoxia was found cumulative negative. The gained knowledge thereafter could be enriched by further assessment. Firstly, deeper understanding of melatonin actions on and interactions with CNS insults, not only from the aspect of its antioxidant and scavenger properties and interaction with free radicals production. Secondly, search for the explanations of the differences in pathogenesis and effect of intermittent normobaric hypoxia and hypobaric continuous hypoxia.

## Souhrn

Poškození mozku jsou spojena i se závažnými funkčními následky. Ty vznikají na základě různých mechanizmů. Ve světové literatuře je u mnoha různých typů poškození uváděn vzestup hladiny volných radikálů. Další práce dokládají, že volné radikály samy o sobě mohou poškozovat tkáň. Vzestup hladin volných radikálů by se tedy mohl nějakým obecným způsobem podílet i na změnách funkcí u různých typů poškození CNS. Kolísání hladin volných radikálů je v živé tkáni běžné, i když jejich hladiny normálně nedosahují úrovně, kdy by mohlo dojít k poškození tkání. Tato homeostáza je také udržována systémy, které volné radikály likvidují. Význam FR se však může prosazovat i za fyziologických stavů, protože existují doklady, že jsou důležitým intra- i extracelulárním signálem.

V práci byly využity modely tří patologických stavů, u nichž víme, že dochází ke změnám produkce volných radikálů. 1. Model fokální fototrombotické ischémie senzorimotorické kůry potkana a dva globální modely poškození: 2. generalizovaný epileptický záchvat vyvolaný flurotylem a 3. intermitentní normobarickou hypoxií. Modelování různých typů působení na CNS umožnilo řešit otázku, zda je role působení volných radikálů v poškození jeho funkcí obecnější.

Dalším hlediskem výběru zásahů bylo, aby intenzita působení mohla být co nejblíže hranici, kde dochází hlavně k poškození funkcí CNS. Vzhledem k použitým behaviorálním metodikám bylo nutné, aby zvířata zásah snadno přežívala. Ke zjištění míry funkčního poškození byly zvoleny behaviorální testy sledující jednak posturální motoriku a motorické učení, a pak testy měřící kognitivní funkce při orientaci v prostoru.

Podstatou práce bylo ovlivnění vzestupu hladin volných radikálů tak, aby ve srovnání s důsledky samotných zásahů bylo možné usuzovat na míru, kterou přispívají ke zhoršení funkčního stavu zvířete. Byly tedy použity scavengery a antioxidanty v dávkách, u nichž byl předpoklad, že zabrání jejich patologickému vzestupu. Pro tento účel byly zvoleny melatonin a tempol, které působí jak extraxcelulárně, tak intracelulárně. Z pohledu experimentálního bylo nasnadě podávat tyto látky preventivně, před zásahem. Z pohledu zajímavějšího pro praktické využití je důležitá i otázka, co se dá udělat po zásahu. Proto byly aplikovány i

následně.

V prvém souboru experimentů byla pozornost upřena na vypracování modelu minimální ischemické léze v oblasti sensorimotorické kůry potkana (soustavně a to i v dalších experimentech byly využíváni mladí bílí samci kmene Wistar o hmotnosti 190 až 250 g). Pro vyvolání této léze byl využit klasický fototrombotický model. Potkanům byla v ketamine/xylazinové narkóze iv aplikována bengálská červeň a potom jim byla transkalvárně ozářena část senzorimotorické kůry paprsky zeleného laseru. V této oblasti vzniklo (podle délky osvitu) na základě vyvolaného trombotického uzávěru cév povrchové ischemické ložisko.

Za 24 hodin po ischemizaci byla zvířata podrobena behaviorálním testům.

V testu beam balance zvířata s ischemicku lézí nevykazovala rozdíly při porovnání s naivními kontrolami a sham operovanými zvířaty.

Ale 48 hodin po vyvolání léze se výkony v testu beam balance signifikantně zhoršily.

Poté byla zvířata všech skupin podrobena testům na rotujícím válci (rotarod) a válci rotujícím opačně (reversní rotarod). Test rotarod ve srovnání s kontrolními skupinami neprokázal změny. Komplikovanější situaci v testu reversní rotarod postižená zvířata zvládala významně hůře než kontroly.

V tomtéž dni byla zvířata testována v Morrisově vodním bludišti. Tento test byl zaměřen pouze na motoriku při plavání, zvířata nemusela hledat ostrůvek, který byl nad hladinou.

Další den bylo zahájeno testování prostorového učení (dosažení skrytého ostrůvku). Učení probíhalo v šesti následujících dnech. U ischemických zvířat bylo prokázáno zhoršení učení spočívající hlavně v poruše strategie vyhledávání ostrůvku. Dále bylo prokázáno nepředpokládané zvýšení rychlosti plavání, které může souviset s hyperaktivitou.

Další dvě skupiny byly testovány stejně, byl jim však aplikován 10 minut po vyvolání léze melatonin nebo tempol. U zvířat po aplikaci tempolu jsme ve srovnání se zvířaty po ischemii nenalezli významné rozdíly. Po aplikaci melatoninu došlo ke

zlepšení výsledků ve většině testů.

U zvířat byly pomocí barvení TTC na živé mitochondrie prokázány povrchové, transkortikálné léze. Po melatoninu se pouze zvýšila variabilita velikosti lézí, zasahující maximálně do IV. a V. vrstvy neokortexu.

Tyto nálezy prokazují, že následná aplikace scavengeru volných radikálů je schopna pozitivně ovlivnit důsledky předchozí ischémie.

Druhý soubor experimentů byl zaměřen na možnosti omezení důsledků flurotylem vyvolaného záchvatu pro učení v Morrisově vodním bludišti.

Již bylo prokázáno, že jeden generalizovaný epileptický záchvat vyvolaný parami flurotylu zhoršuje učení v Morrisově vodním bludišti a že hypobarické hypoxie (1 hodina; 8 000 m) aplikovaná 3 dny před záchvatem dokáže tyto následky flurotylových par výrazně omezit. Předpokládáme, že tento jev může mít některé společné vlastnosti s preconditioningem a aktivací antioxidačních systémů.

Na tomto modelu jsme se snažili prokázat, zda podání vysokých dávek melatoninu těsně před záchvatem a po něm také omezí následné zhoršení prostorového učení.

Experimenty byly prováděny na neoperovaných, volně pohyblivých potkanech. Zvířata byla rozdělena do následujících skupin: naivní kontroly, zvířata po záchvatu, a kombinace aplikace melatoninu 1 hodinu před záchvatem, 150 s po záchvatu a 6 hodin po záchvatu.

Ve všech experimentálních skupinách byl flurotylovými parami vyvolán epileptický tonicko-klonický záchvat a okamžitě po jeho vzniku byly páry odventilovány. Trvání záchvatu bylo maximálně do tří minut. 24 hodin po záchvatu bylo zahájeno testování prostorového učení v Morrisově vodním bludišti. Zvířata hledala ponořený ostrůvek po sedm následujících dnů a byla měřena doba do jeho dosažení.

Kontrolní zvířata se ve srovnání se zvířaty po jednom záchvatu učila lépe, což se zvláště projevovalo v posledních dnech testování.

Preventivní aplikace melatoninu významně zlepšila výkony zvířat a to hlavně v závěrečných fázích testování.

Podání melatoninu 150 s po záchvatu mělo sice menší, ale také podobný významný vliv na důsledky záchvatu.

Podání melatoninu 6 hodin po záchvatu nevyvolalo statisticky významné změny v učení ve srovnání se zvířaty, která prodělala pouze záchvat.

Na základě těchto a předchozích experimentů lze oprávněně předpokládat, že nalezené výsledky jsou spojeny s vlivem melatoninu na vzestup volných radikálů v souvislosti se záchvatem a že se volné radikály podílejí na zhoršení učení po záchvatu. Samozřejmě je nutné brát v úvahu i další možné mechanismy působení melatoninu, jako například ovlivněním receptorů. Je sice známo, že melatonin může působit obecně proti záchvatům, ale to by pak neměl žádný vliv při aplikaci po záchvatu. Spíše musíme uvažovat o kombinaci jeho účinků.

Třetí experiment byl zaměřen na působení hypoxického preconditioningu na důsledky ischemické léze pro prostorové učení.

Tentokrát byla zvolena repetitivní normobarická hypoxie: 8% O2 v experimentální komoře po dobu 30 s, pak 30 s v normální koncentraci O2, vše za normálního tlaku; tento cyklus se opakoval 60x. Ischemickou lézi jsme se snažili prodloužením osvitu kalvy laserem lehce zvětšit, abychom byli připraveni snáze najít předpokládané zlepšení výkonů po preconditioningu.

Experimentální skupiny byly následující: 1) zvířata, která byla vystavena pouze normobarické repetitivní hypoxii 4 dny před testováním v Morrisově vodním bludišti, 2) zvířata jen s ischemickou lézí a od dalšího dne testovaná a 3) zvířata s normobarickou repetitivní hypoxií 3 dny před způsobením ischemické léze a následným testováním.

Všechna zvířata prokázala schopnost učení. Proti naivním kontrolám se významně lišily pouze výkony zvířat s preconditioningem a ischemickou lézí. Byly významně horší.

V tomto experimentu se nesplnil předpoklad, že po vystavení hypoxii dojde ke

zlepšení výsledků učení jako v předchozím experimentu. Naopak, došlo k jejich zhoršení. To si vysvětlujeme tím, že jsme použili delšího působení laseru v kombinaci s jinými faktory, pravděpodobně v kombinaci ischemické léze se závažnějším typem hypoxie. Pro řešení tohoto problému bude nezbytné navrhnout další pokusy s jiným uspořádáním např. chronické s opakovanou repetitivní hypoxií. Na základě prezentovaných výsledků zhoršeného učení v souvislosti s interakcí ischemické léze a repetitivní hypoxie je možné o chronických pokusech v budoucnu uvažovat jako o možném modelu spánkové apnoe.

Dosažené výsledky prvních dvou experimentů výrazně zvyšují předpoklad platnosti stanovené hypotézy, že se volné radikály mohou podílet na negativních funkčních důsledcích ischemie a epileptického záchvatu vyvolaného flurotylem. Bylo dosaženo stanovených cílů, avšak pokus o preconditioning normobarickou repetitivní hypoxií u ischemické léze vyvolal efekt právě opačný. Je však nesporné, že aplikace melatoninu má vliv na zlepšení funkcí postižených zvířat. Výstupem této práce budou dva směry. Jednak další sledování působení melatoninu a nejen z pohledu jeho vlivu na volné radikály, ale také hledání příčin odlišného působení repetitivní normobarické a hypobarické kontinuální hypoxie.

# Content

# 1. Introduction

## 1.1. Preface

Most of detriments of the central nervous system (CNS) are accompanied by increased free radicals (FR) production. It typically occurs as a consequence of reperfusion, hypoxia, injury, inflammation etc. The increase of FR production and their role has already been described in animal models of ischemia (Cao *et al.* 1988; Globus *et al.* 1995), hypoxia (Strasser *et al.* 1997; Tan *et al.* 1999) and epileptic seizures (Shin *et al.* 2011). An intermittent hypoxia that occurs in sleep apnea syndrome is presumed to cause changes also by an increased FR formation. It is assumed that free radicals play role in morphological and functional changes of mentioned models of CNS disorders. Knowledge of this raises the question: are some of the functional manifestations of different types of mentioned detriments connected with FR?

The explanation of this question is possible by either decreasing or blocking the production of FR or blocking their action in the nervous tissue. This could be done by activation of non-specific defence mechanisms involving recruitment of antioxidant enzymes before and after the elicitation of individual models of injuries. For this purposes it was needed to evaluate action of antioxidants and FR scavengers, in terms of prevention of insult consequences but also as treatment. Another option was induction of general tissue tolerance by preconditioning that also modifies FR production (Janoff 1964; Ostadalova *et al.* 1998).

Thus, this work was aimed at possibility to change the behavioral outcome of cerebral ischemia, hypoxia and epileptic seizure. It was evaluated with the use of the ethologic methods of analysis of rat behavior. Basic morphological assessment of the lesion (TTC staining) was also done.

Behavioral methods were chosen to estimate the extension of CNS impact consequences because: they are non-invasive and reflect not only morphological but also plain functional disturbances. This gives an opportunity to determine and monitor relatively subtle changes and also follow their pattern after intervention. There is one more reason for using behavioral methods: the behavioral changes could be the first objective signs that are seen in the stroke and epilepsy patients and what we are trying to manage in order to improve patients' quality of life.

The characteristic changes of CNS function following an insult in animal models could be sufficiently distinguished with the use of battery of sensorimotor test, e.g., the beam balance and the rotarod tests. These tests are sensitive to disorders of dynamic and static motor functions. Changes of cognition are evaluated by the tests in the Morris water maze, which reflect alterations in the spatial learning and memory. Evaluation of those test results can also give an insight into changes of another higher brain function as learning strategy and motivation. The results of behavioral tests were also correlated with morphological findings.

### 1.2. CNS insults – types, mechanisms and models

The human brain is one of the most protected organs against mechanical impact, impact from outside of the body. However, it is also the most sensitive to homeostatic changes. Whenever these changes are below the tolerance threshold – CNS activates non-specific defence mechanisms on the cellular level as well as the ones affecting the whole organism, e.g. activation of the stress response. But if the changes are intense or last long enough, brain damage follows. The extensive morphological injuries have been thought to be repairable only *in vitro* (Oorschot and Jones 1986). Although, some of plain functional disturbances could be quite fast returned to the normal, due to plasticity and redundancy of the CNS (Beck and Yaari 2008; Xian and Zhou 2004), nowadays it was already described morphological plasticity *in vivo* also (Herynek *et al.* 2009). Important role in this type of plasticity is attributed also to stem cells.

Factors that lead to disorders of CNS function and eventual morphological changes could be divided into two large groups: extracorporeal and intrinsic or homeostatic. The first group includes traumatic (Madikians and Giza 2006), infectious (Somand and Meurer 2009), toxic causes as well as other biophysical factors impact. This group is rather broad and will not be discussed in details in this work. The second group includes intrinsic factors that might lead to the nervous tissue damage: ischemia and hypoxia, seizures, genetic, neoplastic, autoimmune, etc. The mechanisms of damage are variant and different, however, all of them if are lasting long enough lead to the changes of function and eventually to cell death.

#### 1.2.1. Trauma

Experimental models of mild concussive head injury are usually based on the

use of different controlled-impact devices. The increase of FR production was described repeatedly (Cristofori *et al.* 2001; Iudice and Murri 2000; Kawamata *et al.* 1997; Tsai *et al.* 2010). There is an attention-grabbing beneficial role of pineal hormone melatonin application in mice models of injury, and its effect in the model was assigned to melatonin antioxidant and scavenger properties (Mesenge *et al.* 1998). However, trauma of the CNS is not the topic of presented work. It has been mentioned to complete the models related to the production of FR.

### 1.2.2. Cerebral ischemia

From the pathophysiological point of view an acute cerebral ischemia can be divided into global ischemia, ischemia of neocortex and focal ischemia (Mares 1995). Global ischemia is going to be just over-viewed but focal ischemia will be discussed in details. Ischemia of the whole neocortex is experimental model of circulatory failure. There is occlusion of vessels that leads to global ischemia of higher brain structures but perfusion of brain stem is preserved.

*Global cerebral ischemia*

In most of the cases of clinical practice, the acute global cerebral ischemia is a result of cardial syncopes, heart arrest or circulatory shock (Harukuni and Bhardwaj 2006). During this type of a short but intense insult, lasting just up to fifteen minutes, the energy substrate in the tissue, ATP, is markedly depleted. This may lead to the massive cell death in sensitive regions. These evident morphological signs of cell death develop within a significant delay after the global ischemic attack. The delay could be extending from hours to days, depending on how intensive and long-lasting was the attack. The functional changes follow the insult invariably.

The experimental models of global ischemia are developed by the decrease of blood pressure that leads to cerebral hypoperfusion. The chosen experimental animals for these models are gerbils. They have been chosen due to their anatomical feature: incomplete circle of Willis. Clamping of both gerbils' common carotid arteries would lead to global ischemia (Laidley *et al.* 2005). In laboratory rat models the 4-vessel-occlusion is used (Pulsinelli and Brierley 1979). To ensure that ischemia is global, both vertebral arteries are electrocauterized prior to clamping common carotid arteries. Both methods are highly invasive. Without precise monitoring of animal condition an unpredictable changes of experimental results may occur. Therefore this

type of cerebral insult was not suitable for purposes of present work.

*Focal cerebral ischemia*

During a focal ischemic insult of the tissue the pathogenetic processes differ from global ischemia in two ways. First of all, at the core of the ischemic lesion the blood flow is almost always higher than during global ischemia, thus longer insults are required to get damage (Lipton 1999). Second of all, there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, passing penumbra, and hence there are different metabolic conditions within the affected site.

Atherosclerosis is known to be the main case of vascular occlusion and stroke in clinical practice. The site of occlusion is at the region supplied by middle cerebral artery (MCA) in majority of cases. Other causes of focal ischemia with lesser incidence are thromboembolic accidents and vessel ruptures. Following an ischemic episode there are two ways of resolution: (1) recanalization of the thrombus leading to reperfusion of affected region and (2) necrosis of ischemic core surrounded by inflammation. In both cases there will be initiation of free radicals cascade and therefore focal increase in FR amount. This effect is mainly the result of oxidative discharge of polymorphonuclear cells. Additional and questionable contributing factor is an increased production of nitric oxide by vascular endothelium in the ischemic zone surrounding necrotic core, i.e. penumbra.

Spontaneous recanalization following thromboembolic accident may occur but at unpredictable time. Kassem-Moussa and Graffagnino (2002) describe in their review that spontaneous recanalization occurs during the first 6 to 8 hours from initial occlusion only in 17% of patients. Thereafter 50% of occlusions would be recanalized by the fourth day following thromboembolic accident (Kassem-Moussa and Graffagnino 2002).

By the time reperfusion of ischemic area occurs, some changes of cell metabolism will be present. Because of the decreased oxygen and nutrient supply cells are forced to start anaerobic pathways of ATP production and gradual depletion of its stores. Focal decrease of pH and increase acidity is supposed to lead to the early damage of neurons. Energy failure at the level of ischemic core develops. This leads to changes of membranes permeability, $K^+/Na^+$-pump failure, and calcium and sodium influx follows. There is also an increased glutamate release in the affected

area which recruits NMDA-receptors, $Ca^{2+}$ membrane channels, and evokes excitotoxicity. This is typical for penumbral ischemic tissue and it potentiates calcium influx. The increased intracellular calcium is thereafter able to potentiate either apoptosis or necrosis cascade. What way the pathogenesis would develop depends on intensity and duration of ischemia. Together with sodium calcium may also induce cellular and organelles' edema, membranes breakdown and necrosis surrounded by inflammation within 12 to 24 hours after ischemia has occurred. In addition, intracellular calcium stimulates the nitric oxide synthetase (NOS) activity and its expression, leading to an increased formation of peroxynitrite, i.e. highly reactive free radical. Excitotoxicity also contributes to the raise of FR production (Cheng *et al.* 2004). Free radicals can irreversibly react with constituents of cell membranes (proteins and phospholipids), and nuclear DNA. These interactions cause lipid peroxidation and membranes' damage, affect cellular regulatory mechanisms, and mutations of the genome.

Apoptosis after cerebral ischemia/reperfusion is one of the major pathways that lead to the process of penumbra cell death (Mattson *et al.* 2001). Reperfusion and therefore reoxygenation of ischemic cells results in increased oxidative load on mitochondria. Its outer membrane is becoming more permeable. Massive calcium influx further damages mitochondria, leading to exacerbation of energy failure. Sequestered mitochondrial $Ca^{2+}$ causes mitochondrial depolarization and swelling. The pro-apoptotic Bax protein is then translocated from the cytosol to the mitochondria and induces cytochrome C release from mitochondrial intermembrane space. This pro-apoptotic protein translocation is controlled by the family of Bcl-2 proteins. The cytosol cytochrome C leads to the formation of the apoptosome, i.e. a complex of apoptotic-protease activating factor-1, procaspase-9, and ATP. The apoptosome initiates autoactivation of pro-caspases and eventually activation of caspase-3, which leads to DNA fragmentation.

Necrosis, in contrast, is more common for more severe ischemia. It does not require any energy consumption but occurs by means of self-digestion when lysosomal and peroxisomal enzymes are released initially to the cytosol and later to interstitium. Damaged mitochondria also produce free radicals that potentiate cellular damage, especially if reperfusion is present, supplying sufficient amount o oxygen. Necrosis typically occurs in the core of ischemia while apoptosis does so in

surrounding penumbra.

The sequential cascade following arterial occlusion ad reperfusion is summarized in the Figure 1.2.1.

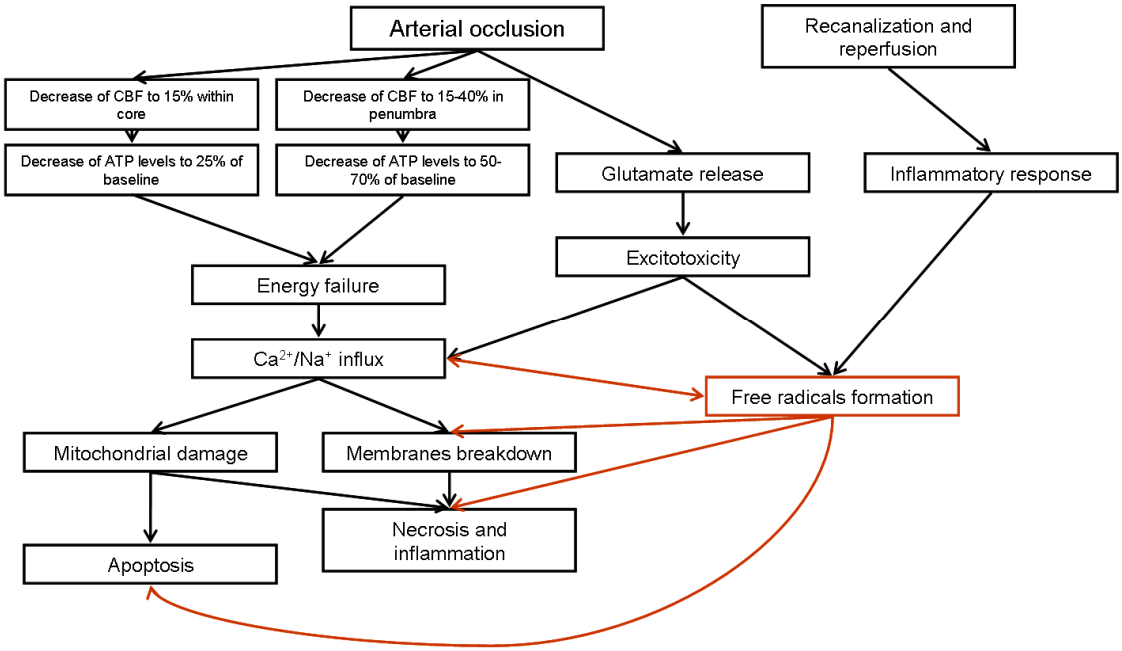



**Figure 1.2.1. Cascade following an arterial occlusion.** For details refer to the text.

The experimental models of the focal ischemic lesion are divided into two basic types – permanent and temporary (Table 1.2.1). The permanent ischemia leads to the ultimate cell death mainly by necrosis with irreversible functional changes. These models are used for evaluation of long-term consequences of cerebrovascular accidents and estimation of possibilities of improvement of the long term quality of life.

Animal models of temporary ischemia are characterized by recanalization and/or reperfusion that follow a thromboembolic occlusion (Schallert and Woodlee 2005). In the temporary restriction of blood flow the vasoconstriction also participates and its relaxation contributes to recanalization. Free radicals that are generated during the phase of restoration of blood flow to ischemic regions potentiate ischemia/reperfusion injury.

**Table 1.2.1. The most frequently used models of focal ischemia in rat.**

Adapted and modified from: SCHALLERT T and WOODLEE MT: Orienting and Placing. The behavior of the laboratory rat: a handbook with tests IQ WHISHAW and B KOLB. Oxford University Press, Inc., New York, 2005, 129-140.

| **Model** | **Preparation** |
|---|---|
| Middle cerebral artery occlusion model | Permanent occlusion can be done by thermo-coagulation of all or part of the middle cerebral artery or by permanent ligation.<br>Temporary - reversible occlusion by ligation or microfilament. |
| Embolism model | Injection of blood clot fragments (temporary ischemia until recanalization) or microspheres (permanent ischemia) into carotid artery. |
| Photochemically induced model | Transcranial and direct cortical intense light beam or laser irradiation after *i.v.* injection of a photosensitive dye. Induction of endothelial damage and platelet aggregation leads to blood clot formation to temporary ischemia until recanalization occurs. |
| Endothelin-1 model | Local application of endothelin-1 causing local vasoconstriction and temporary ischemia of the tissue until vasodilation leads to restoration of blood flow. |

The first two models of focal ischemia described in the Table 1.2.1 have some disadvantage in comparison to the photochemically and endothelin-1 induced models. First of all, thermocoagulation and ligation require extensive surgery, which necessitates accurate monitoring of the vital function, e.g. temperature, arterial blood pressure, arterial blood gases, and blood glucose. Second of all, there is a prolonged animals' recovery period that also has to be extensively monitored. And the last but not the least disadvantage, there is uncertainty whether reperfusion of ischemic lesion occurs and if it occurs then when. Therefore, these models were not the choice for the conducted study.

Photochemically induced ischemia model was introduced and described in 1984 by Dietrich and colleagues (1984). This type of experimental induction mimics naturally occurring process of thrombosis. Transcranial green-light activates circulating photosensitive dye, Rose Bengal that is apart from the others used the most. It triggers endothelial damage by free radicals, with marked platelet aggregation and microvascular stasis (Dietrich *et al.* 1987). In addition to tissue damage by ischemia itself, formation of free radicals during reperfusion phase continues to deteriorate clinical outcome. This model of ischemia has advantage in possibility of precise lesion location and its size gradation.

Focal ischemia induced by endothelin-1 was introduced by Agnati et al.

(1991). Endothelin, as vasoconstrictive oligopeptide synthesized by endothelial cells, was first described by Hickey (1985). It was discovered later on that endothelin is not a single peptide but group of structurally similar peptides: endothelin-1, endothelin-2 and endothelin-3. Endothelin-1 and in lesser degree endothelin-3 is synthesized also in the brain under the normal conditions. Furthermore, endothelin-3 is present in intestines and adrenals, and endothelin-2 was isolated from kidneys and intestines. The endothelins act through G protein–coupled receptor-mediated activation of phospholipase C. Two subtypes of endothelin receptors have been cloned from mammalian cells, $ET_A$ and $ET_B$. $ET_A$ receptors are specific for endothelin-1 and expressed in vascular smooth muscle and connective tissue. Their activation mediates vasoconstrictive and proliferative effects of endothelin-1 (Chabrier 1993). The receptors $ET_B$ bind endothelins with nearly equal affinity and it is expressed on endothelial cells and in epithelial tissues. Activity of $ET_B$ receptors mediates vasodilative response via nitric oxide release from endothelial cells. Studies also indicate important role of both subtypes of endothelin receptors during embryogenesis (Bonano *et al.* 2008; Druckenbrod *et al.* 2008). Although in experimental model of ischemia, endothelin-1 is used. It can be injected directly to the place of interest (corpus striatum, cortex), being delivered to cerebral ventricles or applied to the brain surface in proximity of middle cerebral artery after trepanation. It has been shown that there is a rapid drop in cerebral the blood flow within the minutes of endothelin-1 application. Blood flow decreases by 30-50% and it lasts few hours. It consequentially develops relatively uniform lesion with prominent margin of ischemic penumbra (Krysl 2007).

### 1.2.3. Cerebral hypoxia

Mammalian brains are absolutely dependent on adequate oxygenation to maintain its function. A decrease in the oxygen availability to the tissues, i. e. hypoxia, occurs in hypoxemia (hypoxic hypoxia), anemias of all types, including CO poisoning (anemic or transport hypoxia), or blood flow restriction (circulatory hypoxia), all resulting in the activation of local tissue metabolic response mechanisms. Hypoxemia that would lead to tissue hypoxia develops when the partial pressure of oxygen in the ambient air from the mean sea level value decreases to about 160 mm Hg (dry air). Under the normal conditions the $pO_2$ in arterial blood is about 105 mm Hg (Puchowicz *et al.* 2009). Most of the oxygen is carried by hemoglobin and at these

partial pressures hemoglobin is fully saturated. Hypoxemia develops when the arterial $pO_2$ falls below 90 mm Hg (Puchowicz *et al.* 2009), this can occur through decreased fraction inspired oxygen ($FiO_2$), decreased partial pressure of oxygen in inspired air due to increasing altitude and decreased barometric pressure, or as a result of lung pathology (pulmonary hypoxia).

During hypoxia tissues' compensatory mechanisms are activated in order to adapt to low oxygen delivery. The first response to develop is focal vasodilation to increase capillary surface area and increased oxygen delivery. Further compensatory changes are aim on adaptation to stabilize energy metabolism. The brain's response is dependent on the severity and length of time of exposure to hypoxia (acute and chronic). Acute and severe hypoxia leads to cell death due to the energy metabolism failure and sequent activation of either apoptotic or necrotic pathways. During chronic hypoxia systemic adaptations, such as increased ventilation, heart rate, blood pressure and increased erythropoiesis take place to support adequate oxygen supply to maintain neuronal function. CNS specific changes include a decrease in the of neuronal mitochondria volume density (Stewart *et al.* 1997) and increased cytochrome oxidase activity (Chavez *et al.* 1995; LaManna *et al.* 1996). As the consequence, the resting cerebral metabolic rate and oxygen demand decreases for about 15%. This decrease of brain activity is also related to the activation of hypoxia-inducible factor-1 (HIF-1) pathway that regulate energy metabolism (Chavez *et al.* 2000).

HIF-1 plays a role of an oxygen sensor in the brain (LaManna 2007). An expression of HIF-1 increases during hypoxia. The pathway of its expression is triggered by enzymes from dioxygenases' family, i.e. HIF-prolyl-4-hydroxylases (Appelhoff *et al.* 2004). Raised levels of HIF-1 up-regulate vascular endothelial growth factor and initiate capillary angiogenesis (Pichiule *et al.* 2004). HIF-1 is also a transcription factor that activates genes that have a hypoxic response element in their promoter region. An activation of these genes up-regulate secretion of glycolysis enzymes during hypoxia, leading to restoration of energy homeostasis (LaManna 2007).

Another member of hypoxia-induced factors family is HIF-2. Its role is described in cardiovascular development (Peng *et al.* 2000; Tian *et al.* 1998) and oncology research (Martin *et al.* 2011; Petrella and Brinckerhoff 2009).

There are numerous models of hypoxia. In neuroscience models of hypobaric, normobaric, continuous as well as intermittent hypoxias are widely used. Exposure to hypobaric hypoxia during ontogenesis, short-term continuous and intermittent, was described to play an important role in respect of epileptic seizure development (Maresova 2004; Maresova *et al.* 2005). Hypobaric hypoxia that is used experimentally represents naturally occurring condition that simulates high altitudes. Normobaric hypoxia, when the barometric pressure remains normal but the partial pressure of oxygen is decreased, could be found only under experimental condition. Such a condition can be found when entering the caves at see level. However in this case $pO_2$ is decreased because $pCO_2$ raises and hypoxia would be therefore accompanied by hypercapnia. In experimental settings hypoxia alone can be modeled by increasing partial pressure of nitrogen which has no effect on the cellular function at the see level pressure. Recent model that uses intermittent normobaric hypoxia has been brought into attention, as pathogenetic factor of peripheral sleep apnea (Fletcher 2001; Sica *et al.* 2000).

In experiment settings animals are exposed to hypoxia either acutely or chronically (Figure 1.2.2). Acute hypoxia can either activate programmed cell death

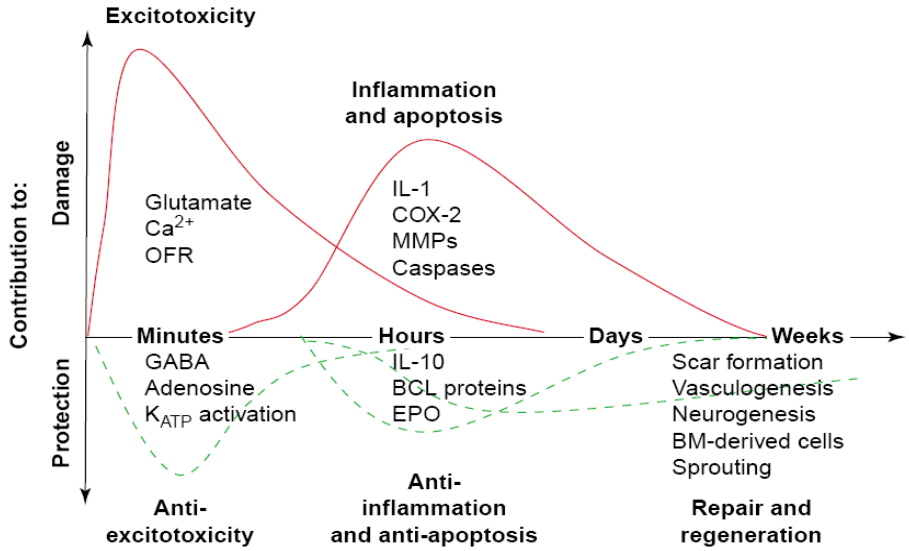



**Figure 1.2.2. Focal cerebral ischemia and hypoxia induces a complex series of mechanisms.** Brain tissue responds to most of the noxious signals by inducing protective mechanisms. In infarcted tissue, destruction overwhelms protection, although tissue around the ischemic core might have been spared by restored substrate delivery (as a result of collaterals, reperfusion, or vasculogenesis) and cellular mechanisms of protection. In this figure, the *x*-axis reflects the evolution of the cascades over time and the *y*-axis illustrates the impact of each element of the destructive (top) and protective (bottom) cascades on final outcome. BM, bone marrow; COX-2, cyclooxygenase 2; EPO, erythropoietin; IL, interleukin; MMPs, matrix metalloproteinase; OFR, oxygen free radicals.

Adapted from: DIRNAGL U, SIMON RP and HALLENBECK JM: Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* **26**: 248-54, 2003

and necrosis mechanisms or activate cellular defence mechanisms leading to tolerance; what way cell death proceeds depends on the intensity and duration of exposure. Chronic exposition to hypoxia in experiment is used to develop of adaptive reactions of organism. Development of adaptation also depends on intensity and duration of hypoxia. Molecular mechanisms of such response to hypoxia are related to those activated by preconditioning (that will be discussed below). Therefore, any type of hypoxia could initiate reactions leading to tissue injury, but it also might initiates neuroprotective mechanisms (Figure 1.2.2).

### 1.2.4. Epileptic seizures

The etiology of epileptic seizures and their classification is rather broad. The causes of epileptogenesis include genetic and post-traumatic causes, toxins, ischemia and hypoxia, fever, etc. Epileptic seizures were classified based on clinical and electroencephalographic signs into following tree groups (1981):

I. Partial seizures
    A. Simple partial seizures (no loss of consciousness)
    B. Complex partial seizures
    C. Partial seizures evolving to generalized tonic-clonic convulsions
II. Generalized seizures
    A. Absence
    B. Myoclonic
    C. Clonic
    D. Tonic
    E. Tonic-clonic
    F. Atonic
III. Unclassified epileptic seizures

Cerebral insult from either type of seizures is a dynamic process and whether the neuronal death occurs depends on the number of factors. The contributing factors involve genetic predisposition, development of excitotoxicity-induced mitochondrial damage, changes in cytokines produced, and oxidative stress (Shin *et al.* 2011). At the cellular level, kindling activity and experimental evoked potentials cause depolarization of cell membranes opening of voltage-gated and NMDA-dependent ion channels leading to increased calcium influx (Van Den Pol *et al.* 1996). Depolarization of the membranes also lead to release of magnesium from NMDA-

receptors, which is replaced by calcium that keeps NMDA-dependent channels open and potentiates calcium influx. The described mechanisms significantly participate in the development of excitotoxicity phenomenon. Elevated intracellular calcium activates biochemical cascades and therefore can lead to neuronal death (Henshall 2007; Mares *et al.* 2004; Mares *et al.* 2005). The sequence of the cell death in the hippocampus leading to gliosis, so called Ammon's horn sclerosis, as a result of status epilepticus and febrile seizures was described already in the 19$^{th}$ century. Ammon's horn sclerosis was also established as the commonest pathology in drug-refractory temporal lobe epilepsy (Meldrum 1997). The pathogenesis of epileptic seizure as well as other neurodegenerative disorders and neuropathologies, e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, involves oxidative stress induced damage (Perry *et al.* 2002). The increase generation of free radicals during and shortly after seizures is related to increased calcium neuronal influx. Increased intra-neuronal calcium, in addition to activation of cascades that were described in the previous sections of the thesis, also activates various enzymes that lead to neuronal death directly and via formation of free radicals, e. g. calpain that increases xanthine oxidase formation, phospholipase $A_{2,}$ protein kinases, nitric oxide synthase, and other (Meldrum 1997).

Experimentally induced epileptic seizures are aimed on understanding their short-term and long-term outcome. Short-term consequences are directly related to neuronal death and changes of energy metabolism. Long-term consequences in animal models are also reflected by cognitive changes, as worsening of spatial learning and memory. The animal models related to epilepsy are covering most of the possible seizure representations. The ways of seizure induction include electrical, hypoxic, thermal stimulation and others (Mares and Zouhar 1988; Stewart *et al.* 2009). There is also broad spectrum of chemicals that can evoke epileptic seizures, for example kainic acid, methotrexate, bicuculline, pilocarpine, etc. For the purposes of this work flurothyl, a volatile liquid convulsant, was used.

Flurothyl was previously used in psychiatry for shock therapy in treatment of intractable depression as an alternative to electroconvulsive therapy (Small *et al.* 1968). At the present, flurothyl is used for experimental modelling of generalized epileptic seizures (Holmes 1991; Veliskova *et al.* 2005) and in status epilepticus models (Sperber *et al.* 1999).

EEG recordings during status epilepticus elicited by flurothyl in study of Sperber et al. (1999) showed simultaneous discharges in the hippocampus and cortex areas. Furthermore, Araki et al. (2002) found presence of high voltage activity in amygdala. Also, Araki et al. had observed so called “transfer phenomenon” between amygdala and hippocampal seizure models, which represents similarity in behavioral manifestations. Although, there is also other type of seizure transfer when increased excitability transferred to the previously inactive areas, it was seen only in seizures induced by electrical stimulation (Michalakis *et al.* 1998; Spiller and Racine 1994). Flurothyl convulsant effect has been reported in relation to antagonism of $GABA_A$ receptor (Krasowski 2000). Benzodiazepine-like compounds, which activate GABA receptors, are shown to inhibit tonic/clonic seizures in Mongolian gerbils (Araki *et al.* 2002) and attenuate high amplitude spike waves in the amygdala, cortex and reticular formation (Aihara *et al.* 1982).

Even though, the exact mechanism of flurothyl neurotoxicity (in the absence of morphological changes (Ni *et al.* 2005)) is not well understood, it is assumed that FR generation during and shortly after seizures plays important role in changes of cognitive function.

### 1.3. Role of free radicals during CNS insults

FR are type of atoms, molecules, or ions with that carry unpaired electrons. Such radicals are highly reactive with very short half-life, $10^{-5}$ seconds or less in aqueous solution. Reactive oxygen species (ROS) are subtype of FR containing oxygen (from *Dorland’s illustrated medical dictionary*). ROS are formed as byproducts of the normal cellular metabolism of oxygen, including superoxide radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen. They play important roles in cell signaling and homeostasis.

However when energy metabolism is failing, an increased amount of ROS could be generated, leading to cellular damage and death. At physiological and mild pathological levels FR and ROS can be scavenged by tissues enzymatic and non-enzymatic antioxidant and scavenger systems. Enzymatic systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and peroxiredoxins (Prxs). To non-enzymatic systems belong vitamin C, vitamin E, and reduced form of glutathione (GSH). However, ROS levels exceed certain limits antioxidant defence ability of tissues fails leading to

oxidative stress. Furthermore, excessive ROS react with nitric oxide (NO) producing reactive nitrogen species, e.g. peroxynitrite ($ONOO^-$) (Figure 1.3.1).

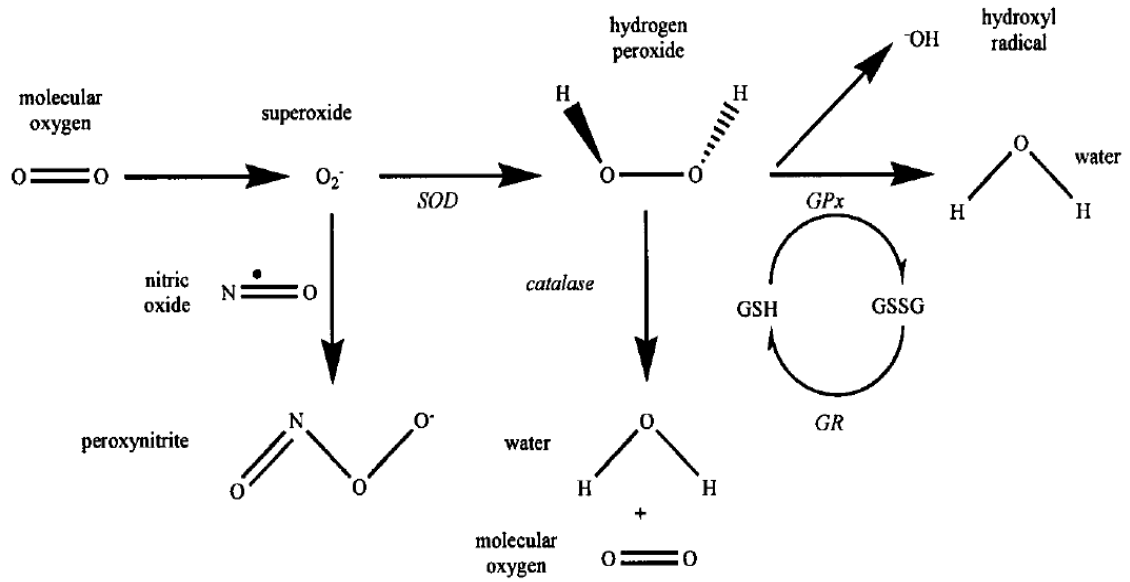



**Figure 1.3.1. Critical FR pathways in mammalian cells.** Molecular oxygen can be transformed into superoxide (O2·-) radicals by various enzymes, particularly in mitochondria. Superoxide can combine with nitric oxide (NO·) to form peroxynitrite (ONOO-), or can be dismutated by superoxide dismutase (SOD) to form hydrogen peroxide (H2O2). Hydrogen peroxide can be neutralized by catalase, by glutathione peroxidase (GPx),or can be transformed to a hydroxyl radical (-OH) by the Fenton reaction (not shown). GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Adapted from: SLEMMER JE, SHACKA JJ, SWEENEY MI and WEBER JT: Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. *Curr Med Chem* **15**: 404-14, 2008.

The mammalian brain is vulnerable to oxidative stress the most, because in comparison to other organs and tissues, it demands the highest amount of oxygen for energy metabolism. There are also other characteristics of the brain that put it at risk of oxidative damage. First of all, it contains high amount of polyunsaturated fatty acids that are prone to lipid peroxidation. Secondly, it is rich in iron that can induce hydroxyl radical formation (Demougeot *et al.* 2000). And the last property is decreased antioxidant defence in terms of low CAT (Kovacsova M. *et al.* 2010).

Oxidative stress results in changes of cells function and can lead to cellular damage. It may also cause cell death by oxidation of lipids, proteins and nucleotides. Oxidation of proteins results in changes of activity of various enzymes. Lipid peroxidation disrupts cell membranes integrity and changes their permeability. Nucleotides oxidation and sequent genom mutation involved in pathogenesis of neurodegenerative disorders and oncogenesis.

Conditions as ischemia, hypoxia, as well as epileptic seizures result in acute oxygen and glucose deficit in neurons and inability to synthesize sufficient amount of ATP by oxidative metabolism. During neuronal energy failure there is inefficient function of ion pumps leading to accumulation of intracellular $Ca^{2+}$. This can cause activation of cascades of apoptosis or necrosis and also to excessive release of the glutamate potentiating excitotoxicity. Homeostatic imbalance stimulates pro-oxidant enzymes (Chan 2001; Chan 2004) and increased ROS generation. During $Ca^{2+}$ overload occurring in ischemia $Ca^{2+}$-dependent enzymes (e.g., neuronal NOS and xanthine oxidase) increase their activity. Some of $Ca^{2+}$-independent enzymes are also activated. Those include cyclooxygenase 1 (COX1), COX2, and NAD(P)H oxidase.

Even more FR are produced after ischemia, when reperfusion occurs and brain oxygen tension increases. This, in combination with increases pro-oxidant enzymatic activity, is the reason for delayed necrosis of neurons in the area of penumbra (Love 1999). Cellular apoptosis is also triggered via redox signaling during reperfusion, contributing to delayed neuronal death hours or days after ischemic accident (Loh *et al.* 2006).

## 1.4. Possibility of neuroprotection during and shortly after a CNS insult

### 1.4.1. Neuroprotection by preconditioning

The term preconditioning, i.e. induced cellular tolerance to the injury was introduced by Janoff (1964). By Czech authors its cardioprotective effect was first described in 1998 on neonatal rats' hearts (Ostadalova *et al.* 1998) and in 2008 was described the effect of hypobaric hypoxia preconditioning to photochemically induced ischemia (Matejovska *et al.* 2008). The mechanism of preconditioning development could be initiated by any noxious stimulus preparing the tissue to further injury. This stimulus could potentially cause tissue damage but in contrast has protective effect when applied at close but below threshold of damage (Petzold *et al.* 2003). Even more, one type of noxious stimulus can be capable to induce cross-tolerance or cross-preconditioning for another following one (Kirino 2002). The stimulus should be detected by cellular sensor, converted into intracellular signals and transduced further to effectors of preconditioning response (Figure 1.4.1). It had been suggested that FR can also trigger preconditioning (Ajamieh *et al.* 2002). Defence mechanisms initiate within minutes of cerebral insult and continue to develop over weeks. Process of

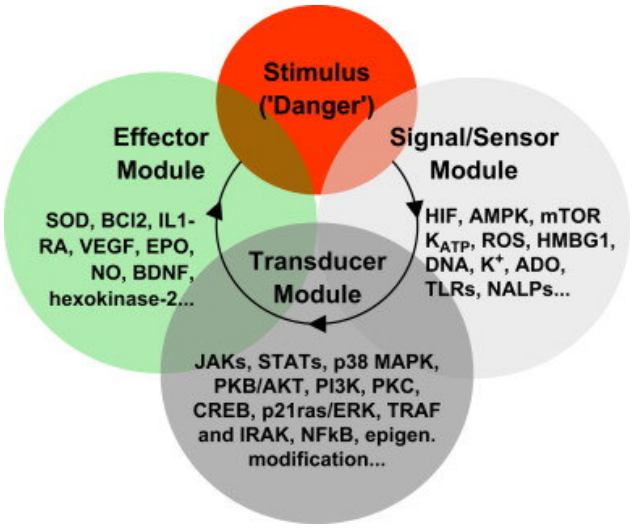



**Figure 1.4.1. A simplified scheme of preconditioning mechanisms.**

Adapted from: DIRNAGL U and MEISEL A: Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? *Neuropharmacology* **55**: 334-44, 2008.

preconditioning development can be divided into rapid and delayed. Rapid preconditioning effect develops within minutes after being triggered, although, its effect does not last for a long and fades with in 14 days. This type is more common for the myocardium (Lochner *et al.* 2009). For the brain is more typical delayed preconditioning (Kirino 2002).

Preconditioning effects of hypoxia or ischemia have no clinical use yet, however animal models provided valuable information on mechanisms of tissue defence and tolerance development. Phenomenon of preconditioning effect induced by ischemia was deeply studied mainly in myocardium (Lochner *et al.* 2009) and brain (Kirino 2002) but also retina (Li *et al.* 2000), kidneys (Fuller *et al.* 2005), and liver (Ajamieh *et al.* 2002) in the experimental settings. Hypoxia can facilitate mechanisms of cellular adaptation with eventual tolerance development mainly via induction of HIF-1, which modulates expression of so called “ischemia/hypoxia related genes” (Jones and Bergeron 2001), and increases secretion of vascular endothelial growth factor (Laudenbach *et al.* 2007), and erythropoietin (Bernaudin *et al.* 2002) in rodents. Intermittent hypoxia is characterized by periodic re-oxygenations with great ROS production as seen during ischemia-reperfusion event (Prabhakar *et al.* 2007). From this aspect intermittent hypoxia causes greater cerebral damage then continuous. However, there is no evidence on preconditioning induction by acute short-term intermittent hypoxia and its potential and this requires further investigations.

### 1.4.2. Neuroprotection by post-conditioning

At the present time the post-insult initiation of tolerance development, i.e. post-conditioning, appears to offer the best hope for functional recovery of the patients suffered brain insult.

Post-conditioning cardioprotective effect was first reported by Zhao (2003). It is

induced by brief cycles of reperfusion and ischemia at the reperfusion phase in the dog model. It has been shown that infarction size of animals subjected to post-conditioning was reduced. This was accompanied by decrease in plasma creatine kinase activity and reduction in edema. There was also lesser neutrophil accumulation in the ischemia area and preserved endothelial function. Neuroprotection by post-conditioning has been shown in the rat model. There was block of apoptotic markers in the penumbra two days after ischemic incident and attenuation of superoxide production (Xing *et al.* 2008). Post-conditioning has advantage in respect of potential clinical use over the preconditioning. It can be applied at the onset of the reperfusion when the insulting factor has been established. Never the less, experimental modeling of neuroprotective post-conditioning has some issues. Surgery techniques of reperfusion on the brain are invasive, there is also need of precise monitoring during and after surgery, and it is expected to be rather expensive (in terms of apparatuses used).

### 1.4.3. Neuroprotection by free radical scavengers and antioxidants

A variety of mechanisms provides defenses against FR damage. As was mentioned above FR scavengers and antioxidants can be synthesized readily within the body. Certain dietary nutrients play important roles as antioxidants. Vitamin C is effective in the plasma and cell cytosol, i.e. aqueous compartments. Vitamin E (tocopherol) and ubiquinol (co-enzyme Q) provide antioxidant protection in the body's lipid phase. Carotenoids also are believed to provide antioxidant protection in lipid rich compartments.

Although organism's natural antioxidant scavenger systems sufficiently balance daily fluctuations of FR levels, their action seems to be inappropriate for complete protection in certain pathological conditions. Therefore natural and synthetic scavengers and antioxidants have been more widely employed for prevention and as adjuvant treatment.

There is broad variety of antioxidants and scavengers on the market. For the present experimental work two FR scavengers and antioxidants – melatonin and tempol – were chosen.

Strong neuroprotective effect of melatonin is supposing due to its potent antioxidant and FR scavenger properties, both intracellular and extracellular (Reiter

1998). It is highly effective in scavenging hydroxyl radicals, nitric oxide and peroxynitrite anion; it also inhibits activity of nitric oxide synthase, directly detoxifies hydrogen peroxide and secondary superoxide anion radical. In addition, melatonin stimulates antioxidative enzymes acting intra- and extracellular, among which are glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase (Nigri *et al.* 2004). Finally, it stimulates the synthesis of glutathione, which is important intracellular antioxidant (Karbownik *et al.* 2001). However, achievement of significant neuroprotection requires very high doses of melatonin (100 mg/kg) (Costa-Lotufo *et al.* 2002; Mevissen and Ebert 1998).

Tempol is a stable, low-molecular weight piperidine nitroxide. It has been shown to be a potent membrane-permeable FR scavenger with SOD-like activity. It also has a potentiating effect of NOS and inhibits the sympathetic nervous system by actions that included activation of ATP-dependent potassium channels (Chen *et al.* 2007). In spontaneously hypertensive rat models tempol showed a reduction in blood pressure and lipid peroxidation (Schnackenberg and Wilcox 1999). In contrast to melatonin in majority of the studies lower dosages (5–30 mg/kg) of tempol were used (Guo *et al.* 2005; Rak *et al.* 2000). The dosage of tempol should be sufficient enough to have desirable effect; however, according to Thiemermann the exposure of the cells to higher concentration of tempol can lead to tissue damage via tempol-induced oxidative stress and apoptosis (Thiemermann 2003). Thus, more investigations are needed to determine appropriate dosage of tempol to have protective effect in ischemia/hypoxia models.

### 1.5. Evaluation of morphologic and functional changes in animal models

Detection of morphological changes are possible by classical tissue staining and microscopy (hematoxylin-eosine, Nissl, etc.) and also by histochemical and special staining analysis (ELISA, CPR, TUNNEL, TTC staining, Fluor Jade B).

Functional changes of CNS are well differentiated with use of cognitive and sensorimotor tests. Animals' behavior observed during these tests is the motor response to neural inputs from sensory system, cognitive and behavioral states (Figure 1.5.1) (Swanson 2000). Of course, it is difficult to determine in what exact level the problem is by analyzing results from a single test, and therefore the battery of behavioral tests needed tests.

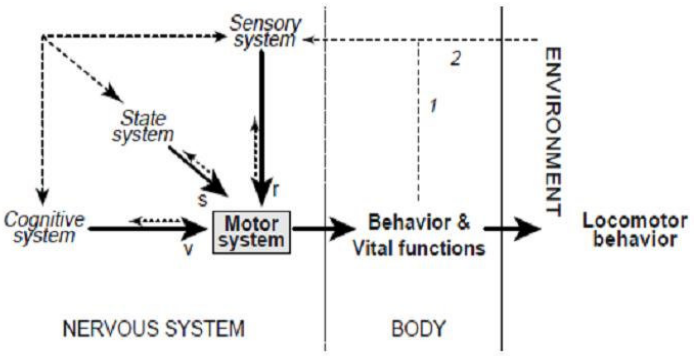



**Figure 1.5.1. A basic schema of loco motor behavior functional organizati on.** It is assumed that the motor system controls behavior and bodily vital functio ns, and that there are three classes of in puts to the motor system: cognitive, whi ch is responsible for voluntary (v) contro l; sensory, which is responsible for refle x (r) control; and behavioral state, which is responsible for state (s) control. Note that the sensory, state, and cognitive sy stems share connections in both directio ns, and that the results of internal (1) an d external (2) behaviors feed back throu gh the sensory system to influence futur e behaviors.
Adapted from: SWANSON LW: Cerebral hemisphere regulation of motivated behavior. *Brain research* **886**: 113-164, 2000.

*Sensorimotor tests*

An assessment of postural control, i.e. body balance, is possible by two ways. First way is the examination of animal's can be ability to maintain a base of support on a narrow rod with minimal movement, static motor control. Second way is observation of animal's ability to perform the task on moving basis while maintaining a stable position, dynamic postural control. Factors that influence postural control include afferent inputs from the somatosensory or proprioceptive, visual, and vestibular systems and efferent response that is affected by coordination, joint range of motion, and muscle strength (Bressel *et al.* 2007). Sensorimotor tests, beam balance and rotarod, require not only unaffected motor coordination but also ability to learn new motor task. Animals have a limited time to process and develop a new motor response to a new sensory input from the environment. In reverse rotarod testing mode animals also must be able to learn to turn to the direction opposite to cylinder rotation and fulfill the time criterion. Worsening of the performance in this test demonstrates role of cortical participation in the motor response development.

1. *Beam balance test* was designed to evaluate static postural reaction (gross vestibular function) (Alexis *et al.* 1995). This test can also reveal mild signs of ataxias.

2. Rotarod tests are frequently used to determine deficiency in motor coordination and it tests dynamic postural reactions (Donát 1999). Developed

by Dunham and Miya (1957) it proven to be the sensitive method for evaluation of motor deficit, vestibulomotor and somatosensory function (Dunham and Miya 1957; Hamm *et al.* 1994).

*Cognitive changes detection by Morris Water Maze tests*

In order to successfully orientate in the environment rat has to create "cognitive map" (O'Keefe and Nadel 1978; Stepankova *et al.* 2003). Process of spatial orientation and cognitive mapping of the environment includes two types of learning processes: allothetic and idiothetic (Bures and Fenton 2000).

During learning process an animal creates system of important elements of the external world and their relations to each other and to the organism. This internal model of the environment is represented by a cognitive map. An animal's behavior during the trials, i.e. spatial navigation is based on this model. In order to construct an adequate cognitive representation of the external world CNS requires information from different sensory inputs. The areas that process acquired information: the hippocampus and the parietal cortex (Roche *et al.* 2005).

An animal during navigation has two sources of information by which it can locate its position in test settings, allothetic and idiothetic (Bures and Fenton 2000; Whishaw *et al.* 2001).

The first source of information comes from a surrounding; an animal uses ambient cues (the sights, sounds, and smells, etc.). The inputs are acquired by exteroreceptors – visual, auditory and olfactory. Those are obtained during movement and exploration of the test setting and also when an animal enters or leaves the trial. These clues are allothetic cues and they are relatively stable stimuli, in terms of position and also last during quite long period of time. They are usually positioned in some distance away from an animal and therefore remain relatively fixed in the relation to an animal as it moves. They are used as reference points during spatial orientation. Allothetic system is highly dependent on visual inputs and requires light and relatively stable surrounding scene.

During the second type of aquisition process, i.e. idiothetic, an animal is processing sensory information from inner environment. There is number of cues that are available to an animal, which help it to track its self-motion. The information obtained from muscle and joint proprioreceptors and from vestibular system. These

inputs inform an animal about its speed of movement, velocities, turning angles and duration of movement. Using this information an animal is able to define its actual position retrospectively to the start point or to the some point on its trajectory of movement. In addition, an animal can learn reference copies of movements used for movement generation or, in the other words creates patter of the movement, i.e. process of motor learning.

1. Place navigation or the test of hidden platform acquisition is designed for evaluation of spatial learning abilities (Stewart and Morris 1993).
2. Probe test is designed for the memory retention evaluation. The strength of the memory is simply evaluated by allowing rat to swim in the absence of escape platform after animal has learned the platform location. This test can provide an index of animal's tendency to persist in searching in the place where the platform had been located during place navigation test, independently from escape latency and speed of swimming.

The development of video tracking software for analysis animal's performance in the Morris water maze tests gives wide range of parameters. It can calculate total distance moved and mean velocity in addition to classically measured escape latency. Also, measuring of total distance to platform during whole trial, meaning summarizing distances measured certain frames per second, gives us useful parameter – search error (Gallagher *et al.* 1993). This parameter helps understand search strategy because it describes how far from to target (platform) the animal was searching. In the other word how effective was that search.

The description of possibilities how to prevent morphologically prominent injury of the central nervous system is in literature relatively frequent. The morphological signs of injury and sequential functional changes were already described in details. Many authors successfully applied different scavengers and antioxidants and other neuroprotective techniques for prevention of worsening of the CNS insult outcome. The behavioral methods of evaluation in such studies are clear and usually related positive results with diminishing of injury. The problem rises though when a week noxious stimulus is use to model mild CNS insult. In this case only minimal or none injury can be detectable by morphologic methods. This is when the behavioral methods of disturbances examination are sensitive and reliable options for evaluation of the consequences of an insult.

## 2. Hypothesis and Aims

The increased generation of FR is described in relation to many processes that are known to lead to CNS disturbances as well as the ones that lead only to increased CNS energy demand.

**Hypothesis:**

FR are involved in functional outcome of certain cerebral insults that lead to neuronal disturbances.

**Aims**

A. Evaluation of functional changes induced by tree different types of pathological processes that are known to lead to increase of FR levels:
    a. Hypoxia
    b. Focal ischemia (mild damage to the cerebral cortex)
    c. Epileptic seizure

B. Examination whether there is possibility of these processes' modulation:
    a. By application of free radical scavengers and antioxidants
    b. Taking into account the assumption that mechanisms, which are playing role in development of hypoxic preconditioning phenomenon, are related to attenuation of FR production, the model of hypoxic preconditioning will be examined on cognitive changes resulting from cortical ischemia.

# 3. General Methods

All experiments were performed according to the guidelines of the Ministry of Health of the Czech Republic. The animal protocols (17658/2007-30 and 17659/2007-30) were approved by the Ethics committee of the Third Faculty of Medicine, Charles University in Prague.

The specific methods of induction of focal ischemia, intermittent hypoxia and flurothyl epileptic seizures will be described in detail in appropriate sections of individual experiments description.

In this section will be described methods of examination of sensorimotor function and spatial learning and memory. The ethologic evaluation following an individual CNS insult was performed for determination of functional changes. The performance of experimental animal groups was always to naïve control group.

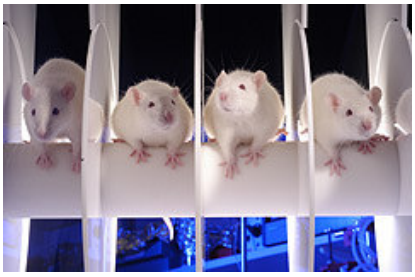
## 3.1. Sensorimotor tests

*Beam balance test*

The beam balance test was designed for evaluation of static postural reaction, i.e. gross vestibulomotor function (Murphy *et al.* 1995). This test also reveals mild signs of ataxias. Rats were positioned on a wooden bar (length – 400 mm, diameter – 10 mm, vertical height – 800 mm); the objective was to remain balancing on the wooden bar for 120 seconds. A maximum of 10 trials were allowed to meet the objective. The number of trials needed to meet the objective was recorded.

*Rotarod tests*

The rotarod test was used to determine deficiency in dynamic postural reactions, motor coordination (Donát 1999). It had been developed and described by Dunham and Miya in 1957 and it was proven to be a sensitive method for evaluation of motor deficit, vestibulomotor and tactile sensory function (Dunham and Miya 1957; Hamm *et al.* 1994). Two modifications of the test were used. In the first, the animals were placed on a cylinder (diameter – 115 mm; speed of rotation – 6 rpm) in an opposing



manner to that of the cylinder rotation. The animals' objective was to remain on the cylinder for 120 seconds in a maximum of 10 trials. In the second, reverse rotarod test, rats were placed on a cylinder oriented in the same direction as that of the rotation. The objective was for the rats to reorient themselves on the cylinder and remain there for 120 seconds. The number of trials needed to meet the objective was recorded.

*Evaluation of swimming in water maze – visible platform acquisition test*

The visible platform acquisition test in Morris water maze (MWM) was used to test the rats' motoric functions. In contract to sensorimotor tests mentioned above it equals motivation factor for all animals. The rats were placed in a water tank (diameter – 1.98 m; water temperature – 19-20 ℃) with a black plastic platform, 10 mm above the waterline. The animals were allowed to swim for 60 seconds; if the rat reached the platform, it was allowed to remain there for 30 seconds before starting the next trial. If the animal failed to locate the platform within 60 seconds, it was manually guided there by the experimenter and allowed to rest for 30 seconds. Eight trials were performed. The latency in platform acquisition, the total distance moved and the mean velocity were calculated in software EthoVision (Noldus, The Netherlands) (Deykun *et al.* 2011).

### 3.2. Spatial learning and memory tests

*Place navigation (hidden platform acquisition)*

This test is designed for evaluation of spatial learning abilities (Stewart and Morris 1993). The test is aimed to assess animals' spatial orientation, in terms of learning the position of a hidden under the water platform with use of extra-maze clues. This test was conducted over consecutive days with eight trials per day until animals learned the platform position. The trial protocol was similar to the visible platform acquisition test described above, with modification of platform that was transparent and hidden 20 mm below the water level. The latency in platform acquisition, the total distance moved, the mean velocity, and the search error (Gallagher *et al.* 1993) were

**Figure 3.2.1.** A snapshot from EthoVision video tracking system (Noldus)that shows an animal's trajectory in the Morris water maze.

calculated using the EthoVision video-tracking system (Figure 3.2.1).

*Probe test*

Twenty-four hours after the last place navigation trial, memory retention was evaluated using the probe test. The strength of the memory is simply evaluated by allowing rat to swim in the absence of escape platform after animal has learned the platform location. Rats were allowed to swim for 60 seconds. The percentage of time spent in the goal quadrant, the quadrant in which the escape platform was positioned during learning, the number of goal quadrant entries and the number of crossings over the area where the platform was previously located were recorded and quantified.

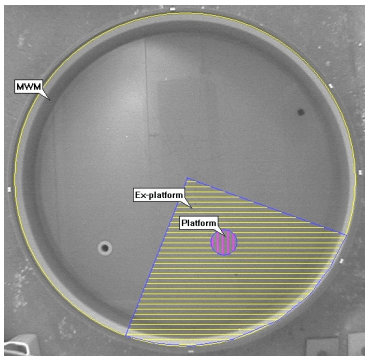



**Figure 3.2.2.** A snapshot from EthoVision video tracking system (Noldus) that shows setting of acquisition zones in the Morris water maze for probe trial.

### 3.3. Statistical analysis

Data were tested for normality of distribution using the Kolmogorov-Smirnov test. The Kruskal-Wallis test followed by Dunn's Multiple Comparison post-test was used as nonparametric test. One way ANOVA tests followed by Tukey's multiple comparison test single and two way ANOVA with repeated measures followed by Bonferroni post-tests were used when appropriate. Differences were considered significant if $p < 0.05$.

## 4. Results

### 4.1. Experiment I – Modulation of focal ischemia outcome

#### 4.1.1. Introduction

The green laser induced photothrombic ischemic lesions (Dietrich *et al.* 1984) were used as model of naturally occurring thromboembolisms in strokes. Laser activation of intravenously injected photosensitive dye induces endothelial damage via free radicals, leading to platelet aggregation and microvascular stasis (Dietrich *et al.* 1987). The functional outcome of focal ischemic lesion of sensorimotor cortex was modified with use of FR scavengers and antioxidants – melatonin and tempol.

### 4.1.2. Methods

The experiment was performed according to the guidelines of the Ministry of Health of the Czech Republic. The animal protocols were approved by the Ethics committee of the Third Faculty of Medicine, Charles University in Prague.

*Animals and reagents*

Sixty male Wistar rats (ANLAB, Czech Republic), 200-250 g, were randomly divided into five groups of 12 animals. One group was subjected to photothrombic cortical ischemia (Ischemia); two other groups, in addition to induction of ischemia, received the FR scavengers – tempol (IschTemp) or melatonin (IschMel). The fourth group was sham operated (Sham) and the fifth was a naïve intact control group (Control). Rats were housed in groups of four under 12-hour light/dark cycles and received food and water *ad libitum*.

All the chemicals used were supplied by Sigma – Aldrich®. Ischemia was induced under the general anesthesia: ketamine 100 mg/kg *i.p.* and xylazine 16 mg/kg *i.m.* The photosensitive die Rose Bengal – 4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetra-iodofluorescein disodium salt, 20 mg/ml/kg, 0.9% NaCl solution, was used. The following ROS scavengers and antioxidants were used: tempol – 4-hydroxy-TEMPO, 50 mg/kg, in 2 ml $H_2O$ solution; and melatonin – N-Acetyl-5-methoxytryptamine, 100 mg/kg in 2 ml of 2% Tween 80 solution.

*Induction of ischemia*

The induction of ischemic lesion was performed under the general anesthesia of 100 mg/kg ketamine *i.p.* and 16 mg/kg xylazine *i.m.* Animals' skulls were exposed and irradiated following *i.v.* application of Rose Bengal. A beam from a high-powered green-light laser (power density = 50 mW/mm$^2$, illuminated area < 1 mm$^2$) was subsequently centered on three points (6 minutes each) of the right side of the skull. Anteroposterior and lateral coordinates of the points were: 0, 5; 0.5, 4.1 and –0.5, 4.1 (Figure 4.1.1, A).

The irradiation was aimed on the following cortical areas: primary motor, premotor, and primary somatosensory, according to Palomero-Gallagher and Zilles (Palomero-Gallagher and Zilles 2004) (Figure 4.1.1, B).

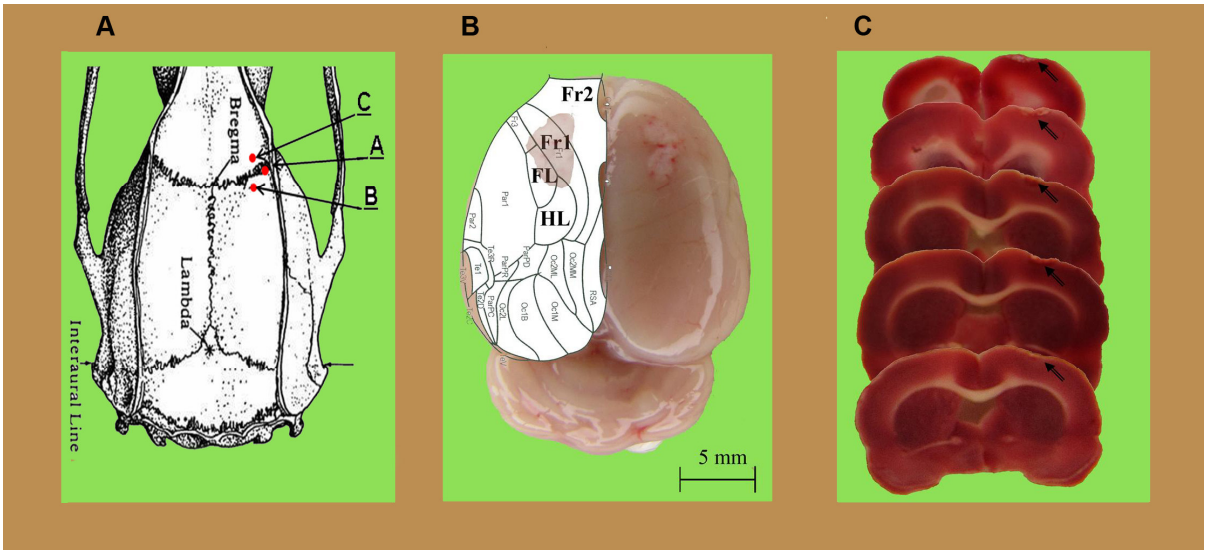



**Figure 4.1.1. Morphological depiction of the irradiated points and ischemic lesion.**
**A.** Figure illustrates the location of bregma and lambda on the laboratory rat's skull as defined by George Paxinos. Anteroposterior and lateral coordinates of the irradiated points (0, 5; 0.5, 4.1 and –0.5, 4.1) are marked red.
**B.** The native photograph of the animal's brain with the ischemic lesion at the frontal part of the right hemisphere. On the left hemisphere were superimposed cortical areas. The shadow area corresponds to the location and extension of the lesion on the opposite hemisphere. The following ares were involved: Fr1 – Frontal cortex, area 1 (primary motor); Fr2 – Frontal cortex, cortex area 2 (premotor, supplementary motor); FL – Parietal cortex, forelimb area (primary somatosensory); HL – Parietal cortex, hindlimb area (primary somatosensory).
**C.** The ischemic lesions on the brain slices are pointed out by arrows.
Adapted from: DEYKUN K, POMETLOVA M, SCHUTOVA B and MARES J: Modulations of behavioral consequences of minor cortical ischemic lesion by application of free radicals scavengers. Gen Physiol Biophys 30: 263-70, 2011.

After the end of laser irradiation, animals' scalps were sutured. The animals were placed for recovery to their home-cages into a dark environment in order to prevent photochemical injury to the retina as well as supporting the potentiation of the effect of melatonin (Talaei *et al.* 2010). The animals from the IschTemp and IschMel groups received an *i.p.* injection of tempol or melatonin respectively ten minutes after the end of irradiation. Sham operated animals received saline solution *i.v.* (0.9% NaCl, 1 ml/kg) and were also subjected to the laser irradiation; otherwise, a similar experimental protocol to the induction of ischemia was used. Intact control animals were kept in a dark environment for the same amount of time as experimental groups, i.e. until the testing phase started 24 hours after induction of ischemia. Thereafter animals were returned to the normal light/dark cycled regime (12 dark /12 light).

*Sensorimotor tests*

Twenty four hours after induction of ischemia animals were tested only with the

beam balance test (BB1) to avoid melatonin-related circadian rhythm influence on their performance.

Forty-eight hours after induction of ischemia, the rats were further tested by the beam balance test 2 (BB2), rotarod test (RT), reverse rotarod test (rRT) and MWM visible platform acquisition.

The tests' objective and experimental settings were described above.

*Spatial learning and memory tests*

Seventy-two hours after ischemic-induction, place navigation testing was started. It was conducted for six consequent days. Twenty-four hours after the last spatial learning trial the probe test was performed.

*Morphology*

Twenty-four hours after the probe test the animals were perfused transcardially with a 0.9% NaCl solution (8-10 ℃) under the general anesthesia. The brains were removed immediately after the perfusion and cut into coronal slices (thickness = 500 μm) at the level of the laser irradiation. The 2,3,5-triphenyltetrazolium chloride reduction test (Khan *et al.* 2000) was used to detect mitochondrial survival. Digital photographs of the slices were taken and evaluated for signs of ischemia.

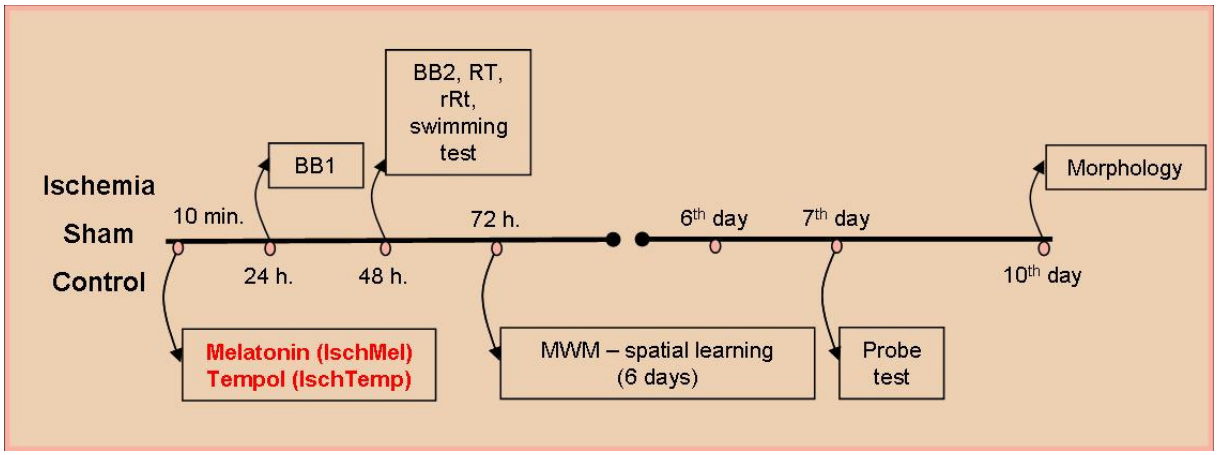



**Figure 4.1.2. Time schedule of Experiment I.** Abbreviations: IschTemp, IschMel – two groups, which in addition to induction of ischemia, received the FR scavengers, tempol and melatonin; BB1 – beam balance test 1; BB2 – beam balance test 2; RT – rotarod test; rRT – reverse rotarod; MWM – Morris mater maze.

### 4.1.3. Results

The morphological evaluation of the ischemic lesions showed minor but distinguishable changes in each animal and were maximally transcortical in the Ischemia group (Figure 4.1.1, C). The animals treated with melatonin and tempol had lesions reaching maximally into the IV-V cortical layers. In such diminished lesions, many times (n = 4), it was not possible to distinguish its borders.

*Sensorimotor changes*

There were no significant differences in the number of trials between either of group in the beam balance 1 (BB1) test performed 24 hours after induction of ischemia.

In the beam balance 2 (BB2) test performed 48 hours after the induction of ischemia (Figure 4.1.3, A), the Ischemia group showed a significant increase in the number of necessary trials compared to the Control ($p \leq 0.001$) and Sham animals ($p < 0.05$). IschMel and IschTemp did not differ significantly in the number of trials needed to meet the objective either from Ischemia or Control and Sham. Also, there was no statistically confirmed difference between Control and Sham animals in their performance.

The rotarod test (RT) performed 48 hours after the induction of ischemia did not reveal any significant disturbances in vestibular and tactile functions in experimental animals. There were no any significant differences in performance of Control, Sham and experimental animals (Ischemia, IschMel, IschTemp). However, in the reverse rotarod test (rRT) (Figure 4.1.3, B) followed RT, the Ischemia group performed more poorly than the Control ($p \leq 0.001$) and Sham ($p \leq 0.01$). There was no difference in performance of Control ans Sham animals. Also, the application of ROS scavenger melatonin decreased the number of trials needed for reaching the objective (IschMel $p \leq 0.001$). The group of animals with tempol application did not differ in performance from either group.

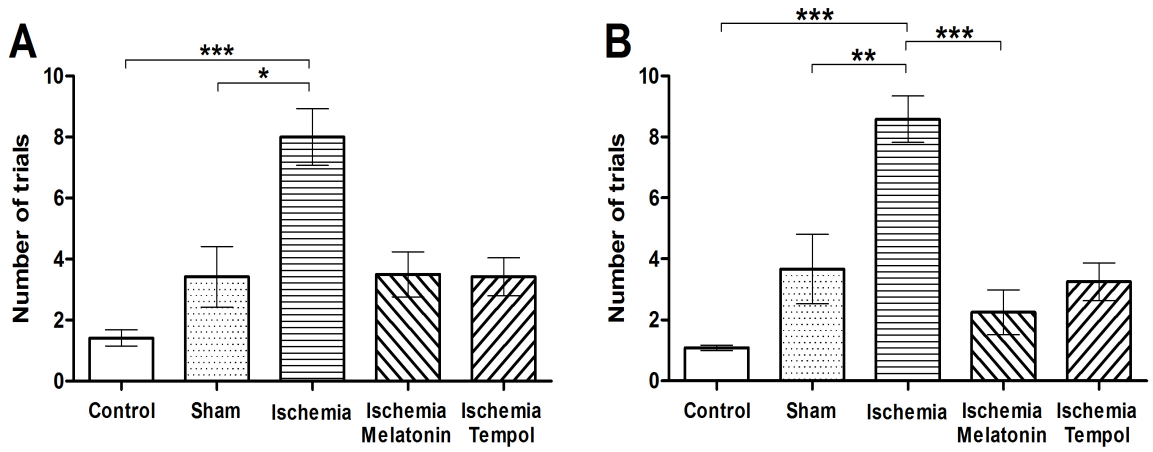



**Figure 4.1.3. Sensorimotor tests results**

**Graph A** illustrates the performance of each group individually in the **beam balance 2** (Mean ± SEM), which was conducted 48 hours after laser irradiation. Animals in Ischemia group needed more trials to meet the objective in comparison to the Control ($p \leq 0.001$) and Sham animals ($p < 0.05$). There were no other statistically confirmed differences in the performance between the groups.

**Graph B** shows performance of each group in the **reverse rotarod test** (Mean ± SEM). The performance of the Ischemia group was worse in respect to Control ($p \leq 0.001$) and Sham animals ($p \leq 0.01$). The group with Melatonin application following the induction of ischemia had lower number of trials needed to meet the objective ($p \leq 0.001$). There were no statistically confirmed differences in the performance between the rests of groups.

In the MWM visible platform acquisition test, we did not find any significant difference in the latencies of platform aquisition.

One way ANOVA of distances traveled to platform exhibited $p \leq 0.001$ and $F_{(4, 55)} = 6.458$. According to Tukey's multiple-comparison test, the IschTemp group showed a significant increase in the distance moved during trial compared to Controls ($p \leq 0.001$), Sham ($p \leq 0.01$) and IschMel ($p \leq 0.01$). No other significant differences in distances moved were determined.

ANOVA of the mean velocity showed $p \leq 0.001$, $F_{(4, 55)} = 6.48$. There was a significant increase of swimming velocity in the Ischemia ($p \leq 0.001$) and IschTemp ($p \leq 0.01$) groups compared to the Control. Ischemia animals also swam faster than their Sham ($p < 0.05$) counterparts. The IschMel group did not display any significant changes compared to the Ischemia or Control groups.

*Spatial learning and memory changes*

All the animals, regardless of treatment, demonstrated learning abilities during

the 6-day trial period, as demonstrated by the decreased latencies ($p \leq 0.001$, $F_{(4, 55)} = 43.41$), the moved distance ($p \leq 0.001$, $F_{(4, 55)} = 44.57$) and the search error ($p \leq 0.001$, $F_{(4, 55)} = 38.34$).

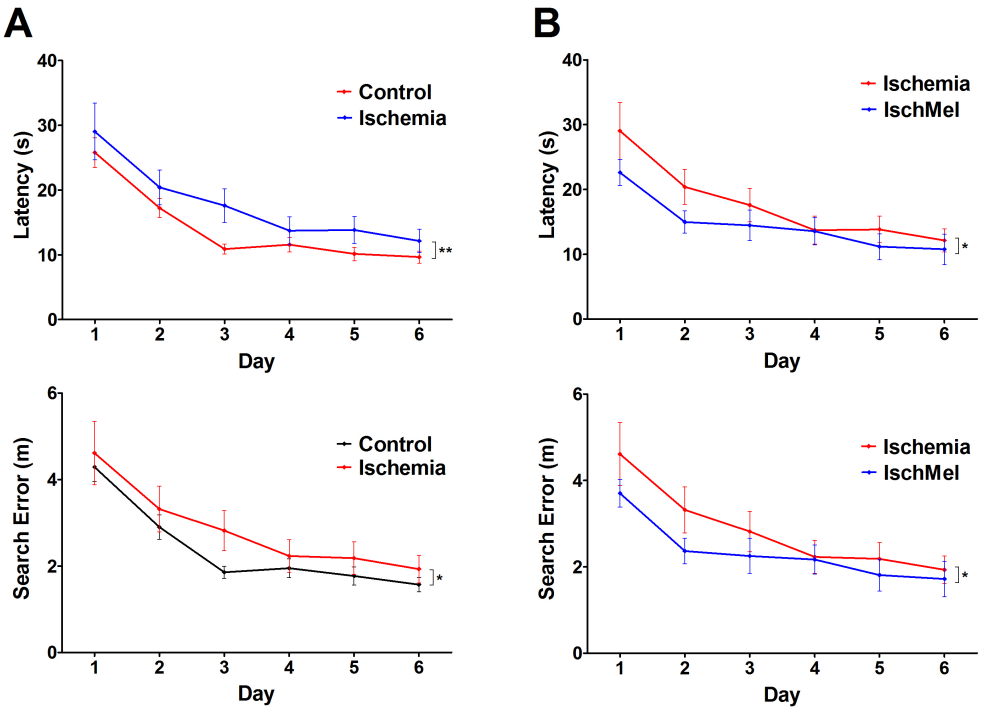



**Figure 4.1.4 – The Spatial learning in place navigation tests**

**Graphs A** illustrate the swimming latencies and cumulative distances (search errors) i n the place navigation test (Mean ± SEM) of Ischemia and Control groups. The animal s with induced ischemia needed a significantly longer time to find the hidden platform ( ANOVA: $p \leq 0.01$, $F_{(4, 55)} = 7.41$). Search errors in ischemic animals were also significa ntly higher in respect to the Control group (ANOVA: $p < 0.05$, $F_{(4, 55)} = 3.95$).

**Graphs B** represent a comparison of the swimming latencies and search errors of isc hemia-induced and Melatonin treated animals (Mean ± SEM). Melatonin treated group had shorter time needed to find the platform (latency) than the Ischemic animals (AN OVA: $p < 0.05$, $F_{(4, 55)} = 4.67$). The search error was also lower in Melatonin treated gr oup (ANOVA: $p < 0.05$, $F_{(4, 55)} = 3.93$).

Overall the Ischemia group performed poorly compared to the Control (Figure 4.1.4, A) and the IschMel (Figure 4.1.4, B) groups. It showed an increase in the latencies, search error (for significance see legend of figure 4,1,4) and the moved distance (Control: $p \leq 0.001$, $F_{(4, 55)} = 15.5$; IschMel: $p \leq 0.01$, $F_{(4, 55)} = 8.45$). Ischemia group also differed from the Sham group in the distance moved ($p \leq 0.01$, $F_{(4, 55)} = 8.61$). Tempol treated animals were significantly worse than the Control in latency to find the platform ($p \leq 0.01$, $F_{(4, 55)} = 6.74$) and distance moved ($p \leq 0.01$, $F_{(4, 55)} = 8.45$). They differed from the Sham ($p < 0.05$, $F_{(4, 55)} = 4.84$) and IschMel ($p < 0.05$,

$F_{(4, 55)}$ = 4.78) groups only in longer distances moved.

Unexpectedly for such a minor cortical lesion, all ischemia induced animals, regardless of treatment, showed an increased mean swimming velocity compared to the Control and Sham groups (Figure 4.1.5). Ischemia induced animals also had higher velocity than the IschMel group.

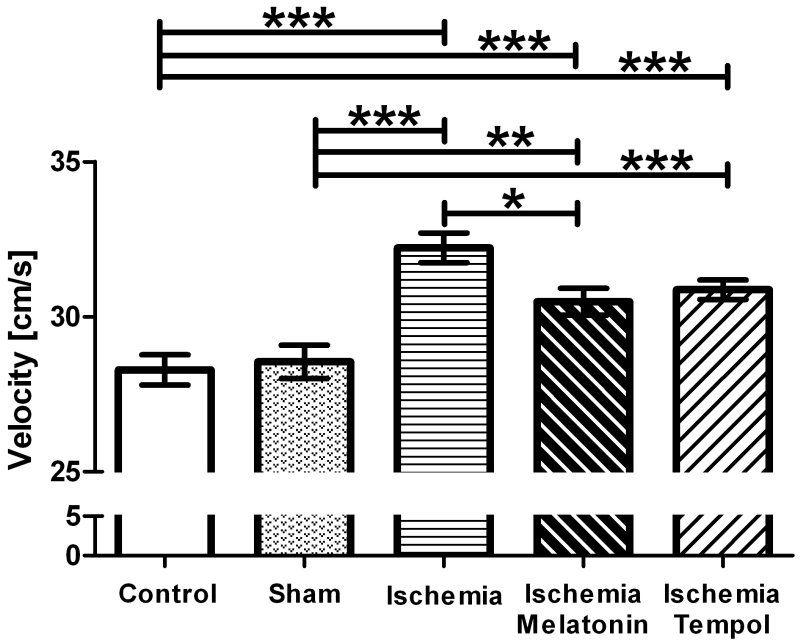



**Figure 4.1.5. Mean swimming velocities**

The graph depicts the mean swimming velocities during the place navigation test (Mean ± SEM, note that the *y* axis is interrupted). The significant differences are indicated by asterisks (*). Regardless of the treatment type, animals with ischemic lesion showed higher velocity compared to the Control and Sham animals. Melatonin treatment after the induction of ischemia caused the significant decrease in swimming velocity in comparison to untreated animals with ischemic lesion.

Significant ANOVA results: Control vs. Ischemia: $p \leq 0.001$, $F_{(4, 55)} = 37.74$; vs. Melatonin: $p \leq 0.001$, $F_{(4, 55)} = 12.39$; vs. Tempol: $p \leq 0.001$, $F_{(4, 55)} = 19.85$). The significance in the Sham treatment vs. Ischemia: $p \leq 0.001$, $F_{(4, 55)} = 27.97$; vs. Melatonin: $p \leq 0.01$, $F_{(4, 55)} = 8.09$; vs. Tempol: $p \leq 0.001$, $F_{(4, 55)} = 13.19$. The significance of the Melatonin treatment vs. Ischemia $p < 0.05$, $F_{(4, 55)} = 5.53$.

The Memory retention conducted by the Probe test in all groups was not affected.

### 4.1.4. Discussion

The presented model of temporary superficial ischemia of the sensorimotor cortex exhibited certain sensorimotor and spatial learning changes. The disturbance of the sensorimotor function was observed when animals had to learn a new motor

skill. The animals also showed modification of the learning strategy that caused an increase in search error during spatial learning. There was also hyperactivity present, in terms of increased swimming velocity in water maze tests.

The conducted study showed that the ischemic damage of sensorimotor cortex develops over 48 hours from onset of ischemia to cause detectable changes in function. The static postural reactions (gross vestibular function) that were examined by beam balance test (Alexis *et al.* 1995), showed to be affected 48 hours after induction of ischemia. The results showed that the Control and Sham animals had been able to learn a new motor skill, while the ischemic group lost this ability. There was evidence of slight disturbances in motor coordination as well. It was also observed that ischemia caused the worsening of the animals' performance in the reverse rotarod test (Žáčková 1984). The animals had to learn to reorient themselves to the direction opposite to the rotation of cylinder, in addition to being able to remain on cylinder for 120 seconds. Deterioration of performance in animals subjected to ischemia in this test clearly demonstrated the role of the sensorimotor cortex in the learning of new motor skills. On the other hand, there were no disturbances in the classic rotarod test assessing dynamic postural reactions of the animals (Donát 1999; Hamm *et al.* 1994) observed in this study. Therefore, superficial cortical lesions did not lead to a significant reduction in gross motor functions followed by this test. The results of sensorimotor tests demonstrated higher sensibility of the motor learning to a minor ischemic insult of the cortex than motor function itself.

The spatial learning ability in the Ischemic animals was also affected. Even though all the animals were able to learn the position of the platform hidden under water, there was a significant increase in the time (latency parameter) needed by the ischemic animals to find the platform. The role of motor learning must be also taken in account for understanding the results from MWM tests.

The hyperactivity was present in animals with the cortical lesion. It was observed 48 hours after ischemia induction in visible platform acquisition test and it lasted through whole spatial learning period. Similar increase in locomotor activity was already described in gerbils with global cerebral ischemia (Cuzzocrea *et al.* 2000; Gerhardt and Boast 1988) and mouse models of focal cerebral ischemia (Kilic *et al.* 2008; Winter *et al.* 2005). In the model of minor and superficial lesion such an increase was unexpected. The possible explanation of the mechanisms of increased

locomotion in the present model is the involvement of the cortico-striatal pathways, which could cause higher levels of noradrenaline in the striatum (Winter *et al.* 2005). The abnormally high firing rates from the damaged neurons would increase swimming velocity of the animals from the experimental groups.

In the two groups that received ROS scavenger treatment ten minutes after induction of ischemia, milder disturbances of function, in comparison to the pure ischemic animals, was observed. The effect of melatonin developed over 48 hours (beam balance test 1 showed no significant changes in comparison to Control and Sham operated) and it lasted over the entire experimental period (9 days in total). However, significant neuroprotection required very high doses of melatonin (100 mg/kg).

Concerning tempol, there was observed the great difference in parameters in respect to ischemic animals the after single dose of tempol (50 mg/kg, i.p.). Despite it, the difference was not statistically significant. Thus, the positive effect of this scavenger was not confirmed either by sensorimotor tests or test of spatial learning and memory. It has been stated by Thiemermann (2003) in his review work on tempol, that the dose needed to have protective effects had to be relatively high (up to 100 mg/kg). On the other hand, Thiemermann describes the exposure of cells to higher concentrations of tempol as potential risk of tissue damage via tempol-induced oxidative stress and apoptosis. According to this author the lower dose of tempol in this experiment could explain the reason why the effects were not impressive in this study. Scavenger activity of tempol was also described with use of lower doses than in this experiment (Rak *et al.* 2000). Thus, more investigations would be needed to determine the appropriate dosage of tempol required in inducing protective effects in ischemia/reperfusion models.

Unpredicted findings were observed in the performance of Sham animals. Minor cortical defects were observed on morphology slices in some animals. In addition, the spatial learning curves analysis showed that the findings in most of the test parameters did not differ from the Control, naïve animals but also from the ischemic group. It was observed that, even though presumed thermal injuries affected spatial navigation tasks, they did not cause hyperactivity, similar to that seen in the ischemic animals. Then the hyperactivity could be related to the ischemic component of the photothrombotic lesion but not only to the tissue defect itself.

## 4.2. Experiment II – Modulation of flurothyl epileptic seizures

### 4.2.1. Introduction

Protection against CNS damage is the goal of many experimental studies. Special attention is given to the consequences severe epileptic seizures. It is necessary to distinguish between protection against seizure onset and protection against the consequences of seizures.

It has been accepted the knowledge that severe and repeating seizures cause morphological damage as well certain functional changes. Classical example of morphological changes is gliosis seen in Ammon's horn sclerosis (Meldrum 1997). Many of animal models of status epilepticus have also shown changes in cognitive functions. The background of these changes was related to morphological damage, to be more specific – cellular death (Mares *et al.* 2005).

The consequences of a single seizure have not been evaluated in detail yet.

Generally, epileptic seizures increase levels of free radicals and a number of negative consequences are associated with this increase. Any significant change can affect homeostasis in various aspects of cell metabolism (Erakovic *et al.* 2000), changing different functions of the CNS, including behavior.

The experiment was aimed on assessing the protective effects of scavenger and antioxidant – melatonin, which was applied before and after the seizure development. The influence of melatonin on increases ROS production during and shortly after seizures was tested on the performance in MWM.

### 4.2.2. Methods

The study was carried out in freely moving animals. All procedures were performed in accordance with the Ethical Guidelines of the Third Faculty of Medicine, Charles University in Prague, Czech Republic and in concordance with the Guidelines of the Animal Protection Law of the Czech Republic, which correspond with respective EU regulations.

The experimental protocol was approved by the Ethical Committee of the Third Faculty of Medicine, Charles University, Prague, Czech Republic and special care

was taken to minimize animal suffering.

*Animals and reagents*

Adult, naïve, male Wistar rats (ANLAB, Czech Republic), 200–250 g, were used in the experiments. The rats were divided into four experimental and one control groups. Experimental groups: (1) flurothyl only – F (n=11) was exposed to increasing concentrations of flurothyl vapors until a tonic–clonic seizure was elicited; (2) melatonin + flurothyl – MF (n=10) was pre-treated with an melatonin 1 h before flurothyl seizure induction; (3) flurothyl + melatonin 150 s – FM1 (n=11) received melatonin 150 s after the seizure; (4) flurothyl + melatonin 6 h – FM2 (n=12) received melatonin 6 h after the seizure. Control group (C; n=12) was tested in the MWM 24 h after sham handling.

Epileptic seizures were induced by flurothyl (SynQuest Laboratories Inc., USA) – Bis(2,2,2-trifluoroethyl)ether, that was infused by the pump at the rate 30 µl/min. The FR scavenger and antioxidant melatonin (Sigma – Aldrich® Inc., Czech Republic) – N-Acetyl-5-methoxytryptamine, was applied *i.p.* at the dose of 100 mg/kg in 2 ml of 2% Tween 80 solution.

*Induction of flurothyl seizures*

The animals from the experimental group F were exposed to flurothyl in an air-tight chamber (volume = 14l) that was administered at a constant rate (30 µl/min), via an infusion pump. The infusion continued onto a filter pad suspended at the top of the chamber, until a tonic-clonic seizure was observed. Immediately after the seizure, the flurothyl-air mixture inside the chamber was evacuated and exchanged with fresh air. The animals were thereafter removed from the chamber and left to recover for 24 hours in their home-cages until the water maze testing started. The animals from three other experimental groups in addition to flurothyl seizure induction received melatonin as scheduled: group MF – 1 hour before induction of seizures, group FM1 – 150 sec after and group FM2 – 6 hours after seizures induction.

*Spatial learning test*

Twenty-four hours after the end of the seizure, place navigation testing was started. It was conducted for seven consequent days. The latency to reach the hidden platform was manually recorded by stopwatch.

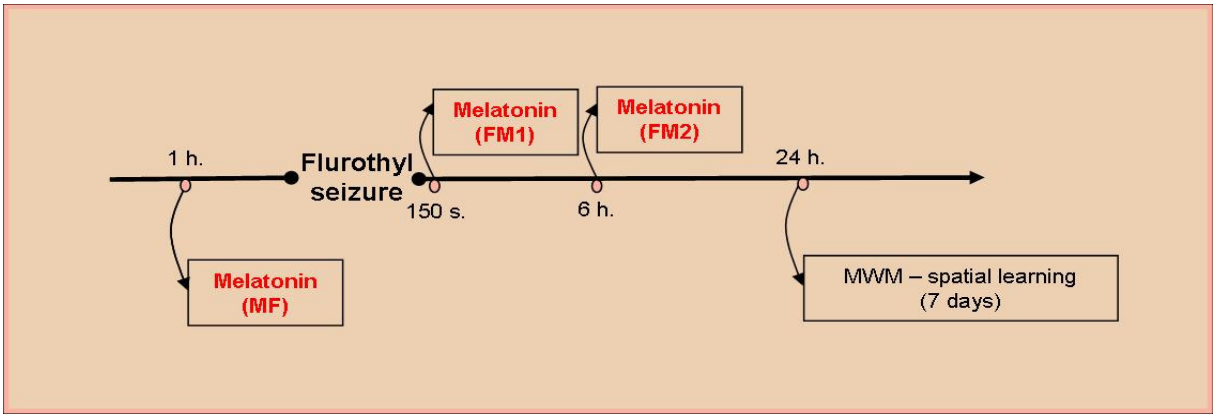



**Figure 4.2.2. Time schedule of Experiment II.** Abbreviations: F – animals exposed to flurothyl only; MF – animals pre-treated with an melatonin 1 h before flurothyl seizure induction; FM1– flurothyl + melatonin 150 s after the seizure; FM2 – flurothyl + melatonin 6 h after the seizure; MWM – Morris water maze.

### 4.2.3. Results

Learning occurred in all groups (Table 4.2.1). The latencies to reach the submerged platform shortened with daily repetition ($F = 340$; $DF = 6$; $p < 0.0001$).

**Table 4.2.1. Significant differences (two-way ANOVA, Bonferroni post-test, $p < 0.0001$) between the groups during the learning. Numbers represent individual days.**

| | | | |
|---|---|---|---|
| **F×C**<br>2, 3, 4, 5, 6, 7 | **F×MF**<br>4, 5, 6, | **F×FM1**<br>6 | **F×FM2**<br>NS |
| | **C×MF**<br>2, 3 | **C×FM1**<br>1, 2, 3, 4, 5, 6, 7 | **C×FM2**<br>2, 3, 4, 5, 6, 7 |
| | | **MF×FM1**<br>2, 3, 4 | **MF×FM2**<br>4 3, 4, 5, 7 |
| | | | **FM1×FM2**<br>2 |

Numbers represent the days when differences ($p < 0.05$) occurred when comparing two individual groups (NS=no significant difference during the whole course of learning – 7 days). Abbreviations for individual groups: C = controls; F = flurothyl application only; MF = melatonin 1 h before the flurothyl seizure; FM1 = flurothyl seizure and melatonin 150 s after the end of the seizure; FM2 = flurothyl seizure and melatonin 6 h after the end of the seizure.

In all groups, learning in the MWM showed significant shortening of latencies until the 4th or 5th day. Afterward, there were no significant changes in latencies in any of the groups. This plateau occurred in all groups, but significant differences were seen in the level of latencies reached. Based on the plateau level, groups could be split into two clusters. One contained controls (C) and melatonin prior to flurothyl (MF) groups, while the second contained flurothyl only (F), as well as both groups in which melatonin was given after the seizure (FM1 and FM2) (Table 4.2.1).

The significant differences between groups exhibited two phases when the MF was compared to C and F groups. At the beginning of learning, group MF had longer latencies to reach the platform. The situation was reversed during the last days of learning when occurred shortening of latencies in comparison with group F and those latencies did not differ from control group (Figure 4.2.1).

### 4.2.4. Discussion

In the experiment was demonstrated that a single, relatively short flurothyl induced tonic-clonic seizure prolongs latencies associated with finding the submerged platform in the MWM test in comparison with the control group. The preliminary study (Mares *et al.* 2005) suggests that it is not related to neuronal death. The neuronal damage or morphological disturbances associated with this model was not found. Therefore the seizure outcome was clearly functional. Some animal studies have demonstrated that learning deficits following status epilepticus or repeated seizures may occur also without any detectable neuronal cell death (Stafstrom 2003).

Epileptic seizures increase normal levels of ROS (Erakovic *et al.* 2000). Negative consequences of seizures are often attributed to this increase (Kovacs *et al.* 2002). ROS influence many neuronal functions and therefore the protective function of scavengers and anti-oxidants is not surprising (Franceschini *et al.* 1999).

Pretreatment with a large dose of melatonin significantly improved performance in rats exposed to the seizure by decrease of learning latencies. Pre-treated group demonstrated decreasing latencies in reaching the submerged platform over the 7-day test protocol. In contrast, when melatonin was applied 150 s after the seizure, it had only slight, but significant effect. When melatonin was applied six hours after the seizure it exhibited almost no effect.

Melatonin has been shown to influence the threshold of seizures elicited by repetitive electrical stimulation in rats, especially in relation to the dose of other antiepileptic drugs (Borowicz *et al.* 1999). It has been demonstrated that melatonin protects against degradation of mitochondrial DNA and lipid peroxidation, and simultaneously limits seizures after kainic acid application (Mohanan and Yamamoto 2002). Never the less, the neuroprotective effect of melatonin is seen when it is applied before seizure but not after. From there facts it can be concluded that the changes in neuronal function probably due to increased ROS develop only during seizure and last long enough to decrease spatial learning ability. Thus, learning impairment after a flurothyl seizure is closely related to an increase in ROS production during seizure. The prevention of learning impairment is possible by melatonin. We can suppose that it is related mostly to its anti-oxidant and scavenger properties.

## 4.3. Experiment III – Evaluation of hypoxic preconditioning

### 4.3.1. Introduction

The endogenous protection gained by the nervous tissue ability to 'condition' itself, has been brought into attention as a powerful new strategy for limiting CNS injury. It was already mentioned (Mares *et al.* 2005) that hypobaric hypoxia exposure has neuroprotective effect over flurothyl epileptic seizure's induced worsening of cognitive function. The similar hypoxic preconditioning effect of hypobaric hypoxia was expected be found in respect to photothrombic cortical ischemia. Never the less, it showed to have only minimal effect (Matejovska *et al.* 2008).

The purpose of this experiment was to assess a possibility of hypoxic preconditioning elicited by intermittent normobaric hypoxia exposition prior to induction of cortical ischemia. This type of hypoxia due to switching of phases of hypoxia and normoxia would simulate the condition of ischemia/reperfusion preconditioning on the heart (Hausenloy and Yellon 2009).

Chronic intermittent hypoxia that models type of hypoxia that is seen in patient with obstructive sleep apnea is known to cause cardiopulmonary and CNS changes (Kalaria *et al.* 2004). Chronic continuous and intermittent hypoxia was shown to have an effect on systemic blood pressure, increase hematocrit and mild changes of cerebrovascular adaptation in relation with NOS inhibition (Barer *et al.* 2006). During

one-hour session of intermittent normobaric hypoxia the increased amount of FR production was expected to be sufficient to facilitate an action of endogenous scavenger and antioxidant systems. As a result the hypoxic preconditioning was expected to be elicited and thereafter to prevent photothrombic ischemic lesion sequential worsening of cognitive function.

### 4.3.2. Methods

The experiment was performed according to the guidelines of the Ministry of Health of the Czech Republic. The animal protocols were approved by the Ethics committee of the Third Faculty of Medicine, Charles University in Prague.

*Animals and reagents*

Forty male Wistar rats (ANLAB, Czech Republic), 200-250 g, were randomly divided into four groups (n = 10). There were three control groups and one experimental. The control groups included: naïve control group (C), ischemia group (Isch) that was subjected to photothrombic cortical ischemia and hypoxia group (H) that was exposed to intermittent normobaric hypoxia. The experimental group (HIsch) was subjected to ischemia after and exposure to hypoxia. Rats were housed in groups of four under 12-hour light/dark cycles and received food and water *ad libitum*.

All the chemicals used were supplied by Sigma – Aldrich®. Ischemia was induced under the general anesthesia: ketamine 100 mg/kg *i.p.* and xylazine 16 mg/kg *i.m.* The photosensitive die Rose Bengal – 4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetra-iodofluorescein disodium salt, 20 mg/ml/kg, 0.9% NaCl solution, was used.

The nitrogen that was used for induction of intermittent normobaric hypoxia was supplied by Messer Technogas, Czech Republic.

*Exposure to the intermittent normobaric hypoxia*

The intermittent normobaric hypoxia was induced in the air-tight chamber with $F_iO_2$ cycled from 21 to 8% and back to 21% every one minute during one hour. The normobaric pressure was achieved by admixture of nitrogen to ambient air. After an exposure to hypoxia animals were placed to their home-cages for recovery. 72 hours later the H group animals were weighted and habituated and 24 hours later proceeded to behavioral testing. The animals from group HIsch were subjected to

photothrombosis as described above 72 hours after an exposure to hypoxia. 24 hours lather they also entered to. Control animals were only weighted and habituated 24 hours prior to behavioral testing.

*Induction of ischemia*

The induction of ischemic lesion was performed by the procedure protocol described in the section 4.1.2 with modification of the duration of laser beam irradiation. The irradiation beam was subsequently centered on the three points for 8 minutes, in contrast to experiment I, where the irradiation for 6 minutes at each of three points. The aim was to induce slightly bigger lesion that would cause more prominent changes in animals' cognition that was described on experiment I. After the end of laser irradiation, animals' scalps were sutured. The animals were placed for recovery for 24 to their home cages, until behavioral testing began.

*Cognitive changes assessment – MWM spatial learning test*

24 hours after ischemia induction in Isch and HIsch group and habituation in groups H and C, place navigation testing was started. It was conducted for seven consequent days. The performance in the test was evaluated by changes in latencies to reach the submerged platform.

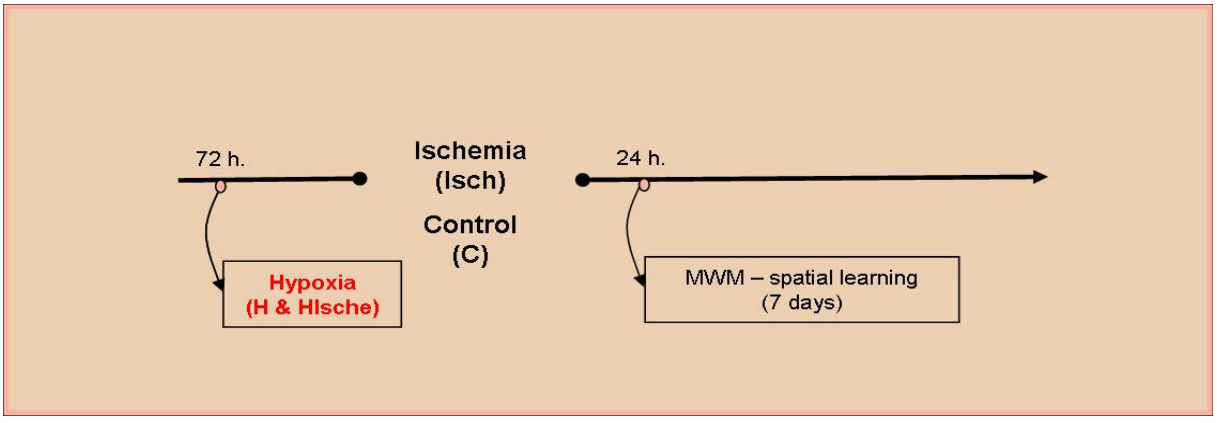



**Figure 4.3.1. Time schedule of Experiment III.** Abbreviations: H – group exposed to intermittent normobaric hypoxia only; Isch – group with photochemically induced ischemic lesion; HIsch – ani mals exposed to hypoxia and 72 hours later had ischemia induced; C – control group; MWM – Mor ris water maze.

### 4.3.3. Results

All the animals, regardless of intervention, demonstrated learning abilities during the 7-day trial period that was demonstrated by the decreased swimming latencies ($p \leq 0.0001$, $F_{(3, 36)} = 222.6$).

Exposure to hypoxia did not affect animals' performance in comparison to control group (Figure 4.3.2).

Overall the Isch group performed poorly compared to the C group (Figure 4.3.3). It showed an increase in the latencies (Two-way ANOVA: $p \leq 0.0001$, $F_{(3, 36)} = 12.94$). However Bonferroni post-tests did not reveal any significant differences within individual days of trials for these two groups.

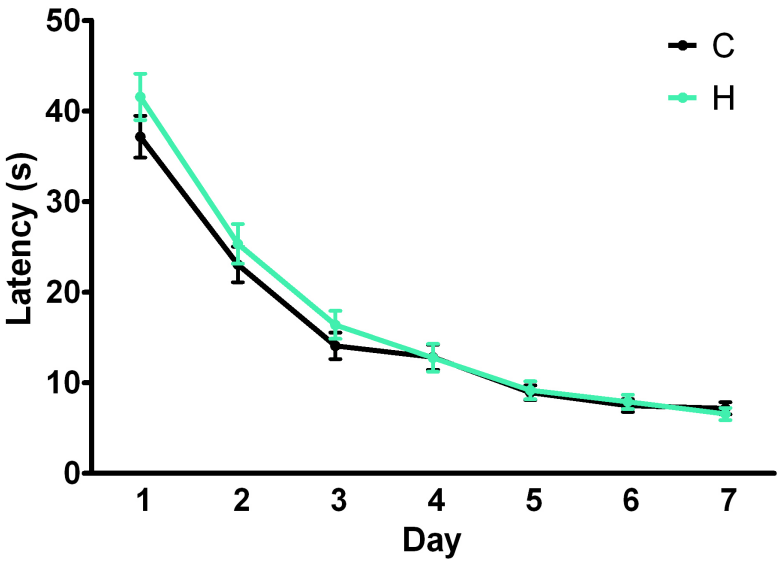



**Figure 4.3.2. Spacial learning curves in plase nevigation test** over 7- days p eriod illustrate the swimming latencies (Mean ± SEM) of control (C) group and t he group exposed to intermittent normobaric hypoxia (H), which did not significa ntly differ.

Unexpectedly, the experimental group of ischemia preconditioned by hypoxia did not show improvement in comparison to Isch group. In contrary, there was worsening in performance comparing to control animals, even greater then C vs. Isch (two-way ANOVA: $p \leq 0.0001$, $F_{(3, 36)} = 32.49$; Figure 4.3.3), especially on trial day 2 (Bonferroni post-tests: $p \leq 0.01$) and trail day 3 (Bonferroni post-tests: $p < 0.05$).

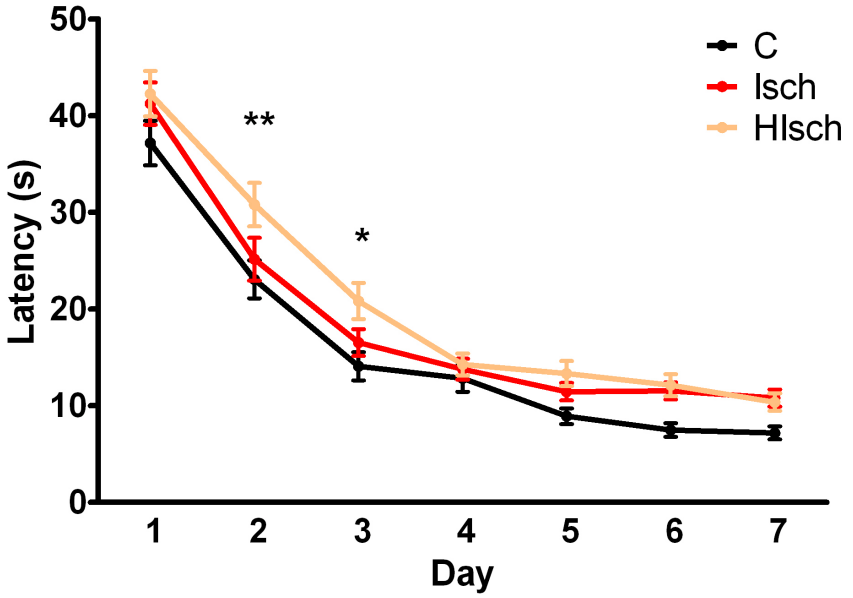



**Figure 4.3.3. Spacial learning curves in plase nevigation test** over 7- days period illustrate the swimming latencies (Mean ± SEM) of control (C) group, group with cortical ischemia (Isch) and the group exposed to intermittent normobaric hypoxia and 72 hours later was subjected to photothro mbic ischemia (HIsch). C shoved better performance then Isch ($p \leq 0.0001$, $F_{(3, 36)} = 32.49$) and HI sch ($p \leq 0.0001$, $F_{(3, 36)} = 32.49$). The significant differences within individual days of trials between C and HIsch ($2^{nd}$ day $p \leq 0.01$; $3^{rd}$ day $p < 0.05$) are indicated by asterisks (*).

HIsch group also overall differed from the Isch group (two-way ANOVA: $p < 0.05$, $F_{(3, 36)} = 5.299$; Figure 4.3.3). Similarly as was demonstrated by comparison of C and Isch, Bonferroni post-tests did not reveal any significant differences within individual days of trials.

### 4.3.4. Discussion

The present experiment did not show development of protective hypoxic preconditioning induced by intermittent hypoxia over ischemic cortical lesion. In contrast, there was worsening of animals' performance in the test of spatial learning. This effect could be explained, first of all, by prolonged laser irradiation then experiment I. The second factor that could attribute to the functional disturbance is combination with more intensive and therefore more severe type of hypoxia than

hypobaric hypoxia (Matejovska *et al.* 2008). Hypobaric hypoxia, which was induced in the mentioned work, did not induce sufficient preconditioning either, however it did not cause worsening of spatial learning in combination with ischemic lesion as intermittent normobaric hypoxia. This type of hypoxia was mimicking slow ascending to a high altitude with continuous decrease of partial pressure of oxygen in inhaled air during 15 minutes. Animals were exposed to hypoxia for 30 minutes and than the pressure was returning to the normal (at see level) during 15 minutes. In contrast to the present work there were no rapid fluctuations of partial pressure oxygen, each minute. Repeated switching between hypoxia and normoxia that is present in intermittent hypoxia is known also to induce stress leading to neuronal death in addition to cardiovascular effects (Prabhakar *et al.* 2005; Xu *et al.* 2004). It would be advantageous the measurement and comparison of stress hormones levels in these two types of hypoxia for understanding pathogenic mechanisms. Unfortunately, it is not possible because the apparatus that was used for modeling of hypobaric hypoxia is not to disposition any more.

To evaluate possibility of development of hypoxic preconditioning initiated by intermittent normobaric hypoxia to other types of cerebral insult the new experimental design should be proposed. The new paradigm of this type of hypoxia use could be chronic with repeated use of intermittent hypoxia of lesser intensity. On the basis of presented results, there is certain interaction of intermittent hypoxia with cortical ischemic lesion that leads to disorder of spatial learning. Chronic, long-term, intermittent hypoxia that is used for modeling the sleep apnea syndrome was described to cause deficiency in cognition (Li *et al.* 2011) and hippocampal neuronal death (Klein *et al.* 2003). It is important to separate two pathogenic reactions: reaction of cardiovascular system to hypoxia (Lefebvre *et al.* 2006) leading to brain ischemia and the neuronal system functional changes in response to intermittent hypoxia (Gozal and Gozal 2001; Wang *et al.* 2010). There was also described an increased incident of comorbidity of sleep apnea and stroke in patients (Rajagopalan 2011).

As to the syndrome of sleep apnea itself, it is a frequent, clinically significant disease associated with higher morbidity and decrease of the life span. It is characterized by the repeated breathing cessations during sleep with consequential intermittent hypoxia. This leads not only to distortion of the sleep continuity and

structure, but also to serious somatic consequences (hypertension, heart failures, arrhythmias, cerebrovascular episodes and endocrine disorders) (Lanfranchi and Somers 2001). The presence of the sleep apnea also worsens prognosis of the patients with cerebrovascular episodes and has facilitating effect on the nocturnal epileptic seizure episodes (Sonka *et al.* 2000) and the development of the interictal epileptic manifestations (Jakoubkova and Sonka 2003).

The known mechanisms of systemic changes caused by the sleep apnea include increased sympathetic activity (Mills and Dimsdale 2004; Prabhakar *et al.* 2007), increased production of reactive oxygen species (Prabhakar *et al.* 2007; Sonka *et al.* 2008). These mechanisms closely resemble the ones that are initiated by intermittent normobaric hypoxia, which was used in the present study. That is why it could be profitable to adapt current model in terms of implementation to the animal model of obstructive sleep apnea syndrome with comorbid cerebrovascular accident development.

## 5. General discussion

The experiments were aiming on evaluation of hypothesis that FR are playing an important role in the pathogenesis of different CNS insults and development of their functional outcome.

The major conclusive result of the experiment I is that the FR scavenger and antioxidant, melatonin, improves performance of animals subjected to ischemia of sensorimotor cortex in behavioral tests. These test evaluated animals' sensorimotor coordination, static and dynamic motor function and spatial learning. It was shown that motor learning capability plays role in the results outcome of these tests. The melatonin was applied shortly after the procedure of induction of ischemia. The neuroprotective effect of melatonin develops over 48 hours after an induction of ischemia. Animals were tested in experimental settings when they first of all had to learn a new skill. Static motor function was evaluated by performance on a narrow rod – beam balance test. Sensorimotor coordination and dynamic motor function was thereafter evaluated in classical rotarod test settings. To successfully perform in reverse rotarod test animals had to adapt to more complex and unusual for them situation. In order to do so, animals had to rapidly precess a new for them dynamic sensorimotor information. The cognition was estimated by spatial learning ability. Melatonin improved animals' performance in all of the mentioned tests. These facts strongly support the conclusion that melatonin not only affects action of FR in the area affected by ischemia but also attenuates further functional changes. It is known that melatonin combines properties of FR scavengers that trap already generated FR with properties of antioxidant that interfere with FR generation cascade (Reiter 1996). In addition, it acts intracellularly as well as extracellularly (Reiter 1998). This dual action important because FR generated during and after ischemia development not only cause structural damage but also act as intracellular signalling molecules. In this role they are able to change cellular energy metabolism and activate self-destruction pathways (Loh *et al.* 2006; Love 1999). That is why it is not surprising that melatonin had the revealed effect on ischemic neurons. It is also important that melatonin was used as a treatment, i. e. after an induction of ischemia. The question remains why the other FR scavenger, tempol, did not show similar effect. Firstly, it is already discussed issue of dosage. Secondly, tempol is stable piperidine nitroxide (stable free radical) of low molecular weight that easily crosses biological membranes. It has

SOD-like activity and scavenges superoxide anions (Laight *et al.* 1997). SOD is known to scavenge superoxide anions, however it is lacking the ability for efficient removal of the hydrogen peroxide (Fridovich 1995). In addition, there is possibility of pro-oxidant activity of SOD as a catalyzer of the hydrogen peroxide conversion to hydroxyl radicals (Yim *et al.* 1990). The question is whether the lack of tempol effect on surviving ischemic neurons can be caused by its possible pro-oxidant activity and whether tempol scavenges superoxide, hydroxyl (Simonsen *et al.* 2009) and nitrous radicals with the same potential.

The results of experiment II confirmed neuroprotective effect of melatonin against the cognitive function disturbances induced by the seizures elicited by flurothyl vapors. The attenuation of these disturbances seen when melatonin was applied an hour before development of seizure and, in lesser degree, when it was applied shortly after. It is thought that mitochondria, which are located in presynaptic neuronal endings, are loaded the most throughout the course of seizure (Langmeier *et al.* 1982). Substantial increase in amount of FR thereafter could be localized to the synaptic contacts and influence their activity. There is one more aspect. Although, morphological damage of the mitochondria is restored to normal within 10 minutes (Langmeier *et al.* 1982), it may lead to longer disturbance of energy metabolism and increase of FR production with following disturbances synaptic conduction. Despite of fast mitochondrial recovery, changes of cognitive function remain, in terms of changes of spatial learning ability, even after single seizure, as was shown in experiment II. Learning as well as seizures is many times mentioned in papers dealing with post-tetanic potentiation (Grant *et al.* 1992). These facts give a raise to the hypothesis and even speculation that seizure induces functional or morphological changes of neurons in two steps. First, it leads to mitochondrial damage, thus local synaptic effects. Second, insufficient recovery of firing neurons due to lack of energy because of damaged mitochondria. As a result, disbalance of neuronal network connectivity develops leading to disruption of the functional integrity of the system, i.e. CNS functional disturbances. It would support an idea of possibility to influence seizures and their consequences by modulating synaptic conductions with FR scavengers (Viggiano *et al.* 2008).

During seizures there is increased demand of oxygen and nutrients by loop-firing neurons and it partially mimics the pathogenic processes of hypoxia and

ischemia. Therefore, in respect to the findings, the mechanisms of melatonin protection of neuronal system's function in a great part are related to its FR scavenger and antioxidant properties. Never the less, there is issue of additional involvement of neuronal melatonin receptors in hippocampus that results in better animals' spatial learning ability after seizures (Stewart and Leung 2005). In this relation, melatonin acts via a receptor-mediated pathway and inhibits proteolytic pathways mediated by calpain and caspases. Calpain and caspase-3 decreased activity is also associated with inhibition of kinases and protease activities giving rise to neuroprotection (Das *et al.* 2010). The involvement of this additional melatonin property is also suggested by the results when effect of melatonin is compared with hypoxic preconditioning effect of hypobaric hypoxia (Mares *et al.* 2012). In this study there was an improvement of spatial learning in animals treated by melatonin and animals exposed to hypobaric hypoxia. However, melatonin did not markedly change the pattern of behavior preceding the seizures, while hypoxia did change the pattern. It can not be, therefore, excluded that activation of melatonin receptors supports mechanisms of neuroprotection in addition to melatonin scavenger and antioxidant activity in hippocampus.

The results of the last experiment III confronted the suggestion that intermittent normobaric hypoxia would activate defence mechanisms in form of hypoxic preconditioning. The animals were exposed to this type of hypoxia only during one hour and, in combination with ischemic lesion of sensorimotor cortex induced tree days later, caused significant worsening of the animals' cognitive function. In contrast, the continuous one-hour hypobaric hypoxia that was also induced three days prior to the induction of ischemic lesion, which did not have these negatively cumulative effects on cognition as intermittent normobaric hypoxia. Therefore, the type of intermittent hypoxia used is more severe cerebral insult due to repeated reoxygenation phases. In general, preconditioning main goal is protection of structural integrity and sequential protection of functional disturbances. It seems that in the situation of intermittent normobaric hypoxia neuronal tissue deals with more general characteristics of preconditioning, which eventually cause worsening of state from the following result. It is assumed that the effect is related to the kind of cumulative effects of two noxious stimuli. Although, it should not be forgotten the other possible mechanism when similar insults would prone tissue to either pro-

apoptotic or antiapoptotic actions depending on intensity and duration of exposure. Therefore rises the question, whether energy demanding process of the cell integrity preservation in this case could not be the cause of functional disturbances. The development of protective preconditioning by intermittent hypoxia showed to be effective in the animal models of ethanol withdrawal syndrome, spontaneous hypertension and myocardial ischemia (Jung *et al.* 2008; Lyamina *et al.* 2011; Ryou *et al.* 2008). However, this should not be the rule for the brain tissue and the synergy of two factors develops. Similar synergy of pathogenic factors is seen in sleep apnea syndrome when distorted sleep associated with chronic repetitive hypoxia and existing comorbidity of high blood pressure. The knowledge of the result from the experiment III opens the opportunity for better understanding of such synergy.

## 6. Conclusions

The acquired results support the hypothesis that free radicals could play important role in the development of negative outcome of focal cortical ischemia and seizures induced by flurothyl. The set aims were achieved, although, the hypoxic preconditioning effect of intermittent normobaric hypoxia was found cumulative negative. Even though, there is no doubt that melatonin application has positive effect on cognitive and sensorimotor function of affected animals.

The gained knowledge thereafter could be enriched by further assessment. Firstly, deeper understanding of melatonin actions on and interactions with CNS insults, not only from the aspect of its antioxidant and scavenger properties and interaction with free radicals production. Secondly, search for the explanations of the differences in pathogenesis and effect of intermittent normobaric hypoxia and hypobaric continuous hypoxia.

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