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PhD Thesis

THE NEUROPROTECTIVE EFFECTS OF CALORIE RESTRICTION ON BIOCHEMICAL AND FUNCTIONAL RESTORATION AFTER STROKE

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Stroke is a major health problem worldwide. In industrialized countries, it is the major cause of long-term disability, and the third leading cause of death after heart disease and all types of cancer. Aged individuals experience the highest incidence of stroke, 75% of stroke victims are above 65 years old, (Rothwell et *al.*, 2005; American Heart Association, 2006) and recover less well from stroke.

The relatively high incidence of stroke may be due in part to the impact of numerous known risk factors in this population (Donnan et al., 2008): arterial hypertension, diabetes, high cholesterol, smoking, alcoholism, obesity, stress, and a sedentary lifestyle. Virtually all drug interventions that have been successful in pre-clinically in experimental stroke have failed to translate this success to the clinical setting. We and others proposed that this is due to the failure of these pre-clinical studies to fully consider the aging and comorbidities for stroke that are present clinically (Murray et al. 2012; Buga et al. 2013; Sandu et al., 2016; Haley and Lawrence, 2016).

It is quite possible that an intervention showing efficacy in abnormal animal may not be effective when co-morbidity exists. Moreover, a number of highly prevalent risk factors such as hypertension, diabetes and atherosclerosis are increasingly understood to act as "silent contributors" to neuroinflammation – not only establishing the condition as a central pathophysiological mechanism, but also constantly fuelling it. Clinical studies have indicated that obesity is characterised by a body mass index (BMI) of at least 30 kg/m2 while those above this limit are labelled as overweight. More importantly it seems that for overweight and obese patients, every 5 kg/m2 are associated with a 40% increased mortality if the patient develops any type of stroke (Goldstein LB, 2011). Therefore, at the present time, AHA recommends weight reduction in those overweight and obese to reduce the risk of stroke (Goldstein LB, 2011). Recently, the same medical organisation recommends a simple rule termed "Life's simple 7" for preventing cardiovascular events including stroke. This includes maintaining normal weight, glucose, blood pressure and cholesterol, being physically active, non-smoking and eating a healthy nutritious diet. However, at least 4 criteria must be met for a good outcome after stroke (Lin MP, 2015). In fact, obesity it is never described as a single abnormality as by itself leads to other pathological complications such as hypertension, diabetes and hypercholesterolemia (Towfighi et al. 2009; Strazzullo et al. 2010; Li et al., 2016). Speakman and Mitchell (2011) have reported that caloric restriction is associated with several physiological changes such as: abolition of the sexual reproductive functions,

decreased body temperature while in parallel there is a reduction in free insulin and glucose indicating a decrease in white adipose tissue mass. In addition, in parallel with a change from carbohydrate to adipose metabolism, it seems that several molecular pathways are activated by caloric restriction: the target of rapamycin (TOR), sirtuin, adenosine monophosphate activated kinase and insulin like growth factor (IGF-1) (Speakman and Mitchell, 2011).
In this context, we have embarked on a thorough characterization of the caloric restriction effects in a murine aged model of ischemic stroke, including all the clinical, behavioural, genetic and biochemical factors at work in this pathological setting.

Caloric restriction, which reduces age-related obesity and hyperglycemia, may represent an efficient and cost-effective strategy for preventing stroke and its devastating consequences. Aged laboratory Sprague Dawley rats fed *ad libitum* become obese and have high insulin levels, and thus represent a "natural" model in which to study the effect of obesity on behavioural recuperation and the severity of cerebral necrosis after stroke.

Experiments reported in this study have been conducted at the Laboratory for Neurobiology of Aging, Chair of Biochemistry, University of Medicine and Pharmacy Craiova. All experiments were approved by the University Animal Experimentation Ethics Board according to the ethical requirements of the National Act on the Use of Experimental Animals and were in accordance with European Union directive. Experiements have been supervised by Prof. univ. dr. Aurel Popa-Wagner.

Previous research has reported that if fed *ad libitum* (AL) these animals develop a pattern of adipose deposition and obesity somewhat similar with humans. Remarkably, this type of obese rats will develop in time pancreatic pathology such as fibrosis, islet hyperplasia, and exocrine atrophy as well mammary and pituitary tumours (Dillberger, 1994). The rats were randomly divided into the control group (young, n=30; aged, AL, n=30) and aged calorie restricted (CR) group (n=30). Of these, three groups of rats (n=7 each) were used to measure body fat mass in young, aged AL and aged CR before surgery. Please note that the animal numbers refers to the subjects who survived 14 days after stroke. Body weights ranged from 290 to 360g for the young rats and from 610 to 700g for the aged rats at the time point when calorie restriction was initiated.
Rats have been kept in standard cages (L=595mm x l=380mm x h=200mm), two rats per cage and the bedding has been changed every other day to prevent infections. Cages with animals

have been kept at a constant room temeprature of 22^{o}C, umidity 40-60% and light between 07.00-19.00, with free acces to water and food. Food was commercial and consisted of a mixture of carbohydrates, lipids and proteins. Ammonia resulted from animal metabolism and carbon doixide were eliminated via air conditioning at 10 cycles/hr. Before surgery animals were kept individually and the bedding was replaced every day to avoid infections. Those rats were no longer fed to minimize the effects of circulating glucose and stroke outcome.

Calorie restriction: The 20-month-old CR rats were fed on 70% of the average amount consumed by AL age-matched rats, in a 2days/week fasting regimen and for 8 weeks before stroke. The regular food pellet had the following composition: 65% carbohydrate, 29%protein, and 6% fat with a physiologic fuel value of 3.3 kcal/g pellet. Food administration was adjusted weekly and the body weight was measured weekly. Young rats were fed ad libitum. Drinking water was available AL.The CR rats were also given a vitamin supplement.

Blood flow through the middle cerebral artery was transiently interrupted in deeply anesthetized rats as previously described (Popa-Wagner et al., 2010). The right middle cerebral artery was slowly lifted with a tungsten hook attached to a micromanipulator until blood flow through the vessel was completely stopped. Both common carotid arteries were then occluded by tightening prepositioned thread loops. The blood flow was monitored with a Laser Doppler (Periflux 5000, Perimed, Sweden) by positioning the optic tube on the temporal bone of rat skull. A decrease in the laser Doppler signal to <20% of controlvalues was considered to indicate successful MCA occlusion. After 90 minutes, the middle cerebral artery and the common carotid arteries were re-opened, allowing full reperfusion of the brain.

Testing procedure involved two persons, one person who did the surgery and was in charge of handling the animals according to group assignment and another one who has tested the animals and was not aware of groups' identity. To evaluate changes in neurological function associated with ischemia, the rats were subjected to a variety of locomotor, sensory, learning and memory tests before and after surgery as previously described by our group (Buchhold et al., 2007; Popa-Wagner et al., 2010). All testing was performed in the morning from 9AM to 11 AM by the same investigator. Results obtained before surgery were used to define 100% functionality for each animal on each test, and functional recovery was expressed as percent recovery relative to the pre-surgery baseline.

Fasting glucose was measured in blood obtained by retro-orbital collection by directing the collection tube gently in a ventro-lateral direction while rotating the tube. For multiple collection times, alternate right and left eyes were used. Kits were used to measure the concentrations of several metabolites in serum: insulin, IGF1 (IBL International, Hamburg, Germany) and free fatty acids (WAKO, Neuss, Germany). At the end of the caloric restriction period, rats were sacrificed (N = 7) and fat pads (mesenteric, retroperitoneal, epididymal, abdominal, and subcutaneous) were dissected, cleaned and weighed. Fat mass was defined as the sum of the adipose pads that were dissected.

The main effects of treatment and time as well as interactions of the two factors were analyzed using two-way ANOVA with repeated measures (GraphPad Software, USA), with treatment as between-subjects variable and time as within-subject variable. For quantitative data the results were expressed as mean ± standard deviation (Mean ± SD). The normality of the distribution of variables was determined by the Lilliefors test. The between-groups analysis was performed using post-hoc tests (Bonferroni) for multiple comparisons. The level of significance was set at $p < 0.05$, two-tailed test. Simple comparisons for normally distributed histological data were analysed using Student's t –tests. The level of significance was set at $p < 0.05$, two-tailed test.

After stroke, the body weight declined progressively in 3-mo and 20-mo ad libitum fed animals (Fig. 1C). While young rats began to regain weight by day 7, the aged *ad libitum* fed animals stopped losing weight by day 10 and barely recovered the pre-surgery weight by day 14. Nevertheless, in contrast to both young and aged *ad libitum* fed animals, the CR aged rats began to regain weight already by day 2 and reached pre-surgery levels by day 14 (Fig. 1C). At the end of the testing period, serum glucose levels increased significantly (x-fold; $p<0.01$) in all groups (Fig. 2A). Fasting serum insulin was significantly decreased (x-fold; $p<0.01$) by calorie restriction in aged animals (Fig. 2B). By day 14 after stroke, insulin levels were significantly increased (x-fold; $p<0.01$) in aged CR rats as compared to the young and AL animals (Fig. 2B). By day 7, the circulating IGF1 levels were significantly decreased (x-fold; $p<0.01$) in *ad libitum* fed aged animals as well as young rats but not in CR animals (Fig. 2D). After stroke, free fatty acid levels decreased significantly (x-fold; $p<0.01$) in *ad libitum* fed animals and by day 14 also in CR animals (Fig. 2C).

The beam-walking task

Because successful performance on the rotating rod requires complex sensorimotor skills, the after effects of the surgical procedure itself were evident in the first 3 days post-stroke, at which time all animals, including the young controls, had difficulty traversing the rotating cylinder. After an abrupt decline in performance on the rotarod at day 3 post-stroke, young rats began improvement and almost recovered by day 14 (Fig. 3A). In contrast, the aged *ad lib* fed rats never recovered fully (Fig. 3A). This was not recorded in aged, CR rats, in which recovery overall performance levels were better than the performance of their counterparts AL aged animals (Fig. 4A, green vs. red line).

Inclined plane

In aged rats, stroke severely impaired performance on the inclined plane test, and the *ad libitum* fed aged rats recovered to a limited extent during the study period (Fig. 4B, red line). Caloric restriction was effective throughout the study period, and allowed aged rats to recover to the levels attained by young rats (Fig. 4B, green line vs. blue line).

The adhesive tape removal test

The asymmetry index test probes for differences between forelimbs in cutaneous sensitivity and sensorimotor integration after stroke. As compared to pre-operative, trained animals, MCAO animals demonstrated a marked difference in post-operative performance for the left (affected) forelimb. By day 3 post-stroke, animals started recuperation and reached significant recovery of function during the entire testing period in the CR fed group as compared to the *ad libitum* fed animals (Fig. 4C, green line vs red line) albeit not to the levels attained by young rats (Fig. 4C, blue line).

Water maze

Over the pre-stroke training period of 7 days, rats learned to locate and climb onto the hidden platform and performance improved significantly during this time. In all groups, the path became shorter as the training sessions progressed (Fig. 4D). Because of the skull injury, we avoided testing the animals in the first week post-stroke. As previously shown, aged rats need more time to recover behaviourally after stroke than young animals (Buchhold et al., 2007). Consequently, the path length required to reach the platform in the 3rd quadrant reached a maximum by day 7 post-stroke. After 7 days, the animals began recovering in this test. The best recovery was seen for the CR group which showed significant improvement of spatial

reference-memory between days 7-14 as compared to the control group.

Immunohistochemical staining of the infarct area at day 14 using an anti-NeuN antibody showed that the infarct volumes were not significantly different between *ad libitum* fed aged animals and aged CR rats.

Upregulated genes: Genes that were upregulated in the perilesional cortex of calorie restricted aged rats as compared to the perilesional area of *ad libitum* fed aged rats, included those associated with glycogen metabolism, IGF signalling, apoptosis, arteriogenesis and hypoxia (Table 2). Remarkably, one of these genes, Prkaac/Prkga1, is localized to specific subcellular structures that corresponded with the expression pattern of glycogen phosphorylase and it has been associated with an increased availability of cellular energy from glycogen stores (Polekhina et al., 2003). The upregulated genes included genes that have a neuroprotective effect (*Igfbp3*) and genes that support angiogenesis (*Igf2*, *Mapkapk2*). The pro-angiogenic genes showed a 2.2-to 4.2-fold increase in CR aged rats as compared to *ad libitum* fed aged rats. Likewise, we noted high-level expression (2.2- to 6.5 fold increases over the perilesional area of ad libitum aged rats) of *Igf2* and *Mapkapk2* that promote both endothelial precursors cells (EPC) recruitment and incorporation into the neovascular area, resulting in an enhanced angiogenesis in vivo subsequent to hypoxia and inflammation (Maeng et al., 2009; Limbourg et al., 2015).

Downregulated genes: Downregulated genes in the perilesional cortex of the caloric-restricted aged rats as compared to the perilesional area of *ad libitum* fed aged rats, included genes involved in energy homeostasis (*Igf1r, Mapk10),* apoptosis *(Camk2g),* vasculogenesis *(Ppp2cb)* or tissue integrity *(Pmds6, Psmc4, Pmc3, Psmb5)*(Table 3).

Weight loss after stroke is a common feature both in rodents and human subjects and is related to impaired feeding, reduced physical activity, sympathetic activation, fever, inflammation, and metabolic imbalances due to insulin resistance, dyslipidemia, or endothelial dysfunction. As a result, accelerated muscle waste and cachexia may occur. Therefore it can be speculated that overweight patients could have a better outcome as they can, at least theoretically, counteract all of the above (Scherbakov et al., 2011). However, the complications determined by obesity including the risk for stroke, outweigh by far the benefits post-stroke.

Before stroke, the CR animals were characterized by a reduction in body weight, adipose tissue mass, circulating insulin, IGF1 and free fatty acids (FFA) levels as compared to the *ad libitum* fed animals. Interestingly, in caloric restriction aged rats we did not record a decrease in body weight after stroke but we noted accelerated body weight re-gain shortly thereafter. This contradicts the clinical observations discussed by Scherbakov et al (2011) but more experiments are needed to fully understand the significance of our findings. However, in our studies, weight gain and behavioural recovery improved in animals subjected to calorie restriction. Before stroke we noted increases in fat mass, serum free fatty acids, insulin and IGF1 levels in *ad lib* fed, aged rats that is suggestive of a state of glucose intolerance with hyperinsulinemia. Usually, glucose intolerance is associated with insulin resistance and insulin secretory defects in aging humans although other factors may contribute to the development of insulin resistance with age, such as obesity and lack of physical activity (Chang and Halter, 2003). Therefore, our experiments have revealed some rather unexpected results in contrast with previously published studies suggesting that the beneficial effect of decreased fat mass on body weight recovery in CR animals is explained by a decreased insulin resistance which is common in both aging rats (Barzilai and Gupta, 1999) and humans (Basu et al., 2003). Moreover, it is known that IGF-1 drops in parallel with increasing age in mice and rat as result of a significant decrease in protein synthesis ability (Sonntag et al., 1999). More recent studies, have reported that caloric restriction reduces plasma IGF-1 levels by 20% in mice (Berrigan et al., 2014).

In patients that were given an acute caloric restriction diet, it was reported a decrease in serum free IGF-I while the IGFBP-1 reached high levels (Henning et al., 2013).
These data are in contrast with our experiments on calorie restricted aged rats that have showed an increase in IGF-1 levels in the first week aftter stroke. However, considering the weight gain and behavioural recovery in CR aged rats, we could state that our results confirm the findings of some epidemiological studies that have noted an inverse relationship between plasma IGF-I levels and the risk of stroke. The same trend has been observed between plasma IGF-1 and the clinical recuperation and outcome after stroke. More specifically, the subjects with low plasma IGF-I levels have a higher risk of stroke and have a worse prognosis after stroke than those patients with high plasma IGF-1 (Bondanelli et al., 2006; Aberg et al., 2011; Denti et al., 2004).

It has been also suggested that in ischemic stroke patients circulating IGF-1 represents a marker of functional performance and outcome (Bondanelli et al., 2006). These conclusions have been subsequently confirmed by Aberg et al (2011). They suggest that during the neurorehabilitation after stroke, a high serum IGF-1 correlates with recuperation of long-term functions (Aberg et al., 2011). Indeed both hemorrhagic and ischemic stroke are associated with low serum concentrations of IGF-1 and IGFBP-3, deficits in neuromuscular performance and selective muscle atrophy (Silva-Couto et al., 2014).

Subsequent to the acute ischemic event, IGF-1 crosses the disrupted blood-brain barrier and can induce differentiation of neural cells in vitro, including neurons, astrocytes, oligodendrocytes, and endothelial cells in vitro and animal models of traumatic brain injury and thus may exert its neuroprotective effects (Mangiola et al., 2015). In parallel, insulin-like growth factor binding protein-3 (IGFBP-3) modulates the bioavailability, transportation, and localization of insulin-like growth factor-I (IGF-I), in animal stroke models especially when administered intranasally (Liu XF et al., 2001). In our model, CR rats had significantly increased serum IGF1 levels in the first week after stroke that coincided with an accelerated recovery of body weight; IGF-1 level was further maintained at day 14 by an increase in serum insulin levels. This setting could be similar with perinatal hypoxia-ischemia which increases cerebral vascular endothelial IGFBP3 expression. IGFBP-3 modulates cell fate by a complex interplay between cells' microenvironments and the presence of cellular IGFBP-3 binding partners and growth factor receptors. However, an increased serum IGFBP3 (by 100%) in CR animals (Olivo-Marston et al., 2014) could explain the elevated gene expression for IGFBP3 in the perilesional area of CR animals. Indeed, recent studies conducted one year after an acute ischemic stroke have indicated that IGFBP3 could represent an independent marker for functional outcome and recovery (Ebinger et al., 2015).

In addition, we have noted a number of genes that were upregulated in the perilesional cortex of caloric restricted aged rats as compared to the perilesional area of *ad libitum* fed aged rats, including genes involved in glycogen metabolism, IGF signalling, apoptosis, arteriogenesis and hypoxia. Downregulated genes in the perilesional cortex of the CR aged rats as compared to the perilesional area of *ad libitum* fed aged rats included genes involved in energy homeostasis (*Ins1, Igf1r, Mapk10),* apoptosis *(Camk2g),* vasculogenesis *(Ppp2cb)* or tissue integrity *(Pmds6, Psmc4, Pmc3, Psmb5)*.

In conclusion, our study shows that in post-stroke, aged calorie restricted Sprague-Dawley rats behavioural recuperation is enhanced as compared with *ad libitum* fed, overweighted rats. In this setting, there is an early gain in body weight and improved behavioural recovery that require complex sensorimotor skills, such as the rotating rod and inclined plane tasks, or cutaneous sensitivity and sensorimotor integration or spatial memory that were associated with increased serum glucose, insulin and IGF1 levels and with specific changes in gene expression including downregulation of genes involved in the ubiquitin proteasome degradation system (*Pmds6, Psmc4, Pmc3, Psmb5*), enhanced vasculogenesis (*Ppp2cb*), neuroprotection (*Mapk10*) and reduced apoptosis (*CaMKIIc*). All of these changes have been recorded in parallel with an increased expression of genes that are neuroprotective (*Igfbp3*), support angiogenesis (*Igf2*, *Mapkapk2*) and allow an increase in available energy (*Prkaac/Prkga1*). However, more experimental studies are needed, in order to fully understand the active complex inter-relationships in this pathological setting.