Research on experimental models of neurodegenerative disorders and radiation-induced brain injury

Summary of PhD thesis

IMOLA PLANGÁR

Department of Neurology

Faculty of Medicine

Albert Szent-Györgyi Clinical Centre

University of Szeged

Szeged

2014

I. Introduction

There may be a considerable number of harmful effects in the background of neuronal damage, caused by several different diseases of the nervous system. These damaging factors may be traumas, vascular disturbances, physical impacts (e.g. irradiation), toxic effects, inflammation and autoimmune, degenerative or tumourous processes.

Neurodegenerative diseases are severe disorders which primarily affect the nervous system. Since the aetiology and precise pathomechanism of these diseases are unknown, the treatment poses a great challenge for basic science and clinical investigations.

Radiation therapy is an important part of the complex management of primary and secondary brain tumours. Sadly, the incidence of brain tumours has become more significant over the years, and therapeutic strategies to prevent or mitigate radiation-induced cognitive impairment have therefore increased in importance.

There are a number of similarities between neurodegenerative disorders and radiation-induced brain injury. Blood-brain barrier (BBB) disruption is one of the major consequences of radiation-induced normal tissue injury (Lee et al. 2012) in the central nervous system (CNS) and disruption of the neurovascular system has been proved in most neurodegenerative disorders too (Lin et al. 2013; Lee & Pienaar 2014). The main mutual feature is that both finally lead to neuronal cell death. The loss of neurones is progressive and irreversible, but the temporal appearance of the consequences may differ considerably. The time which passes from the initiation of neuronal cell death to the appearance of clinical symptoms can vary somewhat, but a period of decades generally passes before an overwhelming number of neurones are affected (Bredesen et al. 2006). Inflammation with varying degrees of severity can be observed in both cases (Flood et al. 2011; Khandelwal et al. 2011; Moore et al. 2013). The other important similarity is the impairment of both motor and cognitive functions. The functional and morphological changes often coexist. However, their distinction can be difficult, and the detection of molecular, cellular and microanatomic changes help to unravel the puzzle and facilitate an understanding of the processes in the background of the disorders.

In the present work, we chose to examine PD, a progressive disorder with functional abnormalities that lead to dementia. The behavioural changes, loss of memory, and histological and cellular disturbances caused by irradiation strongly resemble the changes caused by neurodegenerative processes. In both cases, it is essential to make use of small-animal models in order to perform complex investigations, including neurofunctional and morphological examinations, with potentially neuroprotective, radio-neuroprotective agents.

For these reasons, in the present work we investigated these two at first sight not closely related topics, neurodegeneration and radiation-induced brain injuries, in light of the fact that neuroinflammation and impairment of the BBB may occur in both.

I.1. Parkinson's disease

PD is the second most common progressive neurodegenerative disorder after AD (Tanner & Goldman 1996). Despite intensive research, the distinct cellular and molecular pathomechanism of PD remains largely unclear. It is well known that impairment of the mitochondrial respiratory chain at the level of complex I plays a pivotal role in the pathomechanism (Reichmann & Riederer 1989). MPTP is a neurotoxin precursor to the complex I inhibitor MPP^+, which selectively destroys the dopamine (DA)-ergic neurones in the SNpc in human and non-human primates, with a resulting decline in striatal DA content (Dauer & Przedborski 2003).

I.2. Radiation-induced brain damages

Radiation-induced brain injury is a severe, untreatable and irreversible complication of radiation-treated (RT) malignant brain tumours and prevents the effective treatment of malignant CNS tumours. There is cumulative evidence in the literature that the same factors may play roles in the pathomechanism of both multiple sclerosis (MS) and radiation-related injuries of the brain. In both cases, axonal damage and necrosis are the main features responsible for the neurological disabilities and serious consequences of irradiation. The causes and pathogenesis of axonal damage in MS and late radiation injury are still obscure, but there is a growing body of information indicating that both inflammation and demyelination may play a major role in the process (Na et al. 2013).

I.3. Therapeutic approaches

Recent investigations have attracted attention to the roles of the dysfunction of the BBB and the blood-cerebrospinal fluid barrier (BCSFB) in neurodegenerative disorders. Passage across these barriers is modulated by certain transport proteins and pumps. Many of the efflux pumps belong in the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily (van Veen & Konings 1998). In humans, certain ABC transporters are principal causes of resistance of cancers to chemotherapy and are involved in cystic fibrosis and a range of other inherited human diseases. Increasing attention has recently been paid to the role of MRPs or human ABCCs in neurological disorders (Dallas et al. 2006). The

data obtained from studies using murine models of neurodegenerative diseases, such as AD (Krohn et al. 2011), suggest that MRPs may play an important role in the neurodegenerative process too. However, there are only a few arguments in favour of the involvement of MRPs in PD pathogenesis (Le Couteur et al. 2001). The few available data as regards the role of MRPs in PD led us to investigate the possible roles of MRPs in the neurotoxicity of MPTP/MPP$^+$. As there are no specific and selective inhibitors or stimulators of MRPs that are available for *in vivo* and *in vitro* studies, we applied SIL as an inhibitor of MRP1, MRP2, MRP4 and MRP5, NGN as a stimulator of MRP1, MRP2 and MRP4, SP as an inhibitor of MRP1, MRP4 and MRP5 and a stimulator of MRP2, and AP as an MRP4 stimulator.

Thereafter knowing that the overactivation of excitatory amino acid (EAA) receptors plays an important part in the pathomechanism of several neurodegenerative diseases, we would have been like to investigate the EAA receptor antagonist kynurenic acid (KYNA) and its novel synthetized derivatives.

Following complex research, the dose of 40 Gy and a follow-up time of 4 months are suggested for investigations on neuroradiation modifiers. First of all it was the water-soluble, deacylated phosphatidylcholine derivative L-alpha-glycerylphosphorylcholine (GPC), which could improve both the cognitive functions and the learning and memory capacity.

II. Aims

The aims of our studies were:

(i) an assessment of the roles of certain MRPs (1, 2, 4 and 5) in the neurotoxicity induced by MPTP;

(ii) to examine whether KYNA amides exert any behavioural side-effects in the C57B/6 mouse strain;

(iii) to develop a precise dose delivery technique for partial brain irradiation and to set up a small-animal model of ionizing radiation-induced brain injury;

(iv) to set up a dose–effect curve of radiosensitivity and to establish the most appropriate dose of irradiation for research on radiation modifiers;

(v) to investigate late effects of radiation-induced brain injuries; and

(vi) to apply the model to investigate potential radiation-induced brain injury modifiers.

III. Materials and methods

III.1. Drugs

Naringenin (NGN), silymarin (SIL), sulfinpyrazone (SP), allopurinol (AP), sesame oil, MPTP, DA hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), sodium metabisulphite and sodium octylsulphate were from Sigma-Aldrich Hungary Ltd. (Budapest, Hungary). Perchloric acid, disodium ethylenediaminetetraacetate (EDTA), sodium dihydrogenphosphate, acetonitrile and phosphoric acid were from VWR International Ltd. (Debrecen, Hungary).

L-KYN sulphate (KYNA-11) was from Sigma-Aldrich Hungary Ltd. (Budapest, Hungary). New KYNA amide derivatives (KYNA-1, N-(3-N,N-dimethylaminopropyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KYNA-2) and N-(2-N-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KYNA-6)) were prepared from KYNA and the appropriate amine by using N,N-diisopropylcarbodiimide as coupling reagent.

GPC was produced by Lipoid GmbH (Ludwigshafen, Germany).

III.2. Behavioural studies

Open-field

The spontaneous locomotor and exploration activities were measured by an automated tracking system with an activity chamber. The open black box was connected to a computer which recorded the inquisitive behaviour and locomotor activity of the animal. Each animal were placed individually at the centre of the box (48 x 48 x 40 cm), which was equipped with automated infrared photocells for measurements, and allowed to move spontaneously for 5 (in the case of mice) or 15 (in the case of rats) minutes. The tests were performed at the same time of day so as to minimize changes due to the diurnal rhythm. The movement signals were analysed by Conducta 1.0 (Experimetria Ltd, Budapest, Hungary) software. The analysis resulted in a track record; the locomotor activity was expressed as the total distance moved (cm) in a predetermined period of time, the times spent in movement and at rest (s), the mean velocity (cm/s), and the frequency and duration of rearing.

In the course of the studies of the behavioural effects of KYNA analogues, the mice were examined 2 h after the first treatment (acute), and then on the last day (day 9) of treatment (chronic). In this case, the ambulation time, the mean velocity, the local time and the number of rearings were evaluated. In the other experiments, rats were tested every 2 weeks and the ambulation distance, velocity, immobility time and rearing count were assessed.

Morris water maze test

The MWM protocol of Vorhees and Willams (Vorhees and Williams 2006) was used, visuospatial cues being provided to guide the animals in tests of hippocampal memory. The MWM consisted of a cylindrical white tank with a diameter of 175 cm and a height of 50 cm, containing liquid made opaque with a non-toxic white dye. The tank was filled with water up to 32.5 cm and maintained at 21–24 °C. The pool was divided into four equal quadrants, and a removable transparent Plexiglas platform (10 cm in diameter) that could not be seen by the swimming rats was hidden at the centre of one of the quadrants, with its top 1 cm below the surface. The platform provided the only escape from the water. The position of the platform was constant throughout the 3-day acquisition period. Pictures fixed permanently on the surrounding walls served as distal navigation cues to enable the rats to locate the platform. The distinctive visual cues remained constant throughout the entire course of testing. The first two days were acquisition or training days, and the task was performed on the third day. The training period consisted of 4 trials per day with a 5-min inter-trial interval. Each trial began with the rat in the pool and ended when the rat found the platform or after 120 s. If the animal failed to locate the platform within 120 s, it was guided to the platform manually. Once on the platform, the rat was allowed to rest for 10 s. It was then towel-dried and placed in an inter-trial holding cage where a heating source was provided to maintain the animal's body temperature during the inter-trial interval. During the acquisition phase, measurements were made of the time (s) and the path length (cm) taken to locate the platform. This site navigation test was performed once before the irradiation and subsequently once in the third and once in the fourth month.

III.3. Animal model of PD

Animals

The procedures utilized in this study followed the guidelines of the European Communities Council (86/609/ECC) to minimize animal suffering and were approved in advance by the Ethics Committee of the Faculty of Medicine, University of Szeged. Six-week-old C57B/6 male mice (weighing between 18 and 23 g) were used. The animals were housed in cages (at most 5 per cage) and maintained under standard laboratory conditions, with tap water and regular mouse chow available *ad libitium* on a 12 h light-dark cycle, at 21±1 ℃ and 50±10% humidity.

Treatments

Four groups of mice were used in the first 4 experimental set-ups for the assessment of the 4 substances. The first 2 groups of mice received i.p. injections of SIL (100 mg/kg/day; $n_1=8$, $n_2=9$), NGN (100 mg/kg/day; $n_1=7$, $n_2=7$), SP (100 mg/kg/day; $n_1=8$, $n_2=9$) or AP (60 mg/kg/day; $n_1=10$, $n_2=10$) respectively, once a day, at the same time each day for one week. The second 2 groups of mice received sesame oil (in a volume of 5 ml/kg; $n_3=8$, $n_4=8$; $n_3=7$, $n_4=7$; $n_3=8$, $n_4=9$; $n_3=6$, $n_4=10$) in the same treatment regime. On day 8 of the experiments, 1 h after the regular daily injections, the second and fourth groups received i.p. injections of MPTP (15 mg/kg) 5 times at 2 h intervals (total dosage: 75 mg/kg). The first and third groups received 0.1 M PBS (in a volume of 5 ml/kg) in the same treatment regime. From day 9, the 4 substances were further administered once a day for another week, with the corresponding control injections, according to the treatment regime detailed above.

Sample preparation and chromatographic conditions

Eight days after toxin administration (day 16 of the experiment), all the mice were decapitated and the brains were rapidly removed and placed on an ice-cooled plate for dissection of the striatum. After dissection of both striata from the forebrain block, these were stored at −70 °C until further sample processing. In the next step, the striata were weighed and then manually homogenized in an ice-cooled solution for 1 min in a homogenization tube. The content of the homogenization tube was centrifuged at 10,000 g for 15 min at 4 °C. Thereafter, the supernatant was stored at −70 °C until chromatographic analysis. DA and its metabolites, DOPAC and HVA, were analysed by reversed-phase chromatography, using an Agilent 1100 high-performance liquid chromatography (HPLC) system combined with a Model 105 electrochemical detector under isocratic conditions.

III.4. Study of behavioural effects of some kynurenic acid analogues
Animals

Behavioural experiments were carried out on 6-week-old C57B/6 male mice (n=60). The animals were kept under controlled environmental conditions at 22±2 ˚C under a 12-h light-dark cycle. Food and water were available *ad libitum*. The local Animal Ethics Committee had approved all experiments. The care and use of the experimental animals were in full accordance with the 86/609/EEC directive.

Treatment regime

The mice received i.p. injections of KYNA-1 (200 mg/kg/day, in a volume of 5 ml/kg, dissolved in distilled water, the pH of which was adjusted to 6.5 with 1 N NaOH). For comparison, KYNA-2 or KYNA-6 in equimolar dosages (likewise in a volume of 5 ml/kg, dissolved in distilled water, the pH of which was adjusted to 6.5 with 1 N NaOH) or the vehicle (0.1 M PBS, in a volume of 5 ml/kg) was administered at the same time each day from 6 weeks of age.

In behavioural experiments, the mice were treated once (in acute experiments) or for 9 days (in chronic experiments) according to the regime detailed above.

III.5. Small-animal model of irradiation

Animals

Experiments were performed on 57 adult Sprague-Dawley (SPRD) male rats, weighing on average 210 g (range 176–280 g). The animals were housed in a climate-controlled environment (25 °C) maintained on a 12 h light/12 h dark cycle and were allowed free access to food and water.

Dosimetry of small electron field and irradiation

In order to verify the dose depth curve, the field profile and the lateral dose fall-off of the 2, 4, 6, 8, 10 and 12 mm electron collimators, we measured the absorbed dose of a 6 MeV electron beam irradiating at a dose rate of 300 monitor units (MU)/min in a water phantom. Thus, for evaluation of the dose distribution, the 90% and 70% isodose levels were superimposed on the image of the skull, aligned with the approximate location of the general rat brain structures. Two prone adult male SPRD rats placed nose-to-nose in the irradiation position, with earpin fixation, were imaged in the Emotion 6 CT scanner in order to obtain three-dimensional (3D) anatomical information for planning of the radiation geometry. A 6 MeV lateral electron beam at a 100 cm source-to-skin distance (SSD) was chosen because it has a sharp dose fall-off with depth, confining the radiation dose delivery to the defined volume of the hippocampus, including the corpus callosum of the ipsilateral hemisphere. The planned doses were delivered in a single fraction by means of a linear accelerator, to the following groups of animals: 120 Gy (n=3), 110 Gy (n=3), 100 Gy (n=3), 90 Gy (n=6), 80 Gy (n=3), 70 Gy (n=6), 60 Gy (n=3), 50 Gy (n=6), 40 Gy (n=12) and sham-irradiated (n=12). The irradiation dose rate was 300/900 MU/min and de-escalated doses ranging from 120 to 40 Gy were applied, with 3–12 animals per dose level.

Magnetic resonance imaging (MRI)

Sixty rats underwent 72 MRI procedures prior to or 4–19 weeks after irradiation. Randomly selected animals from each irradiation dose level were examined by means of 1.5 T MRI, using a human head coil with a home-made styrofoam holder containing 6 animals under i.p. chloral hydrate anaesthesia. No contrast agent was used for the MRI images. Twelve animals underwent 3 MRI examinations, at baseline, at mid-term and prior to histology.

Histopathology

Rats were anaesthetized with 4% chloral hydrate and perfused transcardially with 0.1 M phosphate buffer solution (pH 7.0–7.4) before they were fixed with 4% paraformaldehyde buffer solution (pH 7.0–7.4) at 4 °C. The brains were dissected out and postfixed in paraformaldehyde for 1 day before being embedded in paraffin. Serial 30-µm-thick sections were cut with a vibratome. Sections were stained with H&E for histologic evaluation; for the demonstration of demyelination, Luxol fast blue staining was used. All analyses were performed blindly, using coded sections. Evaluations were carried out with a semiquantitative method, independently by 2 experienced histopathologists.

III.6. Application of GPC in the small-animal model of partial brain irradiation

Treatment

A total of 24 adult (6-week-old) male SPRD rats were used in these experiments. The 40 Gy dose level was selected for the investigation of neuroprotection. Rats (weighing from 180 to 220 g) were anaesthetized (4% chloral hydrate, 1 ml/100 g, i.p.) and placed in the prone position, using laser alignment. The dosimetry of the small electron field and the method of irradiation were the same as previously. The following groups of animals participated in the experiment: a sham-irradiated control (CO) group (n=6), an only GPC-treated (GPC) group (n=6), an RT group (n=6), and a both GPC-treated and irradiated (GPC+RT) group (n=6). Beginning from 1 week before the day of irradiation, the rats received GPC (50 mg/kg bw, dissolved in 0.5 ml sterile saline, administered by gavage) or the vehicle at the same time every second day for 4 months.

Histopathology

The preparation for and conductance of the histopathology were the same as reported above. Sections were analysed under an Axio Imager.Z1 light microscope, and photomicrographs

were taken with AxioCam MR5 camera equipment. Digital photos were analysed with the aid of Image-Pro® Plus 6.1 software. All analyses were carried out blindly on coded sections by 2 independent histopathologists. The examined parameters and the scoring system were not altered.

III.7. Statistical analysis

All statistical analyses of HPLC measurements were performed with the help of the SPSS Statistics 17.0 software. We first checked the distribution of data populations with the Shapiro-Wilk W test. We then performed the Levene test for analysis of the homogeneity of variances. The 4 groups in each set of the 4 experiments were compared by using one-way measures of analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test or by a Games-Howell *post hoc* test. The null hypothesis was rejected when the p level was <0.05, and in such cases the differences were considered significant. Data were plotted as means (+ standard error of the mean (SEM)) in the graphs.

For statistical evaluation of the data in the behavioural tests and histopathology, one-way ANOVA was used, followed by Fisher's LSD *post hoc* test with StatView 4.53 for Windows software. These data were expressed as means+SEM. Levels of statistical significance in the behavioural tests were taken as p≤0.05 and p≤0.01.

IV. Results

IV.1. Detection of catecholamines

We first tested the effects of pre- and post-treatment with SIL on the MPTP-induced significant changes in striatal DA, DOPAC and HVA levels. The SIL treatment did not influence these alterations appreciably. The NGN treatment did not significantly alter the reductions caused in the striatal DA and DOPAC levels by MPTP administration, a slight lessening of the DA decrease was observed. In this experiment, the HVA levels were not altered significantly at all. The SP treatment did not influence the MPTP-induced changes, but slightly increased the MPTP-caused lethality in mice: 1 of the 10 mice died in the MPTP-treated group, whereas 3 died in the SP- and MPTP-co-treated group. In the last set of experiments, we tested the effects of pre- and post-treatment with AP on MPTP toxicity. In this set of experiments, MPTP administration caused significant reductions in the striatal DA, DOPAC and HVA levels as compared with the CO values. Although the AP treatment did not influence the MPTP-induced changes significantly, it considerably increased the MPTP-caused lethality in mice, as 1 of the 10 mice died in each of the AP- and MPTP-treated groups,

whereas 7 died in the AP- and MPTP-co-treated group. It is also important to mention that none of the tested compounds themselves altered the CO levels of DA, DOPAC and HVA.

IV.2. Behavioural performances of animals treated with KYNA analogues

Four of the newly synthetized KYNA amides were further investigated in behavioural experiments. Open-field observations were made on 5 groups of animals: a CO group (injected with saline), and KYNA-1, KYNA-2, KYNA-6 or KYNA-11-treated groups. In acute behavioural experiments, the animals were treated with saline (CO) or KYNA amide analogue 2 h prior to the behavioural observations, while chronic treatment was administered on 9 successive days, with observations on day 9, 2 h after the final injection. The animals treated with one or other KYNA amide analogue did not differ greatly in behaviour from those that received the saline vehicle. The ambulation time, the mean velocity and the number of rearings did not exhibit highly significant differences in most cases. Although the ambulation time was somewhat decreased after KYNA-11 administration, the changes were not significant. The mean velocities were nearly the same in each group. Significant changes in performance were observed only in the numbers of rearings. Both KYNA-1 and KYNA-11 decreased the number of rearings in the acute experiments, and KYNA-11 did so in the chronic experiments too.

IV.3. Results of small-animal irradiation

The morphological and functional changes were evaluated at dose levels in the range 90–40 Gy: outside this dose range, either lethal or serious events (120–100 Gy) or no changes (doses <30 Gy; data not reported here) occured during the at most 4-month post-irradiation follow-up period. At the highest dose (120 Gy), all of the animals underwent a rapid severe general and neurofunctional decline and died or had to be euthanized between 25 and 40 days after the irradiation. The rats irradiated at the 110 Gy dose level survived longer, but also deteriorated between 30 and 50 days post-irradiation. At the lower dose levels, there were no signs of a general impairment; the weight gain, eating habits and daily activity did not differ from those of the control rats. All the RT animals suffered hair loss from the site corresponding to the beam entrance within 30 days following the irradiation.

Neurofunctional observations

In the early monitoring, significant differences in the spontaneous locomotor activity of the rats irradiated at 90–40 Gy were not detected with the open-field test, but the motor function

began to decline slightly 8 weeks after irradiation with the 90 Gy dose. The rearing count was significantly reduced in the groups that received 90–60 Gy. The time at which the rearing activity started to diminish was dose-dependent: It was 40–55 days post-irradiation after the 90 Gy dose (mean+SEM, p<0.05). The animals that received 120 Gy died before marked changes could be seen in this parameter.

The MWM test was found to be a highly sensitive tool for the detection of a neurofunctional impairment. A relevant memory deterioration was detected soon after the dose delivery at the 70 Gy dose level and the difference increased with time (p<0.001). A significant cognitive deficit was also observed 8 weeks after the irradiation in the group treated with 60 Gy (mean+SEM, p<0.05). The commencement of the impairment of the learning-memory function proved to be dose-dependent; in the groups irradiated at 50–40 Gy, the first sign of deterioration was detected 30 days post-irradiation and the difference relative to the CO animals was more pronounced after 90 days.

MRI findings

Serial MRI records demonstrated structural damage in the form of cavity formation in the cortical region, with extensive perifocal oedema, which generally appeared 2–4 months following irradiation. We performed the first post-irradiation MRI after 4 weeks in the majority of the cases, since our aim was to investigate late effects. 120 Gy resulted in a serious deterioration within 4 weeks in all rats. Localized radiation-induced cystic necrosis began to appear at approximately 4–8 weeks post-irradiation in one hemisphere of rats irradiated with 120–60 Gy; after a lower dose, the structural changes emerged later, 19–24 weeks after irradiation, in the T2-weighted (T2W) images of the ipsilateral hemisphere, in both the coronal and the sagittal plane.

Histopathological evaluations

No signs of necrosis, i.e. neither reactive gliosis nor any of the other examined histopathological categories, were seen on the H&E-stained slides of the CO animals and the non-irradiated regions of the brain of the RT animals. The following parameters correlated closely with the high (120–90 Gy), medium (80–60 Gy) or low dose (50–40 Gy) level in the irradiated region of the brain: reactive gliosis, vascularization, macrophage density, necrosis and calcification. No significant dose dependence was detected as concerns the extent of haemorrhage. The dose >90 Gy groups displayed severe necrosis that reached the grey and white matter, causing severe demyelination, with destruction of the fibres. The levels of

necrosis, reactive astrogliosis and calcification and the density of the foamy macrophages were markedly elevated in these groups as compared with the CO animals. The extent of the haemorrhage was significantly higher than for the other RT animal groups. Severe-to-moderate necrosis was seen in the 80–60 Gy groups, with severe-to-moderate demyelination, but the fibres could mostly be detected. In comparison with the control group, significant correlations were detected in the following categories: necrosis, macrophage density, vascularization, calcification and reactive gliosis. Moderate haemorrhage was observed in the animals irradiated with the 80 Gy dose. In the 50–40 Gy groups, mild-to-moderate necrosis was detected, with mild-to-moderate demyelination. Significantly increased levels of necrosis, vascularization and reactive astrogliosis were seen. Mild calcification occurred.

IV.4. Treatment with GPC

The 40 Gy RT group exhibited a body weight deficit; their body weight remaining under the normal throughout. The difference between the RT and CO groups did not reach the level of statistical significance.

MWM test

After the 40 Gy irradiation, significant, time-related changes in learning ability were detected in both the RT and GPC+RT groups, but these changes were significantly reduced in the GPC+RT group. The first sign of deterioration was detected 90 days post-irradiation and the difference relative to the CO animals was more pronounced after 120 days ($p<0.001$). A relevant memory impairment was detected in the RT group after 120 days, and a significant cognitive deficit was also observed in the GPC+RT group relative to the CO group ($p=0.0025$). Despite this, there was a significant amelioration after GPC management, which reduced the latency of target finding relative to the RT group ($p=0.012$). The GPC ameliorated the memory of the animals and shortened the latency time of platform finding.

Histopathology

The H&E-stained slides of the CO animals and the non-irradiated regions of the brain of the treated animals exhibited no signs of necrosis, i.e. neither reactive astrogliosis, nor any of the other examined histopathological categories. In the irradiated region of the brain, the following parameters correlated closely with the 40 Gy dose level: necrosis, macrophage density, reactive gliosis, calcification and demyelination. The RT group displayed moderate necrosis that reached the grey and white matter, causing demyelination, with destruction of

the fibres. The grades of reactive astrogliosis and calcification, the density of the foamy macrophages and the degree of demyelination were all significantly elevated in the RT group as compared with the CO animals. Marked protective effects of GPC were detected as concerns the macrophage density ($p<0.001$), reactive astrogliosis ($p<0.001$), calcification ($p=0.012$) and the extent of demyelination ($p=0.035$).

V. Discussion

V.1. The role of MRPs in the MPTP-induced neurotoxicity

The aim of our study was to assess the effects of certain compounds which can modulate the function of MRPs expressed on the BBB and BCSFB (MRP1, 2, 4 and 5) on MPTP toxicity in C57B/6 mice. Two of these compounds, SIL and NGN, are well-known flavonoids (Ross & Kasum 2002), which have already been tested in certain toxin models of PD, but not in the MPTP model. SIL, which is known to exert complex pharmacological action (Saller et al. 2007) including MRP inhibitor properties, is capable of the attenuation of maneb- and paraquat-induced lipid peroxidation (Singhal et al. 2011). However, in our study SIL did not alter MPTP-induced neurotoxicity. The reason for this difference would be that maneb and paraquat were applied in a chronic treatment regime twice a week for 9 weeks, which presumably caused less harm than that in the widely applied acute MPTP model. The other flavonoid, NGN, with MRP1, MRP2 and MRP4 stimulator properties, was moderately protective against 6-hydroxy-DA-induced neurotoxicity as it significantly attenuated the loss of DAergic neurones and the decrease in striatal DA levels (Zbarsky et al. 2005). Accordingly, in our study NGN treatment caused a slight, but not significant preservation of striatal DA content. In contrast with the above flavonoids, the effects of SP have not been tested in toxin models of PD. Although it did not appear to affect the striatal DA concentrations in our study, it slightly increased the MPTP-caused lethality in C57B/6 mice. It should be mentioned here that SP can reduce serum urate levels through its uricosuric effect. In a previous study, oral high-dose administration of the xanthine oxidase inhibitor and MRP4 stimulator AP resulted in a decreased level of urate, which may have an important role in the neuronal antioxidant pool in the striatum, but did not affect the DA level in rats (Miele et al. 1995; Desole et al. 1996). Accordingly, in our study AP considerably enhanced the MPTP-caused lethality, but the striatal DA content was preserved in the survivors. The main explanation for the potentiation of MPTP toxicity by AP would be the decrease of urate concentration by the inhibition of xanthine oxidase. However, an enhanced transport by

MRP4 would also accompany the depletion of urate. Hence, a decrease in serum urate level would accompany the progression of PD (Sun et al. 2012).

In conclusion, these data indicate that the depletion of urate augmented by MRP4 stimulation increases, while the stimulation of MRP1- or 2-mediated transporters (probably GSH-conjugated toxic substances) slightly attenuated MPTP-induced neurotoxicity.

V.2. Effects of some KYNA analogues

It was important that KYNA-1, which had proved neuroprotective in several models (Marosi et al. 2010; Knyihar-Csillik et al. 2008), and partially inhibited NMDA-mediated synaptical transmission in the hippocampus, did not induce significant changes in the behaviour of the tested animals. The results confirmed that none of the studied KYNA derivatives induced major changes in the behaviour of these animals. Only the number of rearings was reduced somewhat after KYNA-1 or KYNA-11 administration. Moreover, as KYNA-1 did not significantly influence the behavioural performance in the open-field arena, KYNA-1 treatment does not appear to have any appreciable side-effects.

V.3. Focal rat brain irradiation model

A simple and effective method was developed for the delivery of a radiation dose to a well-defined area in one hemisphere of the brain, in rats, similarly to human brain tumour radiotherapy, as recommended by others (Kalm et al 2013). It allows the investigation of a maximum of 6 small animals simultaneously and comprises a reproducible experimental model for quantification of the functional and morphological changes occurring due to radiation-induced focal brain damage within a reasonable time frame. We performed a dose-de-escalation from 120–40 Gy in 10 Gy steps. The morphological and functional changes detected were clearly related to the radiation dose. The 2-weekly assessment of open-field tests did not reveal any behavioural alteration, apart from the rats irradiated at 120–110 Gy, which displayed an obvious deterioration. Only the changes in rearing activity indicated the effects of the focal brain injury; these were first observed 40–55 days post-irradiation at the 90 Gy dose level. In our experiments, the slight changes in locomotor activity can be explained by the short time frame of the open-field tests. These revealed that the effects of relevant damage in the motor cortex start to become observable 8 weeks after irradiation, which corresponds well to the development of an irreversible human focal brain injury (Huang et al. 2009, Caceres et al. 2010). The MWM test is widely used for the detection of neurofunctional impairments (Shi et al. 2011). In our model, the memory decline first

appeared between 30 and 120 days after the irradiation, clearly depending on the dose delivered. The behavioural impairment correlated closely with the morphological changes detected by MRI and histology. The radiation-induced morphological changes demonstrated by repeated MRI scans correlated well with the dose, the duration and location of the lesion (Brisman et al. 2003). Single doses of 150 Gy or 100 Gy produced necrosis in the hippocampus within 1–3 months (Liscák et al. 2002) and 75 Gy caused a focal brain lesion within 3–6 months (Jirák et al. 2007). At doses higher than 60 Gy, necrotic changes started to appear within 6 months (Brisman et al. 2003), and 20 months after lower doses, such as 25–50 Gy, delivered to the right frontal lobe of rats, MRI changes were demonstrated in relaxation times T1 and T2 (Ishikawa et al. 1999). We set out to perform simultaneous MRI examinations on 6 rats with the available 1.5 T device and a human brain coil in order to detect and follow up the necrotic changes *in vivo*, and to optimize the time point of histopathologic examinations. The H&E slides showed that the irradiation was localized to a defined small brain volume and the effects in the animals were well reproducible: the damage appeared only in the irradiated region. Significant correlations were detected between the radiation dose and the degree of necrosis, the presence of foamy macrophages, the vascularization and the calcification. Previous studies have indicated that histopathological structural changes involving a decrease in the cell number and demyelination can be expected in the dose range 50–100 Gy (Ernst-Stecken et al. 2007). With such doses, our histopathological analysis revealed measurable 6 x 8 mm necrotic lesions with cysta ex emollition, haemorrhage and a reactive cellular response. In confirmation of earlier data (Kumar et al. 2012), the severity of the radiation damage was strictly dose-dependent.

V.4. Testing radiation injury and potential radiation modifiers

Our earlier study of the dose–response relationship indicated that the chosen model is relevant for studying various aspects of healthy brain protection (Hideghéty et al. 2013).
In our present investigation, this method revealed a promising ameliorative effect of GPC, which can be explained by its role in preserving the cell membranes and cognitive functions in the CNS. Choline and choline-containing phospholipids such as GPC display mainly a cholinergic profile, interfering with phospholipids biosynthesis, brain metabolism and neurotransmitter systems, and are responsible for maintaining the cell membrane integrity and are also precursors of the neurotransmitter acetylcholine, which is involved in a number of brain processes, including learning and memory (Tayebati et al. 2013). After oral administration, GPC can cross the BBB and reach the CNS, where it can exert beneficial

effects in the treatment of the sequelae of cognitive disorders and cerebrovascular accidents. It can incorporate into the phospholipid fraction of the neuronal plasma membrane and can also increase the levels of production and release of acetylcholine in the brain (Tayebati et al. 2013). Our study clearly illustrates the protective effects of GPC at both functional and morphological levels. The cognitive dysfunction resulting from irradiation can be examined by different methods. The MWM has been found to be a highly sensitive tool for the detection of a neurofunctional impairment (Shi et al. 2011; Yoneoka et al. 1999). The MWM task clearly demonstrated the effects of GPC on the working memory and long-lasting reference memory of rats after irradiation at a 40 Gy dose level, the differences in learning ability between the RT and CO groups becoming more pronounced as time passed.

An earlier analysis of the histological changes led to the finding that brain irradiation modified the spine density and also the proportions of the morphological subtypes in the dendrites of the dentate gyrus granule cells and the basal dendrites of the CA1 pyramidal neurones, in a time-dependent manner (Chakraborti et al. 2012). Pathological disturbances such as vascular damage and demyelination are late consequences of irradiation that are likewise revealed by histological examination (Brown et al. 2005). The primary targets of radiation damage include the oligodendrocytes and the white matter, which suffer necrosis (Shen et al. 2012; Valk & Dillon 1991). In our study, the levels of such histopathological deterioration, scored semiquantitatively, were ameliorated significantly by GPC treatment. The changes in cognitive ability correlated closely with the histopathological findings indicative of the radio-neuroprotecive action of GPC.

VI. Acknowledgements

Original publications directly related to the PhD thesis

I. **Plangár I**, Zádori D, Szalárdy L, Vécsei L, Klivényi P. (2013) Assessment of the role of multidrug resistance-associated proteins in MPTP neurotoxicity in mice. Ideggyógyászati Szemle 66: 407-414

    IF: 0.348

II. Nagy K, **Plangár I,** Tuka B, Gellért L, Varga D, Demeter I, Farkas T, Kis Z, Marosi M, Zádori D, Klivényi P, Fülöp F, Szatmári I, Vécsei L, Toldi J. (2011) Synthesis and biological effects of some kynurenic acid analogs. Bioorganic & Medicinal Chemistry 19:7590-7596.

    IF: 2.921

III. Kalincsák J, Farkas R, Kovács P, Aradi M, Bellyei Sz, Weiczner R, Sebestyén Zs, **Plangár I**, Hideghéty K. (2013) Single dose irradiation of defined region of rat brain with stereotactic BrainLab system. Ideggyógyászati Szemle (ahead of print)

    IF: 0.348

IV. Hideghéty K, **Plangár I,** Mán I, Fekete G, Nagy Z, Volford G, Tőkés T, Szabó E, Szabó Z, Brinyiczki K, Mózes P, Németh I. (2013) Development of a small-animal focal brain irradiation model to study radiation injury and radiation-injury modifiers. International Journal of Radiation Biology 89:645-655.

    IF: 1.895

V. **Plangár I,** Szabó ER, Tőkés T, Mán I, Brinyiczki K, Fekete G, Németh IB, Ghyczy M, Boros M, Hideghéty K. (2013) Radio-neuroprotective effect of L-alpha-glycerylphosphorylcholine (GPC) in an experimental rat model. Journal of Neurooncology (under review)

Total impact factor: **5.512**

Publications not directly related to the thesis

I.   Tőkés T, Varga G, Garab D, Nagy Z, Fekete G, Tuboly E, **Plangár I**, Mán I, Szabó RE, Szabó Z, Volford G, Ghyczy M, Kaszaki J, Boros M, Hideghéty K. (2013) Peripheral inflammatory activation after hippocampus irradiation in the rat. Int J Radiat Biol (ahead of print)
IF: 1.895

II.   Torok R, Torok N, Szalardy L, **Plangar I**, Szolnoki Z, Somogyvari F, Vecsei L, Klivenyi P. (2013) Association of vitamin D receptor gene polymorphisms and Parkinson's disease in Hungarians. Neuroscience Letters 551: 70-74.
IF: 2.026

III.   Szalardy L, Zadori D, **Plangar I**, Vecsei L, Weydt P, Ludolph AC, Klivenyi P, Kovacs GG. (2013) Neuropathology of Partial PGC-1α Deficiency Recapitulates Features of Mitochondrial Encephalopathies but Not of Neurodegenerative Diseases. Neurodegenerative Diseases 12: 177-188.

IF: 3.056

IV.   **Plangar I**, Majlath Z, Vecsei L. (2012) Kynurenines in cognitive functions: their possible role in depression. Neuropsychopharmacologia Hungarica 14: 239-44.

V.   Vecsei L, **Plangar I**, Szalardy L. (2012) Manipulation with kynurenines: a possible tool for treating neurodegenerative diseases? Expert Review of Clinical Pharmacology 5: 351-3.

VI.   **Plangár I,** Zádori D, Klivényi P, Toldi J, Vécsei L. (2011) Targeting the kynurenine pathway-related alterations in Alzheimer's disease: a future therapeutic strategy. Journal of Alzheimers Disease 24: 199-209.

IF: 3.745

VII.   Zádori D, Klivényi P, **Plangár I,** Toldi J, Vécsei L. (2011) Endogenous neuroprotection in chronic neurodegenerative disorders: with particular regard to the kynurenines. Journal of Cellular and Molecular Medicine 15: 701-17.

IF: 4.125

VIII.   **Plangár I**, Zádori D, Klivényi P, Toldi J, Vécsei L. (2009) The role of the kynurenine pathway in neurodegeneration and neuroprotection Publication of the Preceedings of the 7th International Congress on the Improvement of the Quality of Life on Dementia, Parkinson's disease, Epilepsy, MS and Muscular Disorders

and Neuroethics. Bologna: Medimond Monduzzi Editore, pp. 21-26 Thessaloniki, Greece.

IX. Varga H, Pardutz A, Vamos E, **Plangar I**, Egyud E, Tajti J, Bari F, Vecsei L. (2007) Cox-2 inhibitor attenuates NO-induced nNOS in rat caudal trigeminal nucleus. Headache 47: 1319-25.

IF: 2.358

<u>Total impact factor:</u> **17.205**

Cumulative impact factor: 22.717