Effects of dietary restriction on ischemic injury, brain remodeling and neuroplasticity after focal cerebral ischemia in mice

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DEDICATION

“This thesis is dedicated to my parents (Carmen e Herminio) who encouraged me to pursue my dreams and for being my strength in every moment”.

“Esta tese é dedicada aos meus pais (Carmen e Herminio) que me incentivaram a perseguir meus sonhos e por serem minha força e meu refúgio em todos os momentos”.

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SUMMARY

LIST OF ABBREVIATIONS

βGluc	β-Glucuronidase
4HNE	4-Hydroxy-2-nonenal
11βHSD2	11-β-hydroxysteroid dehydrogenase
ADP	Adenosine Diphosphate
AKT	Protein kinase B
ALT	Alanine transaminase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDA	Biotinylated dextran amine
BMI	Body mass index
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca	Calcium
Ca^{2+}	Ionized calcium
Cat	Catalase
cDNA	Complementary DNA
CETP	Cholesterol ester transfer protein
CI	Confidence interval
CO_3^-	Carbonate radical
CNS	Central nervous system
Cxcl1	CXC-motif ligand-1
DAB	Diaminobenzidine tetrahydrochloride
Dcx	Doublecourtin
Epo	Erythropoietin
eNOS	Endothelial nitric oxide synthase
GAP43	Growth associated protein-43
GFAP	Glial fibrillary acidic protein
gGT	Gamma-glutamyl transferase
GH	Growth hormone
Glut1	Glucose transporter-1

Glut2	Glucose transporter-2
Gpx3	Glutathione peroxidase-3
GSH	Glutathione
GSK3	Glycogen synthase kinase-3
GSG	Reduced glutathione
GSHPX	Glutathione peroxidase
GSSG	Glutathione disulfide
HPA	Hypothalamic-pituitary-adrenal
HO1	Heme-oxygenase-1
$HO_2^{\bullet}$	Hydroperoxyl radicals
H_2O_2	Hydrogen peroxide
Iba1	Ionized calcium binding adaptor protein-1
ICAM1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IGF1	Insulin-like growth factor-1
IL1RA	Interleukin-1 receptor antagonist
IL1β	Interleukin-1β
IL6	Interleukin-6
IL8	Interleukin-8
IMCES	Imaging Center Essen
iNOS	Inducible nitric oxide synthase
IR or Insr	Insulin receptor
I.p	Intraperitoneal
KO	Knock-out
Ku70	Lupus Ku autoantigen protein p70
LDF	Laser Doppler flow
LDL	Low-density lipoprotein
M1	Macrophage 1
M2	Macrophage 2
MCAO	Middle cerebral artery territory
MCP1	Monocyte chemoattractant protein-1
MIP1α	Macrophage inflammatory protein 1 alpha
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin

Na	Sodium
NAD	Nicotinamide adenine dinucleotide
NFκb	Nuclear factor kappa B
NMDAR	N-methyl-D-aspartic acid
NMDAr	N-methyl-D-aspartic acid receptor
NPC	Neuronal progenitor cells
NO	Nitric Oxide
NO_2	Nitrogen Dioxide
NOS	Nitric Oxide Synthase
Nox4	NADPH oxidase-4
NSCs	Neural stem cells
N_2O	Nitrous oxide
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
$^{\bullet}OH^-$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with triton
PEU	Protein-energy undernutrition
PFA	Paraformaldehyde
PGC1α	Proliferator-activated receptor-γ coactivator-1α
PMN	Polymorphonuclear neutrophil
PVDF	Polyvinylidene fluoride
RNS	Reactive nitrogen species
ROCK	Rho associated kinase
ROS	Reactive oxidative species
rtPA	Recombinant tissue plasminogen activator
RTqPCR	Real-time quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
SHRSP	Stroke-prone spontaneously hypertensive rats
Sirt1	Sirtuin-1
SNAP25	Synaptosomal-associated protein-25

SNS	Sympathetic nervous system
Sod	Superoxide dismutase
Sod1	Superoxide dismutase-1
Sod2	Superoxide dismutase-2
SVZ	Subventricular zone
TBS	Tris-buffered saline
TNFα	Tumor necrosis factor-α
TrkB	Tropomyosin-related kinase B
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
UCP	Uncoupling proteins
VCAM1	Vascular cell adhesion molecule-1
WHO	World Health Organization
WT	Wild type

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ABSTRACT

Low-calorie and low-protein intake can be risk factor for stroke. Severe reduction of calorie and protein consumption aggravates neurological deficits and brain injury as a consequence of undernutrition. In contrast, moderate protein restriction prevents energy deprivation and protects offspring against perinatal hypoxia-ischemia. In our acute stroke studies, male C57BL6/j mice were exposed to energy undernutrition (1300kcal and 20% protein), protein-energy undernutrition (1300kcal and 8% protein) or moderate protein restriction (3541kcal and 8% protein) for 7, 14, or 30 days. Intraluminal middle cerebral artery occlusion was induced and mice were sacrificed 24 h later. In our post-ischemic studies, animals were subjected to focal cerebral ischemia followed by exposure to moderate protein restriction for up to 56 days. In the acute stroke phase, energy undernutrition, protein-energy undernutrition and moderate protein restriction reduced neurological deficits, brain injury, DNA fragmented cells and blood brain barrier disruption. However, energy undernutrition and protein-energy undernutrition induced neuroprotection solely when metabolism reached the compensated stage (14 days diet). All 3 diets reduced the expression of inducible NO synthase, leukocyte infiltration, microglial activation, and brain *IL1β* mRNA gene expression, but these anti-inflammatory effects were not associated with neuroprotection. In the post-ischemic intervention, moderate protein restriction also improved neurological and motor recovery, reduced microglia activation, whole brain, striatal, and corpus callosum atrophy and increased contralesional pyramidal tract plasticity at the level of the red nucleus. In all 3 studies, NAD-dependent deacetylase sirtuin-1 was not directly associated with the neuroprotection effects, but the regulation of this protein was implicated in the activation of anti-oxidant enzymes and inhibition of pro-inflammatory markers. As potential mechanisms, we observed that all 3 diets increased expression of anti-oxidants, such as glutathione peroxidase-3 and superoxide dismutase (1 and 2), showing that these enzymes are possible targets for neuroprotection. Our study suggests that energy undernutrition as well as protein-energy undernutrition provides neuroprotection in a narrow time window, while moderate protein restriction may be an efficacious strategy to enhance neurological recovery, short and long-term brain tissue survival, brain remodeling and plasticity in rodents.

ZUSAMMENFASSUNG

Die verminderte Aufnahme von Kalorien und Proteinen stellen Risikofaktoren für einen Schlaganfall dar. Als Folge von Unterernährung verschlechtert eine sehr starke Reduktion der Kalorien- und Proteinaufnahme neurologische Defizite und die Hirnschädigung. Dagegen verhindert eine moderate Proteinrestriktion Energiemangel und schützt Nachkommen gegen perinatale Hypoxie-Ischämie. In unseren akuten Studien wurden männliche C57BL6/j Mäuse für 7, 14 oder 30 Tage einer Energieunterversorgung (1300 kcal und 20% Proteine), einer Protein- und Energieunterversorgung (1300 kcal und 8% Proteine) oder einer moderaten Proteinrestriktion (3541kcal und 8% Protein) unterzogen. Eine intraluminale Okklusion der mittleren Zerebralarterie wurde induziert und die Mäuse wurden 24 Stunden später getötet. In unseren post-ischämischen Untersuchungen wurden die Mäuse nach einer fokalen zerebralen Ischämie einer moderaten Proteinrestriktion für 56 Tage ausgesetzt. In der akuten Phase nach dem Schlaganfall reduzierten die Energieunterversorgung, die Protein- und Energieunterversorgung und die moderate Proteinrestriktion die neurologischen Defizite, die Hirnschädigung, die Zahl der Zellen mit fragmentierter DNA und die Permeabilität der Blut-Hirn-Schranke. Allerdings induzierten die Energieunterversorgung und die Protein- und Energieunterversorgung eine Neuroprotektion nur dann, wenn der Metabolismus die kompensierte Phase (14 Tage Ernährungsintervention) erreichte. Alle drei Ernährungsformen reduzierten die Expression der induzierbaren NO-Synthase, die Leukozyteninfiltration, die Mikrogliaaktivierung und die zerebrale *IL1β* mRNA Genexpression, allerdings waren diese inflammatorischen Effekte nicht mit einer Neuroprotektion assoziiert. Die post-ischämische Intervention durch die moderate Proteinrestriktion verbesserte ebenso die neurologische und motorische Funktionserholung, reduzierte die Mikrogliaaktivierung, die Atrophie des gesamten Gehirns, des Striatums und Corpus callosum und steigerte die Plastizität des kontraläsionalen Pyramidenbahnsystems auf der Ebene des Nukleus ruber. In allen drei Studien war die NAD-abhängige Deacetylase Sirtuin-1 nicht direkt mit neuroprotektiven Effekten assoziiert, aber die Regulation dieses Proteins spielte eine Rolle bei der Aktivierung antioxidativer Enzyme und der Inhibierung proinflammatorischer Signale. Als potentieller Mechanismus stellten wir fest, dass alle drei Ernähungsformen die Expression von Antioxidantien wie der Glutathionperoxidase-3 und der Superoxiddismutase (1 and 2) erhöhten, was zeigt,

dass es sich bei diesen Enzymen um mögliche Zielstrukturen für die Förderung von Neuroprotektion handelt. Unsere Untersuchungen deuten darauf hin, dass eine Energieunterversorgung, sowie eine Protein- und Energieunterversorgung Neuroprotektion innerhalb eines engen Zeitfensters vermitteln, während eine moderate Proteinrestriktion eine wirksame Strategie sein könnte, um die neurologische Funktionserholung, kurz- und langfristiges Überleben des Hirngewebes, strukturelle Hirngewebe-Reorganisation und Plastizität in Nagetieren zu fördern.

1 INTRODUCTION

Although undernutrition is a risk factor for stroke onset, it is an important predictor for stroke recovery. Reduction of food intake, metabolism changes, chronic diseases and inactivity may aggravate undernutrition, particularly in aged adults [1]. The high prevalence of undernutrition in elderly patients before stroke can range from 6-62%, of which around 16% can be diagnosed with protein-energy undernutrition (PEU). However, after stroke, the incidence of undernutrition in these patients can reach up to 73%, increasing the period of hospitalization and worsening stroke outcome [2].

Previous clinical studies showed that high daily consumption of meat over years or high calorie intake (based on fat and protein-rich foods) increases the incidence of stroke events and can worsen stroke outcome [3-5]. However, studies with undernourished stroke patients did not investigate calorie intake as risk factor. They mostly used well-established markers of undernutrition such as low body weight, body fat, body mass index (BMI) and serum albumin [6, 7].

In humans, consequences of calorie or protein intake for stroke outcome and recovery have never systematically been examined [8]. However, a prospective study already showed that calorie intake of stroke patients (17.4 ± 8.4 kcal/kg for patients with BMI <21.9 kg/m^2) and protein intake (2.72 ± 1.16 kcal/kg protein for patients with BMI <21.9 kg/m^2) is lower than nutritional requirements of stroke patients (≥25 kcal/kg and >4 kcal/kg protein in eutrophic patients) [9, 10]. This lower consumption is strongly correlated with age, gender, admission weight and neurological deficits [10].

Experimental studies using calorie restriction protocols showed inconsistent results regarding neurological deficits and brain injury after focal or global cerebral ischemia [11-18]. Mild calorie restriction reduced pro-inflammatory markers and promoted neuroprotection by stimulating the activity of growth factors, anti-oxidant enzymes, chaperones and NAD-dependent deacetylase Sirtuin-1 (Sirt1) after ischemia [11-13, 18-20].

In contrast, severe protein restriction (0.5-2% protein) impaired functional recovery, neuroplasticity, brain inflammation and neuronal survival, while moderate protein restriction (7-8%) recently induced neuroprotection in a perinatal hypoxia-ischemia model [15, 21-24].

Considering the heterogeneity among previous studies and several open questions, the present thesis aims to investigate the influence of different types of

undernutrition on ischemic injury, neurological deficits, and brain remodeling after focal cerebral ischemia. In two settings we examined how undernutrition before or after stroke influences stroke pathogenesis: 1) mice exposed to undernutrition (energy undernutrition), severe undernutrition (protein-energy undernutrition) or diet restriction (moderate protein restriction) for 7, 14 and 30 days were subsequently submitted to intraluminal middle cerebral artery occlusion (MCAO); and 2) mice submitted to intraluminal MCAO were subsequently exposed to moderate protein restriction for up to 56 days.

2 BACKGROUND

2.1 Undernutrition

Undernutrition is defined as insufficient intake of macro- or micronutrients that meet the individual's needs. The term undernutrition is synonymously used as "malnutrition" in many studies, however, this is a misconception because "malnutrition" in a strict sense can be either undernutrition (inadequate intake) or overnutrition (excessive intake) [25]. Protein-energy undernutrition (PEU) and dietary deficiencies such as anemia, iron, iodine and vitamin A deficiencies are major manifestations of undernutrition [26].

The degree and distribution of undernutrition across life stages are closely related to political and economic situations, food production and access, dietary intake habits or social interactions [27]. According to a recent bulletin from the World Health Organization (WHO), the number of children under 5 years classified as undernourished are 52 millions and classified as severely undernourished are 17 millions. In addition, 462 million adults are underweight [28]. The WHO report did not subclassify adults by age, but a recent study with older adults showed a prevalence of undernutrition about 25–60% in geriatric care facilities, and 35–65% in geriatric hospitals [29].

A particularly severe form of undernutrition is PEU, characterized by inadequate protein and energy intake [27]. In children, PEU can be classified as low weight for age (underweight), low height for age (stunting) or low weight for height (wasting) with severe impact on growth. The direct consequences are marasmus (severe undernutrition), marasmic kwashiorkor (severe undernutrition followed by peripheral edema) or kwashiorkor (undernutrition with peripheral edema) [27]. Poor nutritional status originates from or is associated with malabsorption (marasmus), infectious diseases (kwashiorkor), developmental delay, wasting, anorexia, diarrhea, anemia, skin sores, alopecia, and dehydration (marasmic kwashiorkor) [26].

In adults, PEU is characterized by insufficient energy intake, marked weight loss, loss of muscle mass and subcutaneous fat, liver steatosis, lethargy and edema. These changes are associated with acute phase proteins response such as low blood levels of albumin, prealbumin and transferrin [30, 31].

In elderly patients, though, a common occurrence is anorexia of aging, which consists of a spontaneous reduction of food intake to <70% of estimated needs (typically <1000 calories per day), resulting in undernutrition or PEU [32]. Generally at this age, the nutrient storage and absorption are compromised, drug-nutrient

interactions are disturbed and functional and immunologic responses are decreased. In addition, other facts such as frailty, excessive polypharmacy, digestion problems, depression and cognition loss are also observed [33]. Interestingly, elderly patients who had balanced intake of calories, riboflavin and niacin after stroke showed low degree of anxiety or depression when compared with undernourished elderly patients [34].

In fact, lack of information about the undernutrition state before and after stroke results from the lack of standard methods for nutritional assessment and patient inability to express food habits [35]. In order to mimic undernutrition and to study its role in stroke pathogenesis, experimental studies submitted animals to focal or global cerebral ischemia and used diet protocols that either reduced the total amount of food to 30-60% and protein content to 0.5%-2% or induced fasting [12, 13, 15-19, 21-23, 36-38]. The findings suggest that changes of diet composition markedly influence stroke outcome.

2. 2 Stroke

Clinically, stroke is characterized as an acute focal injury in the central nervous system (CNS), resulting in impaired neurological deficits and loss of brain functions [39]. Major risk factors for stroke are age, arterial hypertension, hypercholesterolemia, cigarette smoking, alcohol abuse, insulin resistance, diabetes, hormonal contraception, physical inactivity, obesity and undernutrition [39].

Earlier studies estimated that 50% of stroke patients are over 75 years and 30% are over 85 years [20]. Recent evidences, however, indicate that stroke incidence is increasing among young people (20-40 years age) as well, representing 10% of all stroke cases. The authors found that 74% of all young stroke patients exhibited inadequate lifestyle and unhealthy diet habits [40].

In general, stroke is classified in two types that are either induced by the thromboembolic occlusion of brain vessels (ischemic stroke) or the rupture of blood vessels (hemorrhagic stroke) [41]. As the incidence of ischemic stroke is higher than hemorrhagic stroke, experimental studies developed surgical methods to mimic the brain vessel occlusion in animal models. In general, global and focal ischemia models exist. The most widely used global occlusion models are two-vessel occlusion or four-vessel occlusion, which mimic consequences of cardiac arrest [42]. In focal ischemia, the middle cerebral artery (MCA) is most often occluded, either

proximally by an intraluminal suture or distally by the clipping or coagulation of the artery via a small craniotomy [43].

The pathogenesis of stroke is defined by a complex cascade of mechanisms related to oxygen and energy (glucose) deprivation, which compromises brain function and activates pathways related to oxidative and nitrative stress, inflammation, necrosis, and apoptosis. At the cellular level, ischemia interrupts oxidative phosphorylation and adenosine triphosphate (ATP) synthesis, induces plasma membrane depolarization, and increases the intracellular sodium (Na) and calcium (Ca) concentration. Additionally, ischemia triggers the release of excitatory neurotransmitters such as glutamate and activates N-methyl-D-aspartic acid (NMDA)-type (NMDARs), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate-type ionotropic receptors [44, 45]. Reactive nitrogen/oxygen species (RNS/ROS) are produced activating downstream pathways that aggravate brain injury [45, 46]. Low energy supply also activates nitrosative and oxidative stress, which induces acidosis in neurons via lactic acid accumulation, increased H^+ concentrations and elevated hydrogen peroxide (H_2O_2) degradation to superoxide anion ($O_2^{\bullet -}$) and hydroperoxyl radicals ($HO_2^{\bullet}$) [47].

In addition, in acute ischemic stroke, endogenous anti-oxidant enzymes (such as superoxide dismutases, glutathione peroxidases and catalase) are unable to prevent free radical formation in brain cells [47, 48]. In cerebral ischemia, mitochondria are the main source of ROS production through the activity of NADPH oxidase (Nox4), phospholipase A2, cyclooxygenase, and the suppression of superoxide dismutase (Sod) activity [49, 50]. The formation of reactive oxygen and nitrogen species is summarized in Figure 1.

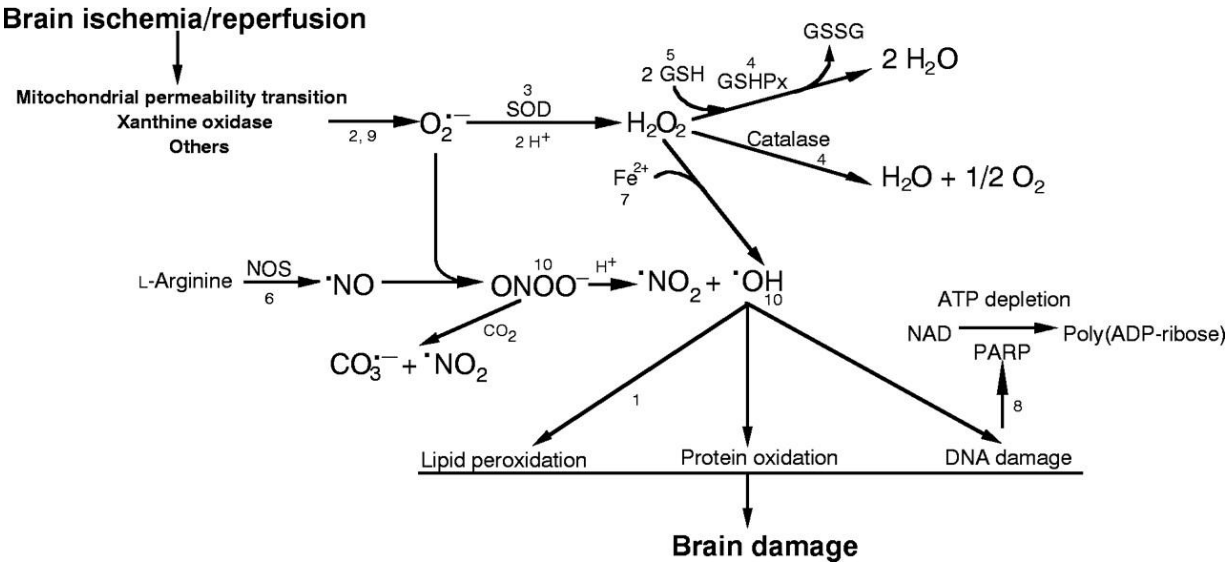

Figure 1: Ischemia/reperfusion induces formation of reactive oxygen/nitrogen species in numerous ways, resulting in tissue injury. Sod, superoxide dismutases; GSHPx, glutathione peroxidases; GSH, glutathione; NOS, nitric oxide synthases; PARP, poly (ADP-ribose) polymerases; $O_2\cdot^-$, superoxide; $CO_3\cdot^-$, carbonate radical; H_2O_2, hydrogen peroxide; GSSG, glutathione disulfide; OH, hydroxyl radical; $\cdot NO_2$, nitrogen dioxide;· NO, nitric oxide; $ONOO^-$ peroxynitrite; NAD nicotinamide adenine dinucleotide. Warner; Sheng; Batinić-Haberle; 2004, Journal of Experimental Biology, p. 3223. Copyright 2004 by The Company of Biologists. Reprinted with permission [51].

Excessive RNS and ROS have profound effects on the cerebral vasculature, cerebral blood flow and the structure of DNA, cytoskeletal, protein and membrane lipids. Excessive ROS and RNS can also affect intracellular Ionized calcium (Ca^{2+}) stores and cell survival [47]. In addition, an overproduction of free radical causes oxidative disruption of macromolecules, namely lipid peroxidation. Products of lipid peroxidation are malonaldehyde (MDA) and 4-hydro-2-nonenal (4HNE), and they are used as a markers of oxidative stress [9]. Compare to MDA, 4HNE has a higher biological activity, being the most reactive aldehyde, which was found to increase infarct volume and reduce the antioxidant responses after MCA occlusion (MCAO) [52].

Cerebral ischemia also alters the pathway that involves peroxisome proliferator activated receptor gamma coactivator-1-alpha (PGC1α), a major regulator of ROS scavenging enzymes [53]. This alteration activates phosphorylation and deacetylation processes that are promoted by silent mating type information regulation 2 homolog 1 (Sirt1 also called sirtuin-1) which is a nicotine adenine dinucleotide (NAD+)-dependent enzyme [54]. Sirt1 is widely expressed in brain cells including neurons, astrocytes, microglial cells and progenitor cells, playing an important role in cell metabolism and endogenous neuroprotection [54, 55]. Experimental studies showed that *Sirt1*$^{-/-}$ mice had larger infarct volumes, in contrast

to animals with overexpression of *Sirt1* that showed less hippocampal injury after stroke [56, 57]. The activation or inhibition of Sirt1 in stroke models directly impacts infarct size via control of different pathways, such as activation of the so-called energy exhaustion pathway (Sirt1-AMPK signaling); the inhibition of apoptotic mediators (p53) and pro-inflammatory factors such as nuclear factor kappa B (NFκB); the modulation of DNA repair Lupus Ku autoantigen protein p70 (Ku70) and the regulation of cerebral blood flow by activation of endothelial nitric oxide synthase (eNOS) [56, 57]. During ischemia, Sirt1 also induces ATP production via glycolysis and gluconeogenesis in the liver, increasing the glucose availability [58].

Apart from the role of Sirt1 in metabolic support after stroke, it is extremely important to understand the individual response of glucose and insulin receptors signaling mainly because these pathways are responsible for energy homeostasis, neuronal survival, and endogenous neurogenesis, which are important factors for stroke recovery [36, 59, 60]. Glucose metabolism disturbance can be associated with reduced glucose transporter (GLUT) activity. This reduction affects glucose uptake, glycolysis and angiogenesis [61]. The 5 subtypes of GLUTs have distinct glucose transport capacity and insulin dependency [62]. Herein, the present work focuses on changes in the mRNA gene expression of GLUT1 and GLUT2 subtypes in the core of the ischemic stroke and in the liver of ischemic animals. GLUT1 is widely distributed in neurons and astrocytes with high efficiency of cellular uptake at very low blood glucose concentration. GLUT2 is mostly present in the liver, kidney, β cells, and small intestine. Downregulations of GLUTs after cerebral ischemia may occur, but glucose uptake can also be induced by insulin via stimulation of cerebral glucose metabolism [62].

Insulin is a peptide hormone released by pancreatic Langerhans islet β cells which plays a main role in maintaining cellular energy supply [62]. Insulin signaling is activated by insulin binding to the extracellular α subunit of the insulin receptors (IRs). In the brain, these receptors are widely detected in neurons and glia. A growth factor with structural homology to proinsulin is named insulin-like growth factor-1 (IGF1), which has its own receptor but also has high affinity to insulin receptors. Once IGF1 binds IRs, insulin-related pathways are activated [62]. After cerebral ischemia, IGF1 activity plays an important role in long-term stroke recovery by reducing infarct volume and ischemic cell injury, promoting neuroplasticity, and suppressing pro-inflammatory transcription factors [36].

Another important target after stroke onset is inflammation. In the ischemic brain, microglia, polymorphonuclear neutrophilic granulocytes (neutrophils), monocytes/macrophages, and regulatory T cells are activated within minutes to hours. In response to this activation, tumor necrosis factor-α (TNFα), interleukins- 1β, 6 and 8 (IL1β, IL6 and IL8), monocyte chemoattractant protein-1 (MCP1) and macrophage inflammatory protein-1alpha (MIP1α) are released, promoting the adhesion and transendothelial migration of additional circulating leukocytes. The infiltrated leukocytes continue to release cytokines and chemokines, accelerating ROS production and activating matrix metalloproteinase 9 (MMP9), leading to the blood-brain barrier disruption (BBB), brain edema, and neuronal death [63]. Nitric oxide (NO) produced by inducible NO synthase is another pro-inflammatory and secondary oxidative stress mediator involved in this cascade [64]. As part of the inflammatory response, astrocytes are activated by upregulating glial fibrillary acidic protein (GFAP). This protein forms the glial scar surrounding the brain injured. Reactive astrogliosis is characterized by the expansion of the cell cytoskeleton resulting in cellular hypertrophy [65]. Indeed, stroke activates several cascades and a successful treatment needs to act on different targets.

2.2.1 Advances in acute stroke treatment

Currently, recombinant tissue plasminogen activator (rtPA) is a well-established and frequently used treatment for acute ischemic stroke. It acts as a fibrinolytic agent, which breaks up the clot allowing reperfusion of brain tissue [42]. rtPA is effective within 4.5 hours after stroke, and this is a limitation, because many patients either do not recognize the stroke symptoms quickly enough or do not receive hospital assistance on time [66]. Also, the number of patients treated with rtPA is limited because of hospital admission steps, such as: blood tests, brain imaging and additional exams for excluding hemorrhagic stroke [66].

Recently, mechanical thrombectomy became another established stroke treatment, in which the blood clot is interventionally removed via a catheter. This method is indicated for patients who did not receive or did not respond to rtPA therapy and can be applied over 6 h after stroke onset or even longer [67]. The current therapies listed above still have several limitations, and the majority of patients has neurological deficits in the long term [68].

In the past decades, a large number of neuroprotection studies have failed in clinical trials [16]. Several reasons to study failures have been identified, such as inadequate drug timing and dosing and insufficient patient follow-up [69].

The poor nutritional conditions of patients including undernutrition, overnutrition or hypercholesterolaemia have insufficiently been considered in ischemic stroke studies. Statins, which are lipid-lowering drugs, already showed beneficial effects on ischemic injury and stroke relapses [70, 71]. However, the effects of food supplementation or restriction on stroke recovery are still undetermined [39, 72].

Pharmacological and cell-based therapies, have recently been used to target endogenous brain responses such as neuroplasticity or neurogenesis [73]. It is still unclear how restorative brain responses are altered by undernutrition.

2.2.2 Neuroplasticity, spontaneous brain repair and post-acute stroke therapy

Neuroplasticity is inhibited in the adult brain. Upon ischemia, neurons sprout, creating suitable environment for recovery of function [74, 75]. This process was confirmed by experimental studies which showed that subtle anterograde Wallerian degeneration of pyramidal tract axons occurs [73, 76]. The degeneration of pyramidal tract axons promotes contralesional pyramidal fiber outgrowth, innervating denervated neurons in the stroke–affected brain hemisphere. Together, all these processes compensate lost functions [73]. Our group proved that after delivering Epo and VEGF, homologous fibers originating from the contralesional motor cortex grow out across the midline [76].

The nutritional status influences neuroplasticity. PEU reduces neuronal cell survival, proliferation, differentiation, and plasticity after ischemia [23, 77]. In addition, undernutrition induces profound alterations in the release of growth factors, such as IGF1, which reduces endogenous neurogenesis and affects neuronal sprouting [78].

2.2.3 Mechanisms involved in post-ischemic undernutrition

Apart from the stroke event, undernutrition before or after stroke activates distinct nutrient-sensing pathways [79]. In general, severe energy depletion inhibits the synthesis of proteins and this negatively affects brain function and whole body metabolism [9]. The interaction between stroke and undernutrition induces a marked metabolic change that directly affects essential metabolic organs such as liver. Shortly after stroke, inflammatory mechanisms promote the rise of liver enzymes

(e.g. alanine transaminase [ALT], gamma-glutamyl transferase [gGT] and aspartate transaminase [AST]) and bilirubin levels [80].

Both undernutriton and stroke induce a global catabolic/anabolic imbalance and stimulate the neuroendocrine sympathetic system, cytokines, immunomodulators and C-reactive protein, resulting in anorexia, natriuretic peptide accumulation, fever, and sarcopenia (see Figure 2). This metabolic response is aggravated when undernutrition and stroke convene with each other [81].

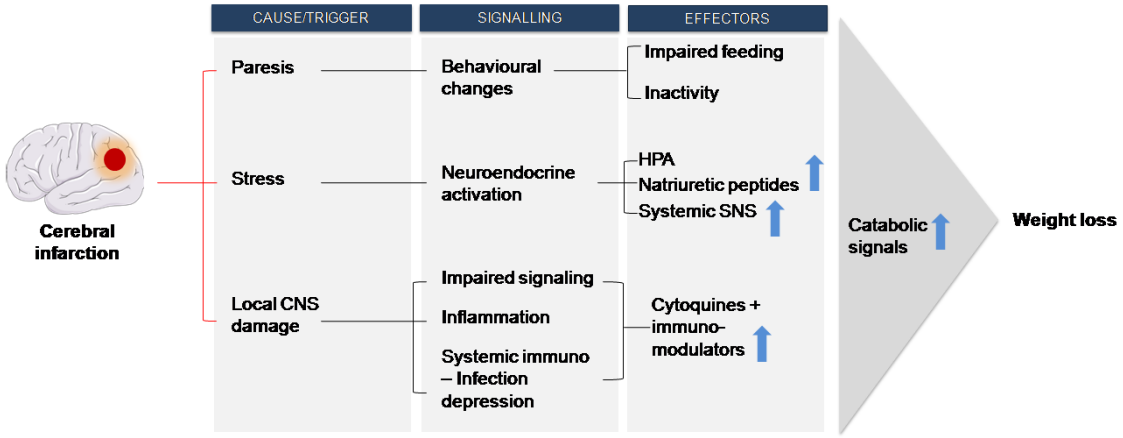

Figure 2: Schematic overview on the complex pathophysiology of systemic metabolic changes and weight loss in ischemic patients. Hypothalamic-pituitary-adrenal (HPA) and Sympathetic nervous system (SNS). Figure adapted from Scherbakov; Dirnagl; Doehner; 2011, Stroke, p. 3648. Bain figure from https://smart.servier.com/ [81].

Recently, studies proved that stroke reduces the levels of Sirt1 and IGF1 in the brain and in liver [54]. Low calorie diets can help to re-establish the intracellular levels of these proteins even though the beneficial effects of low calorie intake occurs solely without undernutrition stage [54]. To conserve energy for survival during undernutrition, circulating IGF1 levels and insulin sensitivity are decreased despite growth hormone (GH) releases are increased, resulting in a growth hormone resistance. This drastic shift occurs in the liver, where IGF1 is produced. To control the hypoglycemia, Sirt1 regulates GH-dependent IGF1 production as an adaptive mechanism. Interestingly, in this stage Sirt1 from brain and liver are recruited to reestablish the GH–IGF1 axis in the liver, showing the important interaction of these proteins under metabolic insults [82].

After stroke, mild calorie restriction and intermittent fasting reduce oxidative stress by activation of heme-oxygenase 1 (HO1), by upregulation of uncoupling

proteins (UCPs) and by deacetylation of PGC1α [83]. On the other hand, undernutrition drastically reduces the endogenous and exogenous anti-oxidant availability, exacerbating ROS production and worsening brain injury and cell survival [84, 85].

Stroke patients commonly have increased levels of plasma catecholamine, glucagon, cortisol, IL6, Interleukin-1 receptor antagonist (IL1RA), acute phase proteins, neutrophils, and white blood cell counts [30, 81, 86]. Similarly, PEU in ischemic animals decreased albumin levels by up to 31%, while serum α2-macroglobulin increased by up to 445%. This worse inflammatory response was associated with impaired walking [19].

Putting all these findings together, undernutrition plays an important role in pathways modulating ischemic injury and stroke recovery.

2.3 Undernutrition as a risk factor for stroke

In earlier life stages, maternal nutritional restriction contributes to the onset of disease in later life, which includes stroke. A poor nutritional status during maternity reduces cerebrovascular abnormalities that include decreased vascular density in offsprings [87, 88]. Previous studies showed that stroke-prone spontaneously hypertensive rats (SHRSP) kept under mild protein restriction (9%) had a lower life span and weight birth, premature and bigger stroke lesions, and higher blood pressure when compared to normotensive rats [89]. The authors suggested that low protein diet during maternity leads to decreased numbers of renal nephrons and renal 11-β-hydroxysteroid dehydrogenase (11βHSD2) activity (responsible for salt sensitivity) and elevated activity of the renin-angiotensin system (RAS) [89, 90]. On the other hand, adult SHRSP rats under dietary restriction (50% calories of control, 10% protein) for 2 weeks showed higher systolic blood pressure, delayed stroke onset and increased life span. In this study, decreased expression levels of *IL1β*, *IL6*, and *TNFα* mRNA in adipose tissues as well as decreased expression of intercellular adhesion molecule-1 (*ICAM1*), vascular cell adhesion molecule-1 (*VCAM1*) mRNA and cluster of differentiation 68 (*CD68*) mRNA were found in the brain. These results indicate that dietary restriction with normal protein levels suppress systemic inflammation and reduce macrophages infiltration into the brain before stroke onset [91]. Based on the studies above, protein intake may be a primary determinant of stroke development.

2.4 Undernutrition as a risk factor for poor stroke recovery

After ischemia, 90% stroke patients do not reach the calorie requirements during their hospital stay [92]. These patients although receiving oral food obtain <50% of their estimated calorie requirements [92]. This lack of food intake is also associated with physical limitations, dysphagia and disturbed taste [92, 93].

Experimental studies proved that severe undernutrition imposed before stroke worsens neurological and motor recovery, brain infarction, astroglial reactivity and NFκB activation after stroke [15, 19, 21, 22]. A drastic correction of overweight to normal weight, on the other hand, is a predictor for good stroke recovery. Obese aged mice under calorie restriction and intermittent fasting for 8 weeks (70% of total amount of food) before their stroke showed an early and quick regain of weight after stroke associated with increased plasmatic glucose, insulin and IGF1 levels. In this case a reduction of food intake can be beneficial for stroke once reversing obesity [17].

Recently, some studies started to investigate how postischemic undernutrition influences stroke outcome. In these studies, PEU was imposed directly after MCAO for one month. The authors observed that after ischemia animals had worse rearing behavior, limb dysfunction, and loss of mature cortical neurons. They associated these negative effects with higher levels of acute phase protein and inflammation [19, 38]. These studies proved that severe energy depletion after stroke is a predictor for poor stroke outcome. Based on the studies above, several features of undernutrition and stroke recovery are still insufficiently investigated. Neurological recovery and brain remodeling were not systematically studied in the past.

3 Scope and general aims

This thesis is based on two published papers (study 1 and 2), and one unpublished paper currently in preparation (study 3). The overall aim was to study the consequences of undernutrition for ischemic injury, stroke recovery and brain plasticity. Thus, two different approaches were used: 1) mice exposed to undernutrition (energy undernutrition), severe undernutrition (protein-energy undernutrition) or diet restriction (moderate protein restriction) for 7, 14 and 30 days were subsequently submitted to intraluminal MCAO; and 2) mice submitted to intraluminal MCAO were subsequently exposed to moderate protein restriction for up to 56 days.

3.1 Specific aims:

- To determine nutrition-related clinical manifestations, murinometrical changes and blood biomarkers (glucose, LDL, triglycerides, cholesterol) in mice exposed to energy undernutrition, protein-energy undernutrition, and moderate protein restriction for 7, 14 and 30 days that were subsequently exposed to MCAO.
- To investigate acute neurological changes, brain injury and blood-brain barrier permeability in animals exposed to energy undernutrition, protein-energy undernutrition, and moderate protein restriction for 7, 14 and 30 days that was followed by MCAO.
- To evaluate neuronal survival and DNA fragmented cells in animals exposed to energy undernutrition, protein energy-undernutrition, and moderate protein restriction for 7, 14 and 30 days that was followed by MCAO.
- To assess leukocyte infiltration, microglia activation, astrogliosis and iNOS formation response in animals exposed to energy undernutrition, protein-energy undernutrition, and moderate protein restriction for 7, 14 and 30 days that was followed by MCAO.
- To determine the role of Sirt1 and downstream proteins (IGF1, IR, Glut1, Glut2, Sod1, Sod2, Cat, NFκB, IL1β, Nox4) in animals exposed to energy undernutrition, protein-energy undernutrition, and moderate protein restriction for 7, 14 and 30 days that was followed by MCAO.
- To evaluate liver responses in animals exposed to energy undernutrition, protein-energy undernutrition, and moderate protein restriction for 7, 14 and 30 days that was followed by MCAO.

- To determine nutrition-related clinical and murinometrical changes manifestations in ischemic mice exposed to MCAO that was followed by moderate protein restriction over up to 56 days post-stroke.
- To examine the blood plasmatic levels of glucose, lipids, hepatic enzymes (ALT and AST), acute phase proteins (albumin and total protein) and urea nitrogen in ischemic animals exposed to MCAO that was followed by moderate protein restriction over up to 56 days.
- To assess effects of post-ischemic moderate protein restriction on neurological deficits, motor performance, and anxiety over up to 56 days post-stroke.
- To evaluate effects of post-ischemic moderate protein restriction on brain atrophy over up to 56 days.
- To analyze neuronal survival, endogenous neurogenesis, and neuroplasticity in animals exposed to MCAO that was followed by moderate protein restriction over up to 56 days.
- To investigate astrogliosis and microglia activation in ischemic animals exposed to MCAO that was followed by moderate protein restriction over up to 56 days.
- To determine the role of Sirt1 and downstream proteins (IGF1, IR, Glut1, Glut2, Sod1, Sod2, Cat, NFκB, IL1β, Nox4) in ischemic animals exposed to MCAO that was followed by moderate protein restriction over up to 56 days.
- To study liver responses in animals exposed to MCAO that was followed by moderate protein restriction over up to 56 days post-stroke.

4 STUDY 1

Title: Neuroprotection induced by energy and protein-energy malnutrition is stage-dependent after focal cerebral ischemia in mice

Author's contributions:

Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%

Contributed to the acquisition of the data:

Animal experiment: TSC – 100%

Histochemical analysis: TSC – 90% / MS, ED – 10%

Molecular analysis: TSC – 80% / EHSM, LMNM, ARSM – 20%

Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 30% / EHSM, LMNM, ARSM, ED – 10%

Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%

Provided final approval of the version to publication: TSC – 50% / DMH – 50%

Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TSC – 50% / DMH – 50%

Authors' contributions:

Tayana Silva de Carvalho and Dirk M. Hermann designed the study. Tayana Silva de Carvalho performed the animal experiments, assisted by Eduardo H. Sanchez-Mendoza and Maryam Sardari. Tayana Silva de Carvalho, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Maryam Sardari conducted histochemical and molecular biological studies. Dirk M. Hermann and Christoph Kleinschnitz provided infrastructural support. Tayana Silva de Carvalho, Eduardo H. Sanchez-Mendoza, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Dirk M. Hermann analyzed the data. Tayana Silva de Carvalho and Dirk M. Hermann drafted the manuscript. All authors concluded it.

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ORIGINAL ARTICLE

Neuroprotection Induced by Energy and Protein-Energy Undernutrition Is Phase-Dependent After Focal Cerebral Ischemia in Mice

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Abstract

Malnutrition predisposes to poor stroke outcome. In animal models, undernutrition protected against ischemic injury in some, but not in other studies. In view of diverse stroke models and food restriction paradigms, the consequences of undernutrition are poorly understood. Herein, we exposed mice to energy-reduced and protein-energy-reduced diets for 7–30 days and subsequently induced intraluminal middle cerebral artery occlusion. Undernutrition phase dependently influenced ischemic injury. Short-lasting 7 days of protein-energy undernutrition, but not energy undernutrition, decreased post-ischemic brain leukocyte infiltration and microglial activation and reduced brain *Il-1β* mRNA, but did not protect against ischemic injury. Fourteen days of energy and protein-energy undernutrition, on the other hand, reduced ischemic injury despite absence of anti-inflammatory effects. Anti-oxidant genes (*Sod-1*, *Sod-2*, and *Cat* mRNAs) were regulated in the liver and, to a lesser extent, the ischemic brain, indicating an adapted, compensated stage. Conversely, 30 days of energy and protein-energy undernutrition caused progressive animal exhaustion associated with post-ischemic hypoperfusion, rise of metabolic markers (*Sirt-1* and *Glut-1* mRNAs, Sirt-1 protein) in the ischemic brain, and reregulation of pro- and anti-oxidant markers (now also *Nox-4* and *Gpx-3* mRNAs) in the liver. In the latter condition, no neuroprotection was noted. Our study suggests an adaptation of metabolic systems that provides neuroprotection in a circumscribed time window.

Keywords Cerebral blood flow · Diet modification · Ischemic stroke · Malnutrition · Neuroinflammation · Neuroprotection

Introduction

Malnutrition predisposes to death, stroke, and poor stroke outcome in humans. In a systematic analysis of 57 prospective studies involving 894,576 adults that were followed up over 13 years, a low body mass index (BMI; < 20 kg/m^2) was associated with increased total and vascular mortality [1]. Malnutrition increases stroke risk in addition to established vascular risk factors; as shown in the U.S. Renal Data System registry, a cohort of 8920 patients with end-stage renal disease, in which three markers of malnutrition, that is, low weight (hazard ratio 1.09 (confidence interval 1.00–1.18) per 25% decrease), low serum albumin (1.43 (1.17–1.74) per g/dl decrease), and investigator judgment of undernourishment (1.27 (1.01–1.61)) independently predicted stroke risk [2]. In 104 acute stroke patients, protein-energy malnutrition increased cortisol stress responses, predisposed them to urinary or respiratory infections, and reduced neurological recovery after 1 month [3].

Undernourishment may have various causes, including disease-associated wasting, denutrition of various causes (including denutrition post-stroke), or intended fasting, which have very different consequences for stroke outcome. Considering the high prevalence of undernutrition in stroke patients, ranging from 6 to 62% depending on hospital environments [4], relatively little is known about how undernutrition affects ischemic brain injury. This is in contrast to the vast literature about the role of overnutrition and obesity in the ischemic brain (e.g., see [5, 6]). Surprisingly, some

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experimental studies showed that continuous or intermittent food restriction reduces post-ischemic neurological deficits [7–11], whereas other studies found exacerbated or unchanged neurological impairments [12–15]. Some studies showed that undernutrition reduced histological brain injury [7–9, 16, 17], whereas other studies observed no effect [10–13]. Some studies reported anti-inflammatory effects related to undernutrition, that is, reduced microglial activation [15] and decreased interleukin (IL)-1β, IL-6, or tumor necrosis factor-α (TNF-α) levels in the brain and blood [9, 16, 17]. Some studies described an upregulation of the NAD-dependent deacetylase sirtuin-1 (Sirt-1) that conferred neuroprotection [8, 16], an upregulation of anti-oxidant heme oxidase-1 and chaperones [7, 17], or an upregulation [17] or downregulation [9] of the growth factors brain-derived neurotrophic factor (BDNF), glial cell–derived neurotrophic factor (GDNF), and fibroblast growth factor-2 (FGF-2).

The above experimental studies strongly differed in their study designs. These differences relate to ischemia models, and the type, duration, and severity of diet modification, which in some cases went along with deficiency of essential micronutrients and vitamins. The diverse nature of these studies precludes more general conclusions about the effect of undernutrition on the ischemic brain. To elucidate how an undernutrition, which affects the amount of energy and protein delivered, but does not withhold essential micronutrients or vitamins, influences ischemic injury, we herein exposed mice to two protocols of diet modification, that is, energy undernutrition or protein-energy undernutrition over 7, 14, or 30 days, and subsequently induced focal cerebral ischemia by intraluminal middle cerebral artery occlusion (MCAO).

Materials and Methods

Legal Issues, Statistical Planning, and Randomization

Experiments were approved by local government authorities (Bezirksregierung Düsseldorf) in accordance with E.U. guidelines (Directive 2010/63/EU) for the care and use of laboratory animals. Sample size calculations determined that 12 animals per group were required for neurological examinations and histochemical studies, given that the effect size was 1.167, the alpha error was 5%, and the beta error (1 statistical power) was 20%. Experimenters were blinded by a third person not involved in the assessments randomizing the animals, weighing, and providing the food pellets.

Food Modifications and Murinometrics

Adult male C57BL6/j mice (8 weeks, 26–30 g; Harlan-Netherlands, Rossdorf, Germany) were randomized to three diets: (a) normal nutrition (C1000; 3518 kcal/kg, 20% protein (i.e., casein); Altromin, Lage, Germany), (b) energy-reduced nutrition (C1012 mod.; 1313 kcal/kg, 20% protein (casein); Altromin), and (c) protein-energy-reduced nutrition (C1003 mod.; 1300 kcal/kg, 8% protein (casein); Altromin). Diets were offered ad libitum over 7, 14, or 30 days. Animals were then submitted to 30 min intraluminal MCAO. Throughout the study, animals were housed in single cages in a 12 h:12 h light/dark cycle. Food consumption and calorie intake were measured daily. Body (i.e., nose-anus) length was determined prior to diet modification. Body weight and BMI were determined weekly. Stool changes and behavioral abnormalities, namely, spontaneous motor hypoactivity, were checked daily.

Experimental Procedures

Mice were anesthetized with 1.0–1.5% isoflurane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. Cerebral laser Doppler flow (LDF) was recorded using a flexible probe (Perimed, Järfälla, Sweden) attached to the skull overlying the core of the middle cerebral artery territory. A midline neck incision was made. The left common and external carotid arteries were isolated and ligated, and the internal carotid artery was temporarily clipped. A silicon-coated nylon monofilament (0.21-mm tip diameter; Doccol, Sharon, MA, USA) was introduced through a small incision of the common carotid artery and advanced to the circle of Willis for MCAO [5, 6]. Reperfusion was initiated by monofilament removal after 30 min. Thirty minutes of MCAO was chosen, since this model induced reproducible injury of the striatum and the most lateral cortex with little animal dropouts. After surgery, wounds were carefully sutured and anesthesia was discontinued. Twenty-four hours later, animals were evaluated using the Clark score [18], which captures general and focal neurological deficits. Immediately before sacrifice, plasma samples were obtained by cardiac puncture after 5 h fasting that were used for analysis of total cholesterol, low-density lipoprotein cholesterol (LDL), triglycerides, and glucose levels (ADVIA® 2400; Siemens, Erlangen, Germany). One set of animals ($n = 12$/group) was transcardially perfused with normal saline followed by 4% paraformaldehyde. The animals' brains were cut into 20-μm-thick coronal sections for histochemical studies. Another set of animals ($n = 6$/group) was transcardially perfused with normal saline. From the animals' brains, tissue samples were collected from the middle cerebral artery territory for Western blots and real-time quantitative polymerase chain reaction (qPCR) studies. For this purpose, a 2-mm-thick coronal brain slice ranging from 1 mm rostral to 1 mm caudal to the bregma was prepared, from which a triangular slice containing the striatum and the most lateral parietal cortex was dissected. This selection strategy was chosen to exclude partial volume effects of infarct

reductions or expansions on gene expression results. From the same animals, liver samples were also obtained.

Infarct Volumetry

Coronal sections collected at millimeter intervals across the brain were stained with cresyl violet. Infarct volume was determined by subtracting the area of healthy tissue in the ischemic hemisphere from that in the contralesional hemisphere [5, 6].

Immunohistochemistry of IgG Extravasation

Brain sections obtained from the rostrocaudal level of the midstriatum were rinsed for 20 min in 0.3% H_2O_2 in 70% methanol/0.1 M phosphate-buffered saline (PBS), immersed in 0.1 M PBS containing 5% bovine serum albumin (BSA) (05470; Sigma-Aldrich, Darmstadt, Germany), and incubated for 1 h in biotinylated anti-mouse IgG (1:100; Santa Cruz, Heidelberg, Germany), followed by diaminobenzidine (DAB) tetrahydrochloride (D5905; Sigma-Aldrich) staining with an avidin-biotin complex peroxidase kit (Vectastain Elite; Vector Labs, Burlingame, CA, USA) [5]. IgG extravasation was analyzed by measuring the area of IgG leakage.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling

Adjacent brain sections were subjected to terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using a commercially available In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) [5, 6]. TUNEL+, that is, DNA-fragmented cells were evaluated under a Zeiss AxioObserver.Z1 microscope equipped with Apotome optical sectioning by counting the total number of labeled cells in the striatum.

Immunohistochemistry for Neuronal, Microglial, Astrocytic, and Inflammation Markers

Adjacent sections were immersed in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 5% normal donkey serum (D9663; Sigma-Aldrich). Sections were incubated overnight at 4 °C in monoclonal rabbit anti-NeuN (1:400; ab177487; Abcam, Cambridge, UK), monoclonal rat anti-CD45 (1:200; 550539; BD Biosciences, Heidelberg, Germany), polyclonal rabbit anti-ionized calcium binding adaptor protein (Iba)-1 (1:500; Wako Chemicals, Neuss, Germany), monoclonal rat anti-glial fibrillary acidic protein (GFAP) (1:200; 130300; Invitrogen, Dublin, Ireland), or polyclonal rabbit anti-inducible nitric oxide synthase (iNOS) (1:100; sc-650; Santa Cruz, CA, USA) antibodies that were detected with Alexa Fluor-488– or Alexa Fluor-594–labeled secondary antibodies (NeuN, Iba-1, GFAP, and iNOS) or biotinylated secondary antibodies followed by DAB staining with an avidin-biotin complex peroxidase kit (Vectastain Elite, Burlingame, CA, USA) (CD45). NeuN, Iba-1, GFAP, and iNOS labelings were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (D9542; Sigma-Aldrich). Sections were evaluated under a motorized Zeiss AxioObserver.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning (NeuN, Iba-1, GFAP, and iNOS) or an Olympus X52 microscope (CD45) by counting the total number of NeuN+, CD45+, or iNOS+ cells in the striatum, in which ischemic injury is most reproducible, or analyzing the area covered by activated microglia (Iba-1) or reactive astrocytes (GFAP). The latter analysis was preferred to cell counting, since individual cells could not always unequivocally be discriminated. The latter data were shown as percent changes.

Real-Time Quantitative Polymerase Chain Reaction

From the brain and liver tissue samples, messenger RNA (mRNA) was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA was converted to cDNA using a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Langenselbold, Germany). Real-time qPCR was performed in a StepOnePlus real-time PCR system using primers selected by the PubMed primer-BLAST tool (https://blast.ncbi.nlm.nih.gov/) (Suppl. Table 1). The efficiency of these primers had been confirmed in melting curves. *β-Glucuronidase* (*β-Gluc*) was used as a housekeeping gene; the brain and liver tissue from healthy mice served as control. Results were quantified using the $2^{-\Delta\Delta Ct}$ method. PCR were performed in triplicate, of which mean values were formed for each mouse.

Western Blots

During the mRNA extraction, protein samples were collected after bromochloropropane (B9673; Sigma-Aldrich) separation. Ethanol was added and samples centrifuged at 12.000*g* for 5 min. This procedure was repeated twice. The resulting pellet was suspended in 4% sodium dodecyl sulfate (SDS) (436143; Sigma-Aldrich). Protein content was measured using the Bradford method. Equal amounts of protein (20 μg) were loaded on 10% SDS-polyacrylamide gels, submitted to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were blocked by 5% non-fat-dried milk (M7409; Sigma-Aldrich) in 50 mM Tris-buffered saline (TBS) containing 0.1% Tween (P9416; Sigma-Aldrich) for 1 h at room temperature, washed, and incubated overnight at 4 °C with monoclonal rabbit anti-Sirt-1 (1:2000; ab32441; Abcam) and polyclonal rabbit anti-β-actin (1:10000; 4967; Cell Signaling, Frankfurt, Germany) antibodies. The next day, membranes were washed

and incubated with secondary donkey anti-rabbit antibody. Blots were revealed using a chemiluminescence kit and scanned using a myECL Imager (Thermo Fisher Scientific). Sirt-1 abundance was densitometrically evaluated in three independent experiments. The relative abundance of Sirt-1 was normalized to protein loading as determined in β-actin blots.

Statistics

Statistical analyses were performed using SPSS for Windows. Nutritional data and LDF recordings were analyzed by two-way repeated measurement ANOVA followed by unpaired *t* tests as post hoc tests. Neurological deficits and histochemical data were analyzed by one-way ANOVA followed by Tukey post hoc tests (for normally distributed results) or Kruskal-Wallis tests (for non-normally distributed results). Real-time qPCR data were compared by pairwise *t* tests. To explore the relationship of calorie intake with infarct volume and neurological deficits, two-tailed Pearson's correlations were computed. Nutritional data, LDF recordings, and real-time qPCR data are presented as mean ± S.D. values. Neurological deficits, histochemical data, and Western blots are shown as median ± interquartile range box plots with minimum/maximum data as whiskers. p values < 0.05 were defined to indicate statistical significance.

Results

Food Modification Induces Weight Loss and Malabsorption Syndrome

Murinometric analyses revealed progressive body weight (Suppl. Fig. 1A–C) and body mass index (BMI) (Suppl. Fig. 1D–F) loss (each by ~ 20%) over up to 30 days in mice exposed to energy and protein-energy undernutrition. Although the total amount of food ingested was elevated in mice on modified diets (Suppl. Fig. 1G–I), calorie intake was consistently reduced at all time points at which ischemia was subsequently induced (Suppl. Fig. 1J–L). Upon diet modification, stool samples progressively increased in size and adopted a pale color (Suppl. Fig. 2; Suppl. Table 2), indicative of malabsorption syndrome. With progressive undernutrition, thin blood beddings were sometimes found on stool samples (Suppl. Table 2). Motor hypoactivity was frequently noted shortly before MCAO (Suppl. Table 2).

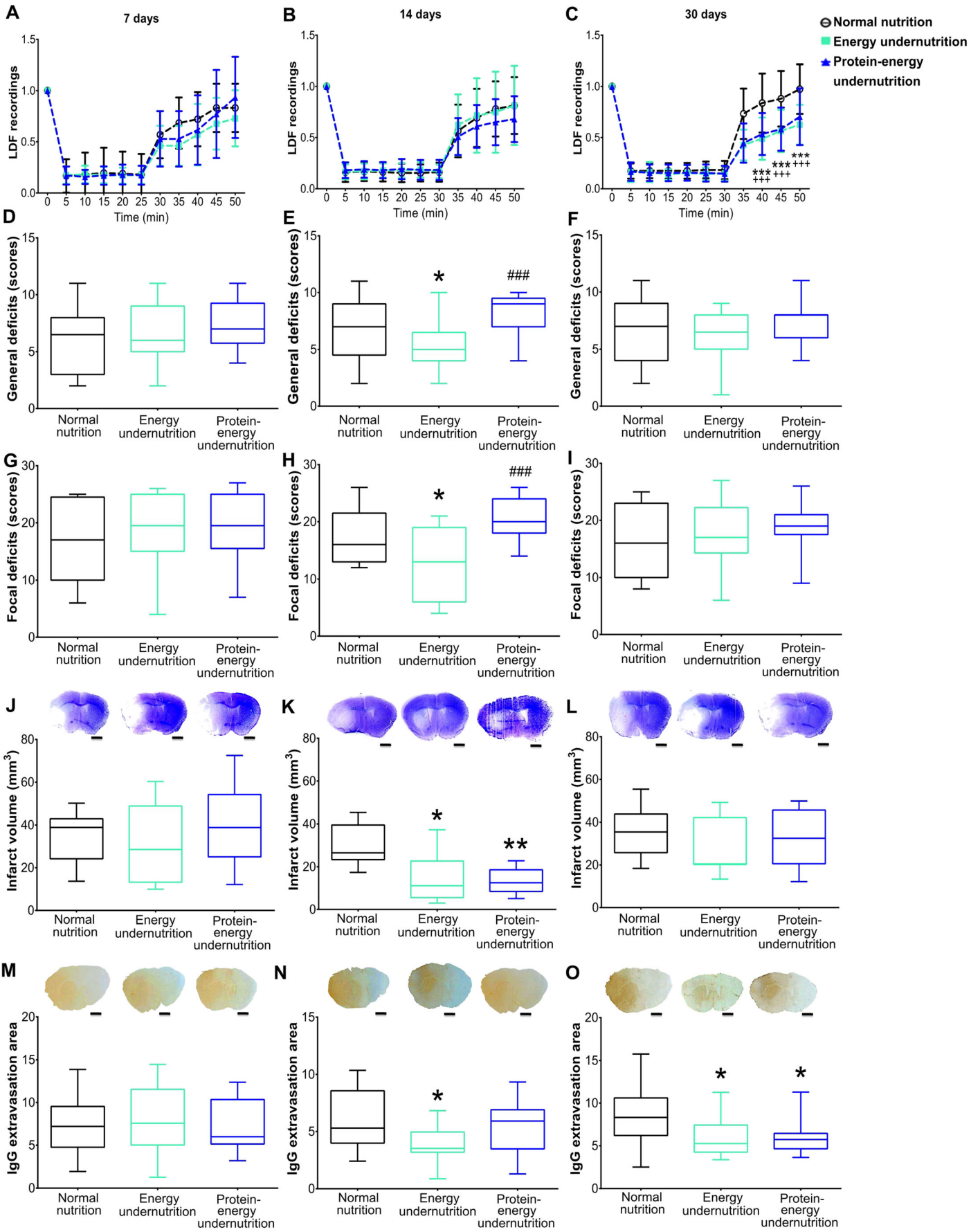
Plasma LDL, which constitutes a comparably small percentage of total cholesterol in mice, since mice lack cholesterol ester transfer protein (CETP) [19], was decreased in mice exposed to protein-energy undernutrition for 7 days, but not for 14 or 30 days (Suppl. Table 3). Total cholesterol, triglycerides, and glucose were not influenced by energy or protein-energy undernutrition (Suppl. Table 3).

Fig. 1 Energy and protein-energy undernutrition reduce ischemic injury in a defined time window. (A–C) Laser Doppler flow (LDF) recordings above the core of the middle cerebral artery territory, (D–F) general neurological deficits evaluated by the Clark score, (G–I) focal neurological deficits examined by the Clark score, (J–L) infarct volume outlined on cresyl violet-stained brain sections, and (M–O) blood-brain barrier permeability in the striatum assessed by IgG extravasation analysis in mice exposed to normal nutrition, energy undernutrition, or protein-energy undernutrition for 7 days (A, D, G, J, M), 14 days (B, E, H, K, N), or 30 days (C, F, I, L, O), followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative cresyl violet stainings and IgG immunostainings are shown. Bars (in (J–O)), 1 mm. $^{***}p < 0.001$ for energy undernutrition compared with normal nutrition/$^{+++}p < 0.001$ for protein-energy undernutrition compared with normal nutrition (in (A-C); $n = 12$ animals/group). $^{*}p < 0.05$/$^{**}p < 0.01$ compared with normal nutrition/$^{\#\#\#}p < 0.001$ compared with energy undernutrition (in (D–O); $n = 12$ animals/group)

Energy and Protein-Energy Undernutrition Attenuate Ischemic Injury in a Limited Time Window

In mice receiving non-modified diet, cerebral LDF decreased to ~ 15–20% of baseline values during MCAO, followed by the restoration of LDF to baseline values within 20 min after monofilament removal (Fig. 1A–C). LDF patterns did not differ in mice exposed to energy or protein-energy undernutrition for 7 or 14 days (Fig. 1A, B), whereas decreased reperfusion indicative of hemodynamic impairment was noted in mice exposed to prolonged energy and protein-energy undernutrition for 30 days (Fig. 1C). Neurological examination 24 h after MCAO revealed that energy undernutrition but not protein-energy undernutrition for 14 days decreased general and focal neurological deficits (Fig. 1E, H), whereas diet modification for 7 or 30 days did not influence neurological performance (Fig. 1D, F, G, I). Both energy and protein-energy undernutrition significantly reduced infarct volume, when imposed for 14 days, but not 7 or 30 days (Fig. 1J–L). IgG extravasation, a measure of disturbed blood-brain barrier integrity, was reduced by energy undernutrition for 14 or 30 days and protein-energy undernutrition for 30 days (Fig. 1M–O).

Our data were indicative of three stages of undernutrition: an initial adaptation stage (that is, 7 days after diet modification), in which ischemic injury is not influenced by energy and protein-energy undernutrition, which is followed by a compensated stage (that is, 14 days after diet modification), in which brain tissue is protected against ischemia, and an exhausted stage (that is, after 30 days), in which neuroprotection is lost as a consequence of post-ischemic hemodynamic impairments. Pearson's correlations revealed that calorie intake in the adaptation stage was negatively correlated with general neurological deficits ($r = -0.275$, $p = 0.02$), but not with infarct volume or focal deficits (Suppl. Fig. 3). In the compensated stage, calorie intake was positively correlated with infarct volume ($r = 0.572$, $p < 0.001$), but not with


A
7 days
B
14 days
C
30 days
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
LDF recordings
Time (min)
D
E
F
General deficits (scores)
G
H
I
Focal deficits (scores)
J
K
L
Infarct volume (mm3)
M
N
O
IgG extravasation area
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
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neurological deficits (Suppl. Fig. 3). In the exhausted stage, no correlations of calorie intake with infarct volume or neurological deficits were found (Suppl. Fig. 3).

Undernutrition Differentially Influences Neuronal Survival, Brain Leukocyte Infiltration, and Microglial Activation

Immunohistochemical studies showed that energy and protein-energy undernutrition for 14 days increased the density of surviving NeuN+ neurons in the ischemic striatum (Fig. 2B) and that protein-energy undernutrition reduced the density of DNA-fragmented, that is, irreversibly injured TUNEL+ cells (Fig. 2E). Undernutrition did not influence neuronal survival (Fig. 2A, C) or cell injury (Fig. 2D, F), when imposed for 7 or 30 days. Protein-energy undernutrition decreased brain leukocyte infiltration, as assessed by CD45 immunohistochemistry (Fig. 3A), and microglial activation, as evaluated by Iba-1 immunohistochemistry (Fig. 3D), when imposed for 7 days. Energy undernutrition for 7 days reduced

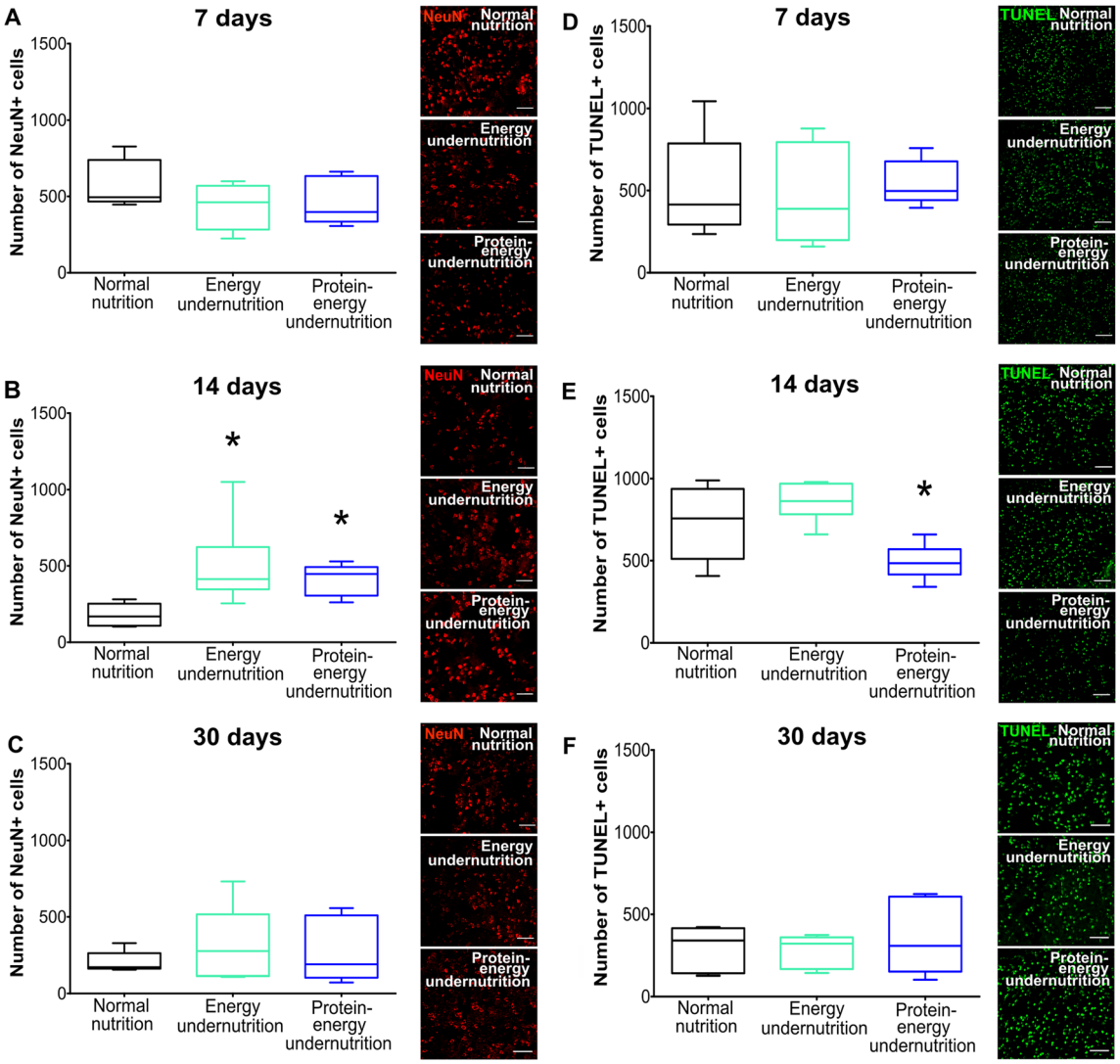
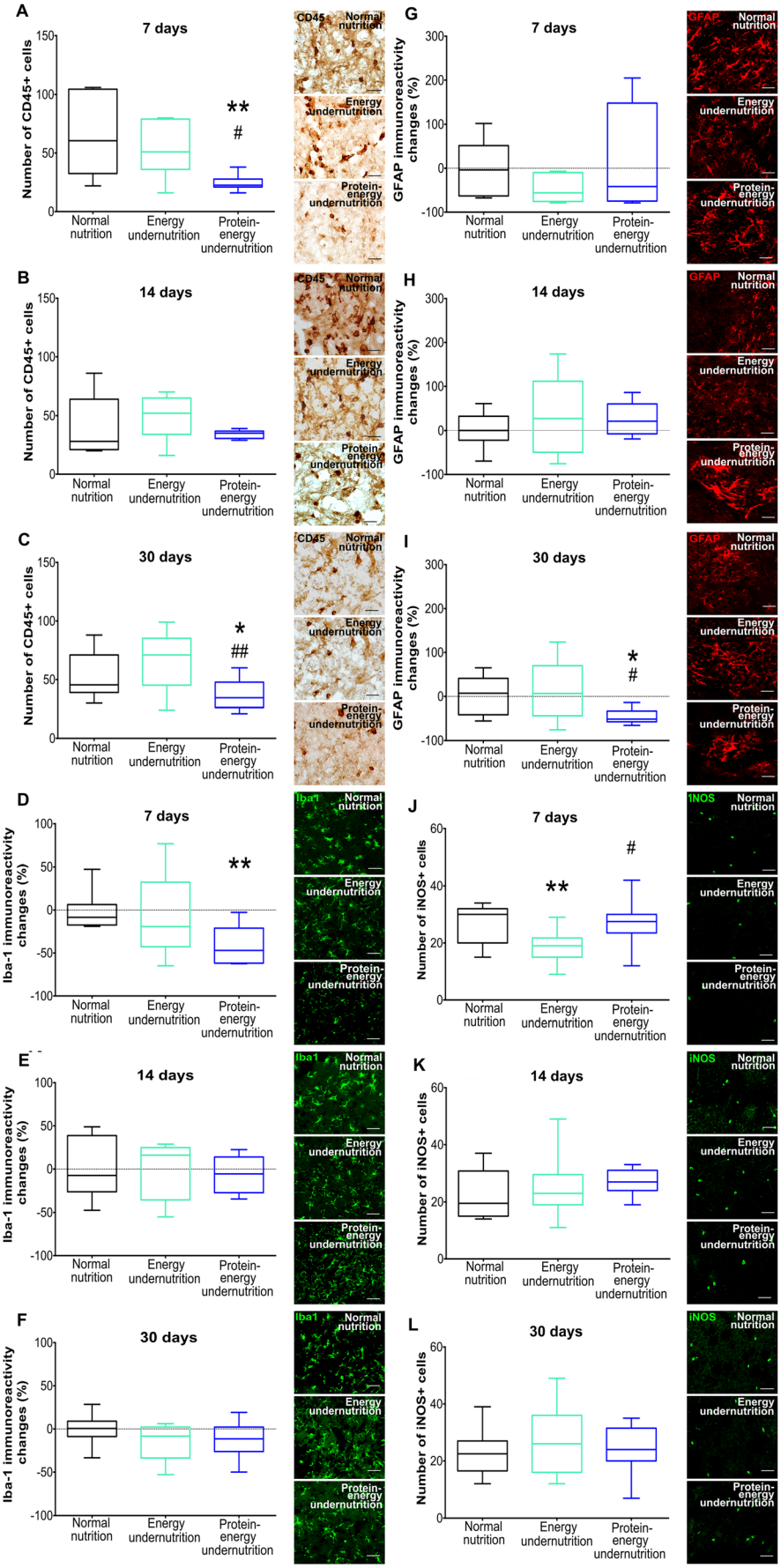


Fig. 2 Undernutrition increases neuronal survival in a limited time window. (A–C) Number of NeuN+ surviving neurons and (D–F) number of DNA-fragmented, that is, irreversibly injured TUNEL+ cells in the ischemic striatum of mice exposed to normal nutrition, energy undernutrition, or protein-energy undernutrition for 7 days (A, D), 14 days (B, E), or 30 days (C, F), followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative microphotographs are shown. Bars, 100 μm. $*p < 0.05$ compared with normal nutrition ($n = 12$ animals/group)

Fig. 3 Undernutrition differentially influences brain leukocyte infiltration, microglial activation, and astroglial reactivity. (A–C) Number of CD45+ leukocytes, (D–F) immunoreactivity for microglia marker Iba-1, (G–I) immunoreactivity for astrocytic marker GFAP, and (J–L) number of iNOS+ cells in the ischemic striatum of mice exposed to normal nutrition, energy undernutrition, or protein-energy undernutrition for 7 days (A, D, G, J), 14 days (B, E, H, K), or 30 days (C, F, I, L), followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative microphotographs are shown. Bars, 100 μm. $*p < 0.05$/$**p < 0.01$ compared with normal nutrition/$^{\#}p < 0.05$/$^{\#\#}p < 0.01$ compared with energy undernutrition ($n = 12$ animals/group)


A
7 days
Number of CD45+ cells
150
100
50
0
**
#
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
CD45
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
G
7 days
GFAP immunoreactivity changes (%)
300
200
100
0
-100
GFAP
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
B
14 days
H
14 days
C
30 days
*
##
I
30 days
*
#
D
7 days
Iba-1 immunoreactivity changes (%)
**
Iba1
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
J
7 days
Number of iNOS+ cells
60
40
20
0
**
#
iNOS
Normal nutrition
Energy undernutrition
Protein-energy undernutrition
E
14 days
K
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F
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L
30 days

the density of iNOS+ cells (Fig. 3J), which had the size and shape of microglia. Interestingly, diet modification for 14 days did not alter brain leukocyte infiltration, microglial activation, or iNOS formation (Fig. 3B, E, K). Protein-energy undernutrition for 30 days reduced the brain infiltration of CD45+ leukocytes (Fig. 3C) and decreased astrocytic GFAP immunoreactivity (Fig. 3I).

Undernutrition Regulates Metabolism-Related, Inflammatory, and Anti-Oxidant Genes in Ischemic Brain Tissue

Real-time qPCR showed that undernutrition regulated metabolism-related, inflammatory, and anti-oxidant genes in the ischemic brain. *Sirtuin-1* (*Sirt-1*) mRNA, which encodes for an NAD-dependent deacetylase that stabilizes mitochondrial function and metabolism partly by deacetylating the transcription regulator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [20], and *glucose transporter-1* (*Glut-1*) mRNA, which encodes for a membrane transporter promoting glucose uptake in brain cells [21], were upregulated in the ischemic brain of mice exposed to 7 days energy undernutrition (Table 1), as was Sirt-1 protein, as shown in the Western blots (Fig. 4A). *Il-1β* mRNA was downregulated by 7 days protein-energy undernutrition (Table 1). Only subtle gene expression changes were noted in the ischemic brain of mice exposed to 14 days undernutrition, that is, an elevation of *superoxide dismutase* (*Sod*)*-1* mRNA, which encodes for a dismutase degrading superoxide anions, in mice exposed to protein-energy-reduced diet (Table 1). *Sirt-1* and *Glut-1* mRNAs (Table 1) as well as Sirt-1 protein (Fig. 4B) did not differ from mice receiving non-modified diet, indicating that the metabolic needs of the tissue were adapted to the reduced energy supply. In the ischemic brain of mice exposed to prolonged energy or protein-energy undernutrition for 30 days, *Sirt-1* mRNA (Table 1) and Sirt-1 protein (Fig. 4C) were increased, and in the ischemic brain of mice exposed to prolonged protein-energy undernutrition for 30 days, *Glut-1* and *Sod-1* mRNA (Table 1) were elevated. Conversely, *insulin-like growth factor-1* (*Igf-1*) mRNA, which encodes for a growth factor with insulin-like properties, *Il-1β* mRNA, and *nuclear factor-κb* (*Nf-κb*) mRNA, which encodes for a transcription factor, were reduced by energy or protein-energy undernutrition (Table 1). The responses of *Sirt-1*, *Glut-1*, and *Igf-1* were interpreted as effort to maintain brain tissue glucose supply and confine metabolic needs in face of energy reserves which faded.

Undernutrition Regulates Metabolism-Related, Pro-oxidant, and Anti-oxidant Genes in the Liver

Real-time qPCR showed that protein-energy undernutrition over 7 days downregulated *catalase* (*Cat*) mRNA, which encodes for a protein that degrades peroxides formed by dismutases, in the liver (Table 2). After 14 days protein-energy undernutrition, *Sod-1* mRNA was increased, whereas *glucose transporter-2* (*Glut-2*) mRNA, *Sod-2* mRNA, which encodes for another dismutase, and *Cat* mRNA were reduced, as was *Sod-2* mRNA after 14 days energy undernutrition (Table 2). Prolonged energy and protein-energy undernutrition over 30 days downregulated *NADPH oxidase-4* (*Nox-4*) mRNA, which encodes for a protein that catalyzes the production of superoxide free radical by transferring one electron to oxygen from NADP, and anti-oxidant *Sod-2* mRNA (Table 2). Prolonged energy undernutrition over 30 days upregulated

Table 1 Undernutrition regulates metabolism-related, inflammatory, and anti-oxidant genes in the ischemic brain

Brain tissue	*Sirt-1*	*Igf-1*	*Insr*	*Glut-1*	*Il-1β*	*Nf-κb*	*Sod-1*	*Gpx-3*
7 days								
Normal nutrition	1.13 ± 0.60	3.60 ± 3.26	1.44 ± 0.61	1.18 ± 0.44	6.14 ± 3.26	1.40 ± 0.65	0.89 ± 0.34	0.13 ± 0.10
Energy undernutrition	1.87 ± 0.91*	3.94 ± 2.88	1.87 ± 0.86	1.99 ± 0.74*	3.68 ± 2.77	1.87 ± 0.69	1.38 ± 0.61	0.18 ± 0.09
Protein-energy undernutrition	1.12 ± 0.30	2.44 ± 1.83	1.37 ± 0.49	1.65 ± 0.50	1.71 ± 0.37*	1.30 ± 0.36$^{\#}$	1.15 ± 0.54	0.12 ± 0.03
14 days								
Normal nutrition	0.60 ± 0.23	1.15 ± 0.58	1.33 ± 1.07	1.17 ± 0.44	3.50 ± 1.59	2.91 ± 1.23	0.90 ± 0.27	0.28 ± 0.15
Energy undernutrition	0.51 ± 0.21	1.86 ± 0.83	0.97 ± 0.33	1.11 ± 0.55	3.16 ± 1.62	2.32 ± 1.10	0.90 ± 0.32	0.19 ± 0.05
Protein-energy undernutrition	0.70 ± 0.36	2.01 ± 2.88	1.15 ± 1.11	0.87 ± 0.47	3.00 ± 2.69	3.30 ± 2.42	1.76 ± 0.86*	0.26 ± 0.14
30 days								
Normal nutrition	0.28 ± 0.11	1.84 ± 1.12	0.69 ± 0.28	1.09 ± 0.56	5.72 ± 3.18	2.75 ± 1.34	0.57 ± 0.12	0.48 ± 0.50
Energy undernutrition	0.63 ± 0.27*	0.77 ± 0.37*	0.74 ± 0.17	1.72 ± 0.75	1.11 ± 0.45**	1.44 ± 0.42*	0.71 ± 0.24	0.45 ± 0.37
Protein-energy undernutrition	0.66 ± 0.25*	2.11 ± 0.80$^{\#}$	0.98 ± 0.61	2.02 ± 0.76*	1.50 ± 0.68**	1.39 ± 0.35*	0.99 ± 0.39*$^{\#}$	0.63 ± 0.45

*$p < 0.05$/**$p < 0.01$ compared with corresponding normal nutrition; $^{\#}p < 0.05$ compared with corresponding energy undernutrition ($n = 6$ animals/group, analyzed in triplicate)

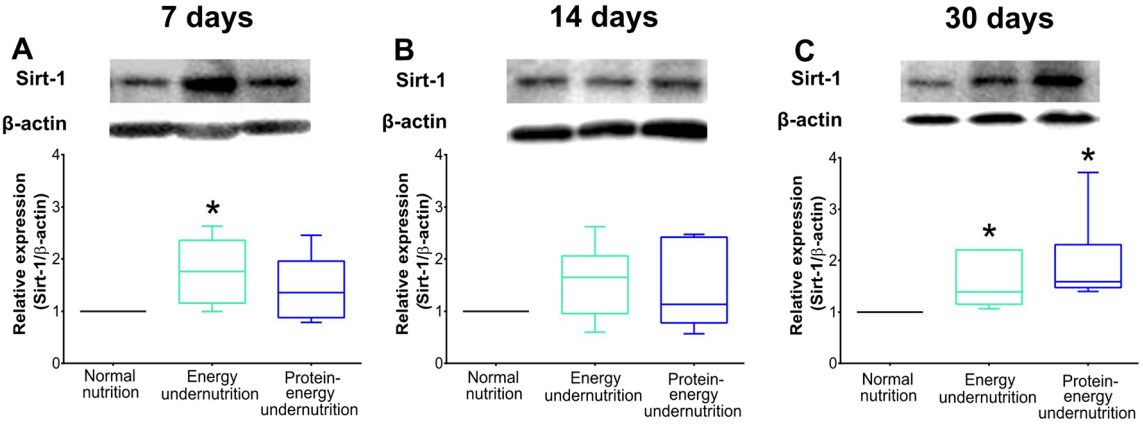

Fig. 4 Undernutrition regulates Sirt-1 protein in the ischemic brain. Western blot analysis of Sirt-1 protein in ischemic brain tissue of mice exposed to normal nutrition, energy undernutrition, or protein-energy undernutrition for (A) 7 days, (B) 14 days, or (C) 30 days, followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative Western blots are also shown. $*p < 0.05$ compared with normal nutrition ($n = 6$ animals/group)

glutathione peroxidase-3 (*Gpx-3*) mRNA (Table 2), which encodes for another peroxidase.

Discussion

By exposing mice to energy-reduced or protein-energy-reduced diets for 7, 14, or 30 days, which were subsequently submitted to intraluminal MCAO, we show that energy and protein-energy undernutrition influences ischemic injury in a phase-dependent way. When exposed to modified diets for 7 days, combined protein-energy undernutrition reduced leukocyte infiltration and microglial activation and decreased the pro-inflammatory cytokine *Il-1β* mRNA in ischemic brain tissue. The brain was not protected against ischemia. Conversely, 14 days of energy or protein-energy undernutrition reduced ischemic injury and in the case of energy undernutrition decreased neurological deficits. Anti-inflammatory effects were absent in the ischemic brain. Genes encoding anti-oxidant enzymes (*Sod-1*, *Sod-2*, and *Cat* mRNA) were profoundly regulated in the liver and, to lesser extent, the brain. With prolonged energy or protein-energy undernutrition for 30 days, an exhausted stage evolved characterized by disturbed post-ischemic reperfusion, rise of metabolism markers in the brain (*Sirt-1* and *Glut-1* mRNA, Sirt-1 protein), downregulation of inflammatory markers in the brain (*Il-1β* and *Nf-κb* mRNA), and reregulation of pro-oxidant and anti-oxidant markers in the liver (now including *Nox-4* and *Gpx-3* mRNA), in which the brain was not protected against ischemia.

Previous studies in animal models of focal cerebral ischemia found that undernutrition reduces brain injury [7–9, 16, 17], whereas other studies in models of focal or global ischemia showed that ischemic injury was unchanged by undernutrition [10–13]. A variety of studies in models of focal or global ischemia observed a significant reduction of stroke-induced neurological deficits [7–11], whereas other studies, again in focal or global ischemia, noted exacerbated or unchanged deficits [12–15]. Major differences in these studies relate to the type of undernutrition. As such, undernutrition was induced by (a) reducing food access to 60 or 70% of the average amount of control animals for 4 weeks to 3 months [8, 10, 13, 16], (b) reducing food protein content to 0 to 12% for 6 days to 4 weeks [9, 12, 14, 15], or (c) intermittent fasting on alternate days or twice per week for one to several months [7, 11, 17]. While reducing the amount of food access similarly reduces protein and energy consumption, the reduction of protein content to 0–2% results in a reduction in the total amount of food ingested, since the animals refuse this chow [9, 12, 14, 15]. In such animals, combined protein-energy malnutrition is noted. In order to prevent exhaustion of the animals' energy state, we decided to use a strategy, in which we applied modified diets ad libitum to mice. Essential minerals, micronutrients, and vitamins were adequately complemented in these diets. The use of an energy-reduced and protein-energy-reduced diet allowed us to discriminate both types of undernutrition.

Previous studies reported that neuroprotection in response to food restriction or undernutrition involves anti-inflammatory effects, that is, inhibition of microglial activation [15], downregulation of *Il-1β*, *Il-6*, *Tnf-α*, *Cxc-motif ligand-1* (*Cxcl-1*), and *intercellular adhesion molecule-1* (*Icam-1*) mRNA [9] or downregulation of IL-1β, IL-6 and TNF-α protein [16, 17] in the brain and the blood. Interestingly, in our study, diet modification reduced leukocyte infiltration, microglial activation, and *Il-1β* and *Nf-κb* mRNA levels in the ischemic brain under conditions not associated with neuroprotection, i.e., when modified chows were imposed for 7 or 30 days. After 14 days energy or protein-energy undernutrition, when neuroprotective effects were noted, anti-inflammatory effects were absent in the brain.

Table 2 Undernutrition regulates metabolism-related, pro-oxidant, and anti-oxidant genes in the liver

Liver tissue	*Sirt-1*	*Igf-1*	*Insr*	*Glut-2*	*Nf-κb*	*Nox-4*	*Sod-1*	*Sod-2*	*Gpx-3*	*Cat*
7 days										
Normal nutrition	0.94 ± 0.45	0.75 ± 0.43	0.40 ± 0.48	0.53 ± 0.64	1.66 ± 0.87	1.15 ± 0.83	0.74 ± 0.26	1.07 ± 0.42	1.40 ± 1.29	0.54 ± 0.37
Energy undernutrition	1.31 ± 0.70	0.80 ± 0.63	0.33 ± 0.41	1.03 ± 0.76	1.57 ± 0.80	1.34 ± 1.02	1.04 ± 0.35	1.72 ± 0.98	1.70 ± 0.73	0.38 ± 0.34
Protein-energy undernutrition	1.09 ± 0.43	0.77 ± 0.70	0.26 ± 0.45	0.88 ± 0.78	1.45 ± 1.05	0.97 ± 0.88	0.80 ± 0.31	1.76 ± 1.04	2.72 ± 2.35	0.20 ± 0.20*
14 days										
Normal nutrition	0.45 ± 0.45	0.59 ± 0.37	0.50 ± 0.68	1.07 ± 0.26	1.05 ± 1.13	0.72 ± 0.51	0.97 ± 0.48	0.99 ± 0.22	4.23 ± 3.15	0.43 ± 0.47
Energy undernutrition	0.25 ± 0.15	1.01 ± 0.81	0.69 ± 0.71	0.68 ± 0.65	0.75 ± 0.43	0.62 ± 0.37	0.91 ± 0.21	0.59 ± 0.15*	3.72 ± 2.10	0.40 ± 0.36
Protein-energy undernutrition	0.27 ± 0.23	0.75 ± 0.47	0.23 ± 0.37	0.30 ± 0.33*	1.18 ± 1.36	0.86 ± 0.81	2.04 ± 0.72*##	0.46 ± 0.17**	3.48 ± 3.91	0.02 ± 0.02*
30 days										
Normal nutrition	0.74 ± 0.24	0.48 ± 0.47	0.24 ± 0.26	0.98 ± 0.65	0.67 ± 0.28	1.25 ± 0.36	1.04 ± 0.09	2.50 ± 1.86	0.84 ± 0.53	0.69 ± 0.54
Energy undernutrition	0.68 ± 0.39	0.43 ± 0.09	0.22 ± 0.19	0.56 ± 0.30	0.60 ± 0.36	0.61 ± 0.26**	1.13 ± 0.92	0.57 ± 0.07*	2.31 ± 0.76*	0.67 ± 0.47
Protein-energy undernutrition	1.07 ± 0.93	0.70 ± 0.78	0.29 ± 0.25	0.70 ± 0.58	1.20 ± 1.28	0.42 ± 0.11***	1.10 ± 0.84	0.62 ± 0.19*	2.60 ± 2.47	0.46 ± 0.27

*$p < 0.05$/**$p < 0.01$/***$p < 0.001$ compared with corresponding normal nutrition; ##$p < 0.01$ compared with corresponding energy undernutrition ($n = 6$ animals/group, analyzed in triplicate)

Thus, diet-induced neuroprotection dissociated from anti-inflammation. That food deprivation reduces the brain infiltration of leukocytes has to the best of our knowledge not been shown.

Earlier studies found that undernutrition increases brain levels of the NAD-dependent deacetylase Sirt-1 [8, 16]. Sirt-1 has a large variety of actions in the healthy and injured brains, stabilizing mitochondrial function and metabolism in response to energy deprivation partly by deacetylating the transcription regulator PGC-1α [20]. Via multiple downstream targets, mitochondrial energy coupling is promoted, reactive oxygen species formation reduced, anti-oxidant capacity increased, and DNA repair enhanced [20]. In our study, *Sirt-1* mRNA and *Sirt-1* protein were elevated when energy or protein-energy undernutrition had been imposed for 7 or 30 days. Under these conditions, no neuroprotection was noted. Interestingly, Sirt-$1^{-/-}$ was previously found to exacerbate ischemic injury in mice exposed to intraluminal MCAO but failed to abolish protective effects of energy restriction [16]. The combined evidence of these data suggests that Sirt-1 acts as a regulator of metabolism-related, pro-oxidant, and anti-oxidant genes, but does not contribute to diet-induced neuroprotection. In fact, *Sirt-1* mRNA and Sirt-1 protein elevation in our study were associated with the regulation of a broad set of downstream mRNAs, namely *Igf-1*, *Glut-1*, *Il-1β*, *Nf-κb*, *Sod-1*, and *Gpx*. Upregulation of anti-oxidant heme oxidase-1 has previously been reported in ischemic brains of mice exposed to alternate fasting or protein-energy undernutrition [7, 17]. The regulation of *Igf-1*, *Glut-1*, *Nf-κb*, *Sod-1*, and *Gpx* mRNAs in ischemic brains of animals exposed to energy or protein-energy undernutrition is new.

In our study, surprisingly modest metabolic, anti-inflammatory, and anti-oxidant changes were noted in animals exposed to 14 days energy or protein-energy undernutrition, which exhibited neuroprotective effects. In the brain, metabolic, anti-inflammatory, and anti-oxidant changes were lacking. It should be noted that histochemical and gene expression changes were determined at the same time point, at which brain injury was assessed, i.e., at 24 h post-MCAO. Our data cannot exclude earlier histochemical or gene expression changes that had already disappeared by then. At 24 h post-MCAO, neither brain leukocyte infiltration nor microglial activation, which are mediators of secondary brain injury [22], were altered in response to energy or protein-energy undernutrition. Our data suggest that the previously reported neuroprotection in models of energy and protein-energy undernutrition may represent a state of ischemic tolerance rather than a true neuroprotective state. The preceding regulation of metabolism-related genes (*Sirt-1*, *Glut-1*) indicates that tissue energy demands had been adjusted which enabled the tissue to survive ischemic injury. Ischemic tolerance can similarly be induced by intermittent fasting in young and aged rats [7, 11, 17], suggesting that this type of endogenous protection might

also be induced in elderly humans by repeated short-lasting food restriction episodes. Diet modification induces a series of physiological and biochemical responses that we did not examine in this study, such as changes in blood gases, as well as changes of plasma glucose and lipids that we evaluated prior to animal sacrifice but not at baseline. It should be noted that plasma glucose and lipid levels are influenced by ischemic stroke and anesthesia. All these factors were adequately controlled for in animals on normal diet.

Notably, the diet-induced neuroprotection vanished with progressive exhaustion of the animals' nutrition state, that is, after 30 days of energy or protein-energy undernutrition, when post-ischemic hypoperfusion prevented survival-promoting effects. As such, in advanced undernourishment, observations in animals do not contradict clinical experience in human patients that malnutrition impairs stroke outcome. With this respect, malnutrition apparently resembles its opposite state, that is, overnutrition and obesity, for which it has been assumed for many years that it enhances ischemic stroke outcome [23]. This so-called obesity paradox has meanwhile been refuted [24]. In view of its clinical relevance, future studies should more stringently examine consequences of nutrition modifications for ischemic stroke and stroke recovery.

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Author Contributions TSC and DMH designed the study. TSC performed the animal experiments, assisted by EHSM and MS. TSC, LMNM, ARSM, ED, and MS conducted histochemical and molecular biological studies. DMH and CK provided infrastructural support. TSC, EHSM, LMNM, ARSM, ED, and DMH analyzed the data. TSC and DMH drafted the manuscript. All authors finalized it.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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5 STUDY 2

Title: Moderate protein restriction protects against focal cerebral ischemia in mice by mechanisms involving anti-inflammatory and anti-oxidant responses

Author's contributions:
Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%
Contributed to the acquisition of the data:
Animal experiment: TSC – 100%
Histochemical analysis: TSC – 90% / MS, ED – 10%
Molecular analysis: TSC – 80% / EHSM, LMNM, ARSM – 20%
Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 30% / EHSM, LMNM, ARSM, ED – 10%
Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%
Provided final approval of the version to publication: TSC – 50% / DMH – 50%
Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TSC – 50% / DMH – 50%
Authors' contributions:
Tayana Silva de Carvalho and Dirk M. Hermann designed the study. Tayana Silva de Carvalho performed the animal experiments, assisted by Eduardo H. Sanchez-Mendoza and Maryam Sardari. Tayana Silva de Carvalho, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Maryam Sardari conducted histochemical and molecular biological studies. Dirk M. Hermann and Christoph Kleinschnitz provided infrastructural support. Tayana Silva de Carvalho, Eduardo H. Sanchez-Mendoza, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Dirk M. Hermann analyzed the data. Tayana Silva de Carvalho and Dirk M. Hermann drafted the manuscript. All authors concluded it.
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Moderate Protein Restriction Protects Against Focal Cerebral Ischemia in Mice by Mechanisms Involving Anti-inflammatory and Anti-oxidant Responses

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Abstract

Food composition influences stroke risk, but its effects on ischemic injury and neurological deficits are poorly examined. While severe reduction of protein content was found to aggravate neurological impairment and brain injury as a consequence of combined energy-protein malnutrition, moderate protein restriction not resulting in energy deprivation was recently suggested to protect against perinatal hypoxia-ischemia. Male C57BL6/j mice were exposed to moderate protein restriction by providing a normocaloric diet containing 8% protein (control: 20% protein) for 7, 14, or 30 days. Intraluminal middle cerebral artery occlusion was then induced. Mice were sacrificed 24 h later. Irrespective of the duration of food modification (that is, 7–30 days), protein restriction reduced neurological impairment of ischemic mice revealed by a global and focal deficit score. Prolonged protein restriction over 30 days also reduced infarct volume, brain edema, and blood-brain barrier permeability and increased the survival of NeuN+ neurons in the core of the stroke (i.e., striatum). Neuroprotection by prolonged protein restriction went along with reduced brain infiltration of CD45+ leukocytes and reduced expression of inducible NO synthase and interleukin-1β. As potential mechanisms, increased levels of the NAD-dependent deacetylase sirtuin-1 and anti-oxidant glutathione peroxidase-3 were noted in ischemic brain tissue. Irrespective of the protein restriction duration, a shift from pro-oxidant oxidative stress markers (NADPH oxidase-4) to anti-oxidant markers (superoxide dismutase-1/2, glutathione peroxidase-3 and catalase) was found in the liver. Moderate protein restriction protects against ischemia in the adult brain. Accordingly, dietary modifications may be efficacious strategies promoting stroke outcome.

Keywords Cerebral metabolism · Ischemic stroke · Middle cerebral artery occlusion · Neuroprotection · Oxidative stress · Protein intake

Introduction

Alimentation with protein-rich animal products has greatly fostered human development. At the same time, protein-rich nutrition, specifically with red meat, might elevate health risks, as indicated by a number of population-based studies. Regarding stroke, the Nurses' Health Study and Health Professionals Follow-Up Study, which prospectively examined 84,010 women and 43,150 men more than 20 years, reported that one serving of red meat per day increases the incidence of stroke events by a factor of 1.13 (95%-confidence interval (CI) 1.04–1.22) [1]. This observation was later confirmed in a meta-analysis on 329,495 subjects followed up over 8–26 years, which found a 1.11 (1.06–1.16)-fold increase of stroke events per red meat serving [2].

The question how food composition influences the severity of ischemic injury and neurological impairment, once a stroke has occurred, has major importance for stroke management. Yet, little data exist on this issue. It is well established that severe malnutrition characterized by low body mass index (BMI) and hypoalbuminemia is associated with poor stroke recovery [3]. How more moderate changes in food intake that are not associated with BMI changes influence ischemic

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injury is unknown. Randomized studies are lacking on this issue. The diversity of human nutrition habits possibly precludes insights from clinical cohort studies [1, 2].

In rats and gerbils, the delivery of diets containing 0.5–2% protein 3 to 4 weeks in models of global [4–6] or focal [7] cerebral ischemia compromised neurological recovery, increased brain inflammation, increased neuronal injury, and reduced brain plasticity. Yet, such severe protein restriction results in a reduction in the total amount of food ingested, since the animals refuse this chow [4–7]. In such animals, combined protein-energy malnutrition is noted. A single rat study so far examined consequences of a more moderate diet containing 7% protein, which, when administered during pregnancy and lactation to mothers, reduced brain injury but augmented sensorimotor deficits of offspring exposed to unilateral cerebral hypoxia-ischemia at 7 days post-birth [8]. How such moderate protein restriction influences ischemic injury and neurological deficits in the adult brain was unknown. By exposing mice to a normocaloric diet containing 8% protein, which we subsequently exposed to intraluminal middle cerebral artery occlusion (MCAO), we now examined this question. Our hypothesis was that moderate protein restriction reduces neurological deficits and brain injury.

Materials and Methods

Legal Issues, Statistical Planning, and Randomization

Experiments were approved by local government authorities (Bezirksregierung Düsseldorf) in accordance with E.U. guidelines (Directive 2010/63/EU) for the care and use of laboratory animals. Sample size calculations determined that 12 animals per group were required for the neurological examinations and histochemical studies, given that the effect size was 1.167, the alpha error was 5%, and the beta error (1–statistical power) was 20%. Experimenters were blinded by a third person not involved in the assessments randomizing the animals, weighing and providing the food pellets. All animals survived the stroke surgery. To minimize the use of laboratory animals, all animals were used both for behavioral studies and histochemical or molecular biological analyses.

Animal Nutrition and Murinometrics

Thirty-six adult male C57BL6/j mice (8 weeks, 26-30 g; Harlan-Netherlands, Rossdorf, Germany) were randomized to two diets: (a) normal nutrition (C1000; 3518 kcal/kg, 20% (by weight) casein, 13% (by weight) fat; Altromin, Lage, Germany) and (b) protein-reduced nutrition (C1003; 3541 kcal/kg, 8% casein, 13% fat; Altromin). Diets and water were delivered ad libitum over 7, 14, or 30 days. These durations were chosen to obtain a thorough understanding of the animals' adaptation to the food modification. Animals were then submitted to 30 min intraluminal MCAO. Throughout the study, animals were housed in single cages (Green line IVC Sealsafe PLUS mouse; Tecniplast, Hohenpeißenberg, Germany) in a 12 h/12 h light/dark cycle, in order to quantify food intake. Food consumption and calorie intake were measured daily. Body weight and BMI were measured weekly. Body (i.e., nose–anus) length was determined prior to diet exposure for evaluating BMI (in kg/cm^2) [9].

Experimental Procedures

Mice were anesthetized with 1.0–1.5% isoflurane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. Cerebral laser Doppler flow (LDF) was recorded using a flexible probe (Perimed, Järfälla, Sweden) attached to the skull overlying the core of the middle cerebral artery territory. A midline neck incision was made. The left common and external carotid arteries were isolated and ligated, and the internal carotid artery was temporarily clipped. A silicon-coated nylon monofilament (0.21 mm tip diameter; Doccol, Sharon, MA, USA) was introduced through a small incision of the common carotid artery and advanced to the circle of Willis for MCAO [10, 11]. Reperfusion was initiated by monofilament removal. Wounds were carefully sutured and anesthesia was discontinued. MCAO was induced during the day cycle of the animals in the surgery facility of the NeuroScienceLab. Behavioral abnormalities and clinical manifestations were checked eight-hourly until 24 h after MCAO. Twenty-four hours later, animals were evaluated using the Clark score [12], which captures general and focal neurological deficits. Immediately before animal sacrifice, plasma samples were obtained after 5 h fasting by cardiac puncture that were used for analysis of total cholesterol, low-density lipoprotein cholesterol (LDL), triglycerides, and glucose levels (ADVIA® 2400; Siemens, Erlangen, Germany). One set of animals ($n = 12$/ group) was transcardially perfused with normal saline followed by 4% paraformaldehyde for histochemical studies. Another set ($n = 6$/ group) was transcardially perfused with normal saline for Western blots and real-time quantitative polymerase chain reaction (qPCR) studies. Brains were cut into 20-μm-thick coronal sections.

Infarct Volumetry

Coronal sections collected at millimeter intervals across the brain were stained with cresyl violet. Infarct volume was determined by subtracting the area of healthy tissue in the ischemic hemisphere from that in the contralesional hemisphere [11]. Considering that infarct volume was the most rigid stroke readout, infarct volume was defined as primary readout of this study.

Immunohistochemistry of IgG Extravasation

Brain sections obtained from the rostrocaudal level of the midstriatum were rinsed for 20 min in 0.3% H_2O_2 in 70% methanol/ 0.1 M phosphate-buffered saline (PBS), immersed in 0.1 M PBS containing 5% bovine serum albumin (BSA) (05470; Sigma-Aldrich, Darmstadt, Germany), and incubated for 1 h in biotinylated anti-mouse IgG (1:100; Santa Cruz, Heidelberg, Germany), followed by diaminobenzidine tetrahydrochloride (DAB) (D5905; Sigma-Aldrich, Darmstadt, Germany) staining with an avidin-biotin complex peroxidase kit (Vectastain Elite; Vector Labs, Burlingame, CA, U.S.A.) [11]. IgG extravasation was analyzed by evaluating the area covered by IgG in the ischemic brain.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling

Adjacent brain sections were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a commercially available In Situ Cell Death Detection kit (Roche, Mannheim, Germany). TUNEL+, that is, DNA-fragmented cells were assessed under an inverted microscope equipped with Apotome optical sectioning (Zeiss Axio Observer.Z1) by counting the total number of labeled cells in the ischemic striatum [11].

Immunohistochemistry for Neuronal, Microglial, Astrocytic, and Inflammation Markers

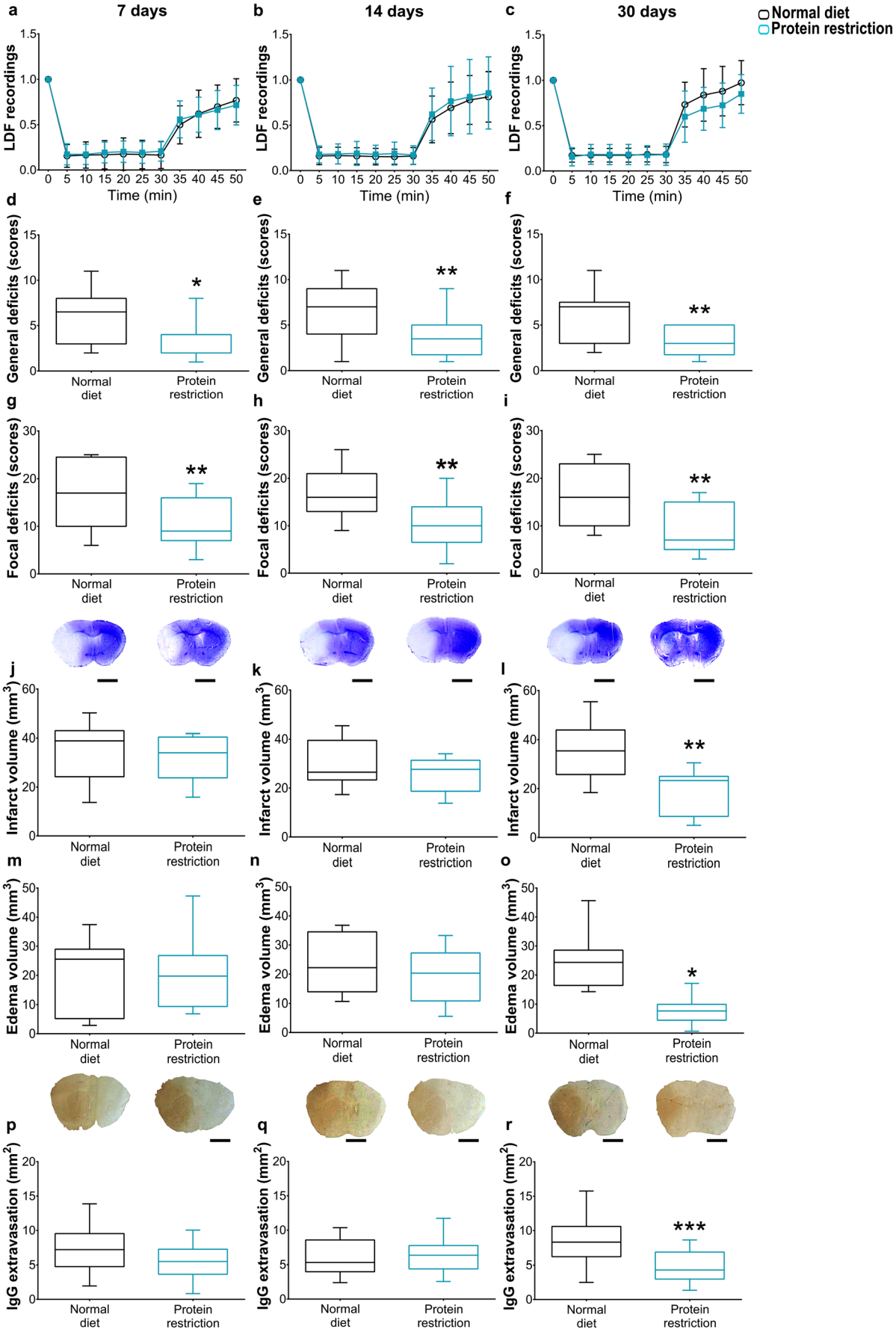
Adjacent sections were immersed in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) and 5% normal donkey serum (D9663; Sigma-Aldrich, Darmstadt, Germany). Sections were incubated overnight at 4 °C in monoclonal rabbit anti-NeuN (1:400; ab177487; Abcam, Cambridge, UK), monoclonal rat anti-CD45 (1:200; 550,539; BD Biosciences, Heidelberg, Germany), polyclonal rabbit anti-ionized calcium binding adaptor protein (Iba)-1 (1:500; Wako Chemicals, Neuss, Germany), monoclonal rat anti-glial fibrillary acidic protein (GFAP) (1:200; 130,300; Invitrogen, Dublin, Ireland), or polyclonal rabbit anti-inducible nitric oxide synthase (iNOS) (1:100; sc-650, Santa Cruz, Heidelberg, Germany) antibodies that were detected with Alexa Fluor-488 or Alexa Fluor-594-labeled secondary antibodies (NeuN, Iba-1, GFAP, iNOS) or biotinylated secondary antibodies followed by DAB staining with an avidin-biotin complex peroxidase kit (Vectastain Elite; Vector Labs, Burlingame, CA, U.S.A.) (CD45). NeuN, Iba-1, GFAP, and iNOS labeling were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (D9542; Sigma-Aldrich, Darmstadt, Germany). Sections were evaluated under a motorized Zeiss Axio Observer.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning (NeuN, Iba-1, GFAP, iNOS) or an Olympus X52 microscope (CD45) by counting the total number of NeuN+, CD45+, or iNOS+ cells in the striatum or analyzing the area covered by activated microglia (Iba-1) or reactive astrocytes (GFAP). The latter analysis was preferred to cell countings, since individual cells could not always unequivocally be discriminated. The latter data were shown as percent changes.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

From tissue samples harvested from the ischemic middle cerebral artery territory and liver, messenger RNA (mRNA) was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA was converted to cDNA using the high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, U.S.A). Real-time qPCR was performed in a StepOnePlus real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA) using primers designed or selected by the PubMed primer BLAST tool (https://blast.ncbi.nlm.nih.gov/) (Suppl. Table 1). Melting curves were used to confirm the efficiency of the primers. β-glucuronidase (β-Gluc) was used as housekeeping gene; brain and liver tissue from healthy mice served as control. Data were finally normalized that animals on normal diet were set as 1. Results were quantified using the $2^{-\Delta\Delta Ct}$ method [13]. PCR was performed in triplicates, of which mean values were computed for each animals.

Western Blots

During mRNA extraction, protein samples were collected after bromocholoropropane (B9673; Sigma-Aldrich, Darmstadt, Germany) separation. Ethanol was added and samples centrifuged at 12,000×*g* for 5 min. This procedure was repeated twice. The resulting pellet was suspended in 4% sodium dodecyl sulfate (SDS) (436,143; Sigma-Aldrich, Darmstadt, Germany). Protein content was measured using the Bradford method. Equal amounts of protein (20 μg) were loaded on 10% SDS-polyacrylamide gels, submitted to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked by 5% nonfat-dried milk (M7409; Sigma-Aldrich, Darmstadt, Germany) in 50 mM Tris-buffered saline (TBS) containing 0.1% Tween (P9416; Sigma-Aldrich, Darmstadt, Germany) for 1 h at room temperature, washed and incubated overnight at 4 °C with monoclonal rabbit anti-sirtuin-1 (Sirt-1; 1:2000; ab32441; Abcam, Cambridge, UK), polyclonal rabbit anti-glutathione peroxidase-3 (Gpx-3; 1:2000; ab59524; Abcam, Cambridge, UK), and polyclonal rabbit anti-β-actin (1:10000; 4967; Cell Signaling, Frankfurt, Germany) antibody. The next day, membranes were washed and incubated with secondary donkey anti-rabbit antibody. Blots were revealed using a


a
7 days
b
14 days
c
30 days
Normal diet
Protein restriction
LDF recordings
Time (min)
d
e
f
General deficits (scores)
g
h
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Focal deficits (scores)
j
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Infarct volume (mm3)
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Edema volume (mm3)
p
q
r
IgG extravasation (mm2)
Normal diet
Protein restriction

◀ **Fig. 1** Protein restriction decreases neurological deficits, infarct volume, brain edema, and blood-brain barrier permeability. **a**–**c** Laser Doppler flow (LDF) recordings above the core of the middle cerebral artery territory, **d**–**f** general neurological deficits evaluated by the Clark score, **g**–**i** focal neurological deficits examined by the Clark score, **j**–**l** infarct volume and **m**–**o** edema volume outlined on cresyl violet-stained brain sections, and (**p**–**r**) blood-brain barrier permeability in the striatum assessed by IgG extravasation analysis in mice exposed to normal or protein-reduced diet for 7 days (**a**, **d**, **g**, **j**, **m**, **p**), 14 days (**b**, **e**, **h**, **k**, **n**, **q**), or 30 days (**c**, **f**, **i**, **l**, **o**, **r**), followed by 30-min intraluminal MCAO and 24 h reperfusion. Representative photographs are shown. Data are presented as mean ± S.D. values (**a**–**c**) or median ± interquartile range box-blots with minimum/maximum data as whiskers (**d**–**r**). Bars in (**e**, **f**, **g**, **h**, **i**, **l**), 1 mm. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ compared with corresponding normal diet ($n = 12$ animals/group)

chemiluminescence kit and scanned using amyECL Imager (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Sirt-1 and Gpx-3 abundance was densitometrically evaluated in three independent experiments. The relative abundance of Sirt-1 and Gpx-3 was normalized to protein loading as determined in β-actin blots.

Statistics

Statistical analyses were performed using SPSS for Windows. Murinometric data, nutritional data, and LDF recordings were analyzed by repeated-measurement ANOVA followed by

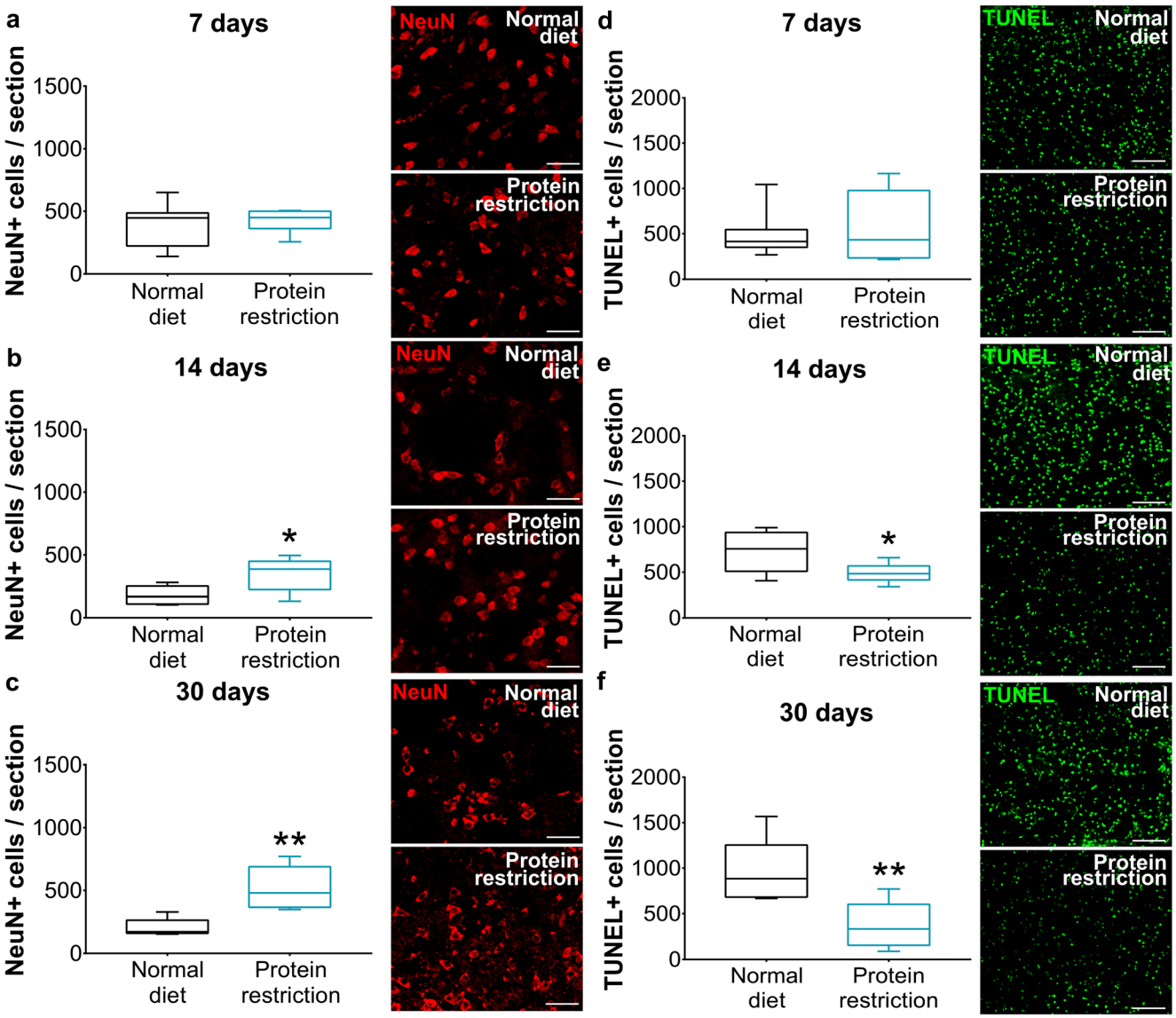
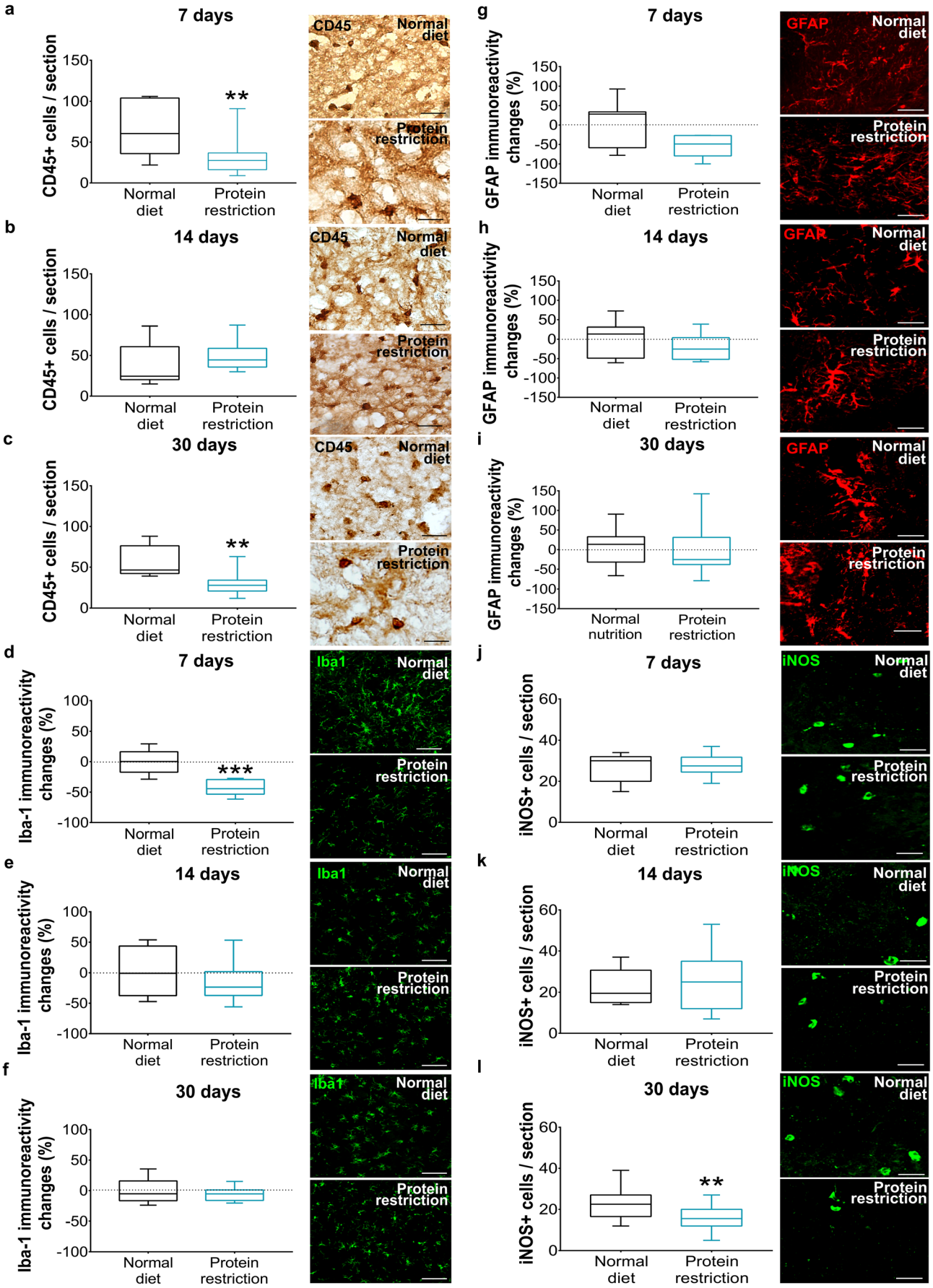


Fig. 2 Protein restriction promotes post-ischemic neuronal survival. **a**–**c** Number of NeuN+ surviving neurons and **d**–**f** number of DNA-fragmented, that is, irreversibly injured, TUNEL+ cells in the ischemic striatum of mice exposed to normal or protein-reduced diet over 7 days (**a**, **d**), 14 days (**b**, **e**), or 30 days (**c**, **f**), followed by 30-min intraluminal MCAO and 24 h reperfusion. Representative microphotographs are shown. Data are median ± interquartile range box-blots with minimum/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◀ **Fig. 3** Protein restriction differentially influences brain leukocyte infiltration, microglial activation, and iNOS formation. **a**–**c** Number of CD45+ leukocytes, **d**–**f** immunoreactivity for microglia marker Iba-1, **g**–**i** immunoreactivity for astrocytic marker GFAP, and **j**–**l** number of iNOS+ cells in the ischemic striatum of mice exposed to normal or protein-reduced diet for 7 days (**a**, **d**, **g**, **j**), 14 days (**b**, **e**, **h**, **k**), or 30 days (**c**, **f**, **i**, **l**), followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative microphotographs are shown. Data are median ± interquartile range box-blots with minimum/maximum data as whiskers. Bars, 100 μm. $^{**}p < 0.01$; $^{*}p < 0.05$ compared with normal diet ($n = 12$ animals/group)

unpaired *t* tests as post hoc tests. Neurological deficits, histochemical data, Western blots, and real-time qPCR data were analyzed by *t* tests. To explore the relationship of protein and calorie intake with infarct volume and neurological deficits, two-tailed Pearson's correlations were computed. LDF recordings, murinometric, nutritional, and real-time qPCR data are presented as mean ± S.D. values. Neurological deficits, histochemical data, and Western blots are shown as median ± interquartile range box-blots with minimum/maximum data as whiskers. *P* values < 0.05 were defined to indicate statistical significance.

Results

Moderate Protein Restriction Does Not Alter Major Murinometric and Nutritional Variables but Reduces Plasma LDL and Triglycerides

Murinometric assessments did not display any changes in body weight (Suppl. Fig. 1A-C) or BMI (Suppl. Fig. 1D-F) over up to 30 days in mice exposed to protein restriction compared with mice receiving normal diet. Likewise, the total amount of food ingested (Suppl. Fig. 1G-I) and calorie intake (Suppl. Fig. 1J-L) prior to MCAO did not differ between groups. After MCAO, until animal sacrifice, animals receiving the protein-reduced diet exhibited higher food (Suppl. Fig. 1G-I) and calorie (Suppl. Fig. 1J-L) intake than animals receiving the normal diet. This stabilized food and calorie intake resembles observations previously made in ischemic mice exposed to intermittent fasting prior to MCAO [14]. Food and calorie intake post-MCAO in animals on protein restriction was very similar to pre-stroke food and calorie intake. This was not the case in animals on normal diet. In mice exposed to 30, but not 7 or 14 days protein restriction, plasma cholesterol [158.3 ± 19.5 vs. 252.1 ± 120.0 mg/dl, $p < 0.05$], LDL [13.6 ± 14.1 vs. 32.7 ± 29.7 mg/dl, $p < 0.05$], and triglyceride [177.1 ± 74.6 vs. 259.3 ± 147.2 mg/dl, $p < 0.05$] levels were significantly lower than in mice on normal diet (Suppl. Table 2). Glucose levels did not differ between groups (Suppl. Table 2). The appearance, color, and size of stool samples did not differ between groups (not shown). Behavioral abnormalities (e.g., hypoactivity) or fur changes were absent in mice exposed to protein restriction.

Protein Restriction Decreases Neurological Deficits, Ischemic Injury, Brain Edema, and Blood-Brain Barrier Permeability

Cerebral LDF recordings during and after MCAO did not differ between groups. LDF reproducibly decreased to ~ 15–20% of baseline values during MCAO, followed by the return of LDF to baseline values within 20 min after reperfusion (Fig. 1a–c). Irrespective of the duration of food modification (7, 14, or 30 days), general (Fig. 1d–f) and focal (Fig. 1g–i) neurological deficits after 24 h were significantly reduced by protein restriction. Conversely, infarct volume (Fig. 1j–l), brain edema (Fig. 1m–o), and blood-brain barrier permeability assessed by serum IgG extravasation (Fig. 1p–r) was significantly decreased by protein restriction when administered over 30, but not 7 or 14 days. The number of surviving NeuN+ neurons in the ischemic striatum was significantly

Table 1 Responses of metabolism-related, inflammatory, and anti-oxidant genes in the ischemic brain of mice exposed to protein restriction

Brain	*Sirt-1*	*Igf-1*	*Insr*	*Glut-1*	*Il-1β*	*Nf-κb*	*Sod-1*	*Gpx-3*
7 days								
Normal diet	1.13 ± 0.60	3.60 ± 3.26	1.52 ± 0.74	1.18 ± 0.44	5.40 ± 3.61	1.55 ± 0.60	0.90 ± 0.34	0.13 ± 0.11
Protein restriction	1.11 ± 0.40	2.30 ± 1.65	1.14 ± 0.44	1.05 ± 0.42	4.12 ± 3.70	1.05 ± 0.40	0.80 ± 0.31	0.09 ± 0.01
14 days								
Normal diet	0.60 ± 0.23	1.64 ± 1.33	1.32 ± 1.11	1.17 ± 0.44	3.50 ± 1.60	3.00 ± 1.23	1.01 ± 0.34	0.30 ± 0.15
Protein restriction	0.50 ± 0.20	1.83 ± 1.22	0.80 ± 0.30	0.90 ± 0.55	3.16 ± 1.95	2.25 ± 0.73	1.12 ± 0.46	0.30 ± 0.42
30 days								
Normal diet	0.30 ± 0.11	1.84 ± 1.12	0.70 ± 0.30	1.14 ± 0.63	5.72 ± 3.20	2.35 ± 1.65	0.70 ± 0.30	0.40 ± 0.47
Protein restriction	0.60 ± 0.20*	0.83 ± 0.83	0.63 ± 0.20	0.90 ± 0.24	1.64 ± 0.92*	1.74 ± 0.66	0.63 ± 0.14	5.17 ± 4.00*

Data are real-time quantitative polymerase chain reaction (qPCR) results, expressed as mean ± SD values

$^{*}p < 0.05$ compared with normal diet ($n = 6$ animals/group; analyzed in triplicates, for which mean values were formed)

increased (Fig. 2a–c), whereas the number of irreversibly injured, that is, TUNEL+ cells was significantly reduced (Fig. 2d–f) by 14 days and more pronounced 30 days protein restriction.

To further evaluate links between daily protein intake with infarct volume and neurological deficits, Pearson's correlations were calculated. These Pearson's correlations revealed that after 30 days [r = 0.387, $p = 0.001$], but not 7 days [r = 0.004, $p = 0.350$] or 14 days [r = 0.071, $p = 0.206$] of protein restriction, protein intake was positively correlated with infarct volume (Suppl. Fig. 2). Independent of the duration of protein restriction (7, 14, or 30 days), protein intake was positively correlated with general and focal neurological deficits (Suppl. Fig. 2).

Protein Restriction Differentially Influences Brain Leukocyte Infiltration and Microglial Activation

Protein restriction significantly decreased the invasion of CD45+ leukocytes into ischemic brain tissue, when imposed to 7 or 30 days, but not 14 days (Fig. 3a–c). On the other hand, microglial activation, evaluated by Iba-1 immunohistochemistry, was significantly reduced by 7 days, but not 14 or 30 days protein restriction (Fig. 3d–f). Astrogliosis, examined by GFAP immunohistochemistry, was not influenced by protein restriction (Fig. 3g–i). The number of cells immunopositive for iNOS, a marker of pro-inflammatory M1 macrophages/microglial cells [15], was significantly reduced by protein restriction, when imposed for 30 days, but not 7 or 14 days (Fig. 3j–l). Based on their size and shape, the iNOS+ cells had the appearance of microglial cells.

Protein Restriction Upregulates the NAD-Dependent Deacetylase Sirtuin-1, Downregulates Interleukin-1β, and Upregulates Glutathione Peroxidase-3

Real-time qPCR showed that protein restriction did not alter the expression of metabolism-related, pro-inflammatory, and anti-oxidant genes in ischemic brain tissue, when imposed for 7 or 14 days, but increased the level of *sirtuin-1* (*Sirt-1*) mRNA, which encodes a NAD-dependent deacetylase that stabilizes mitochondrial function and metabolism partly by deacetylating the transcription regulator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [15]; reduced the level of *interleukin-1β* (*Il-1β*) mRNA, which encodes a pro-inflammatory cytokine strongly expressed by M1 microglial cells [15]; and increased the level of *glutathione peroxidase-3* (*Gpx-3*) mRNA, which encodes an anti-oxidant enzyme that degrades hydrogen peroxide [16, 17], in ischemic brain tissue, when imposed for 30 days (Table 1). Western blots revealed that the abundance of Sirt-1 and Gpx-3 proteins was increased after protein restriction over 30 days and in case of Gpx-3 less pronounced also over 14 days (Fig. 4). The metabolic markers *insulin-like growth factor-1* (*Igf-1*) mRNA, which encodes a growth factor with insulin-like properties; *insulin receptor* (*Insr*) mRNA; and *glucose transporter-1* (*Glut-1*) mRNA [18] were not influenced by protein restriction, as was *nuclear factor-κb* (*Nf-κb*) mRNA, which encodes a transcription factor deacetylated by Sirt-1 that controls *Il-1β* expression [19], or *superoxide dismutase-1* (*Sod-1*) mRNA, which encodes a dismutase degrading superoxide anions to hydrogen peroxide [16] (Table 1).

Protein Restriction Regulates Metabolism-Related, Pro-Oxidant and Anti-Oxidant Genes in the Liver

Real-time qPCR showed that protein restriction regulated genes encoding metabolism-related genes, pro-oxidant and anti-oxidant enzymes in the liver within 7 days. Thus, *Sirt-1* mRNA was upregulated, *NADPH oxidase-4* (*Nox-4*) mRNA, which encodes a protein that catalyzes the production of superoxide free radicals by transferring electrons to oxygen from NADP [20], was downregulated, and *superoxide dismutase-2* (*Sod-2*) mRNA and *catalase* (*Cat*) mRNA, which encodes another peroxidase [17], were upregulated by protein restriction (Table 2). After 14-day exposure to protein-reduced diet, liver levels of *Sirt-1* mRNA, *Nox-4* mRNA, and *Cat* mRNA were reversed to levels in mice receiving normal diet, and liver levels of *Sod-1* mRNA, *Sod-2* mRNA, and *Gpx-3* mRNA were increased (Table 2). Prolonged 30-day protein restriction reregulated metabolism-related and anti-oxidant markers. Thus, *Sod-1* mRNA and *Sod-2* mRNA were restored to levels in normal diet mice, and *Sirt-1* mRNA, *Igf-1* mRNA, and *Gpx-3* mRNA were elevated (Table 2).

Discussion

By exposing adult mice to intraluminal MCAO that had been submitted to a protein-reduced diet for 7, 14, or 30 days, we show that protein restriction protects against focal cerebral ischemia. Irrespective of the duration of food modification (7–30 days), post-ischemic neurological deficits were reduced by protein restriction. In contrast to these behavioral improvements, only prolonged protein restriction over 30 days reduced infarct volume, brain edema, and blood-brain barrier permeability. Neuroprotection by protein restriction went along with increased neuronal survival in the ischemic striatum, reduced brain infiltration of CD45+ leukocytes, and reduced the expression of iNOS and interleukin-1β. As potential mechanism, increased expression of the NAD-dependent deacetylase Sirt-1 and increased expression of anti-oxidant Gpx-3 were noted in ischemic brain tissue. Robust responses

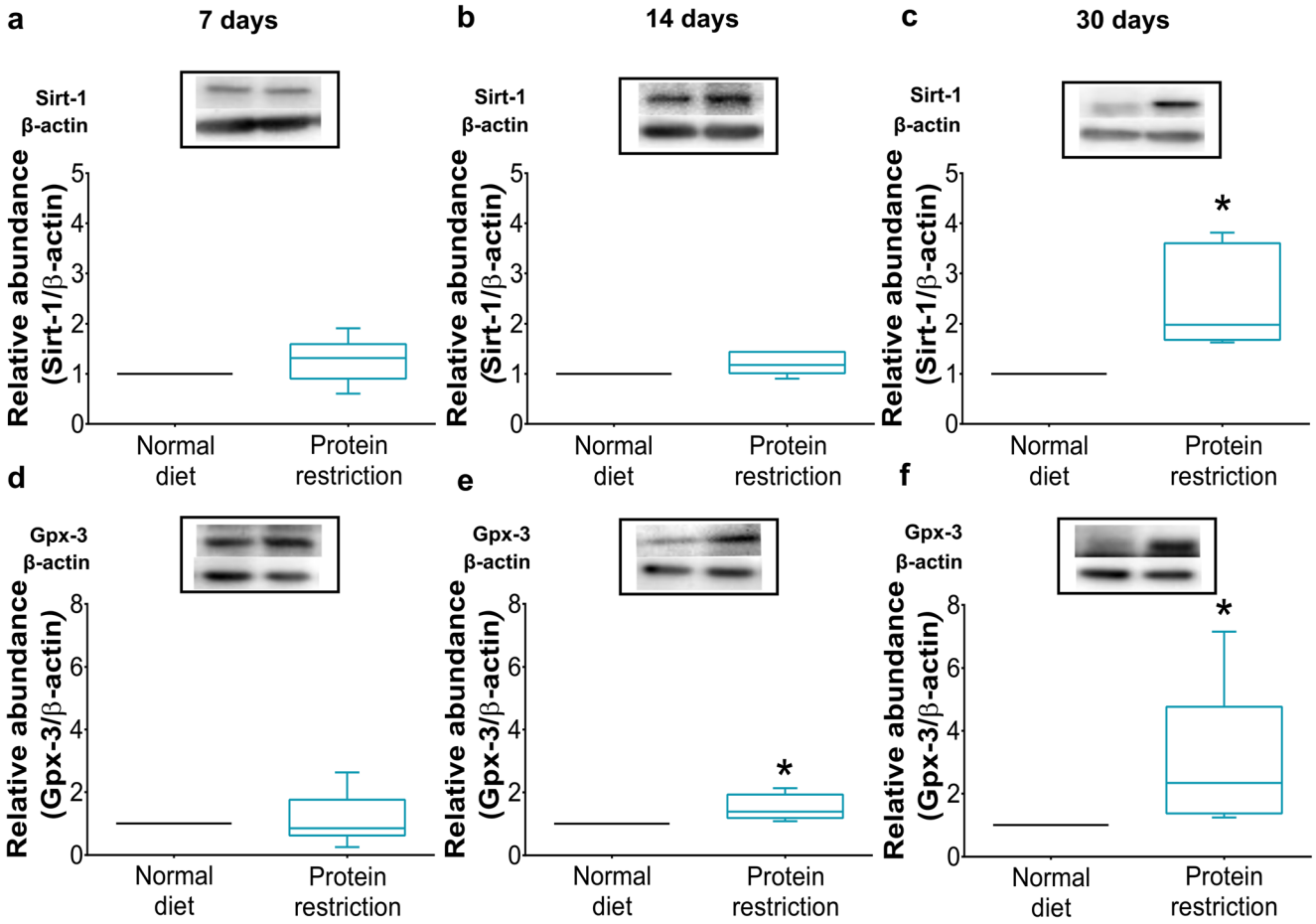

Fig. 4 Protein restriction increases sirtuin-1 and glutathione peroxidase-3 abundance in ischemic brain tissue. Western blot analysis of **a**–**c** sirtuin-1 (Sirt-1) and **d**–**f** glutathione peroxidase-3 (Gpx-3) protein in ischemic brain tissue of mice exposed to normal or protein-reduced diet for **a**, **d** 7 days, **b**, **e** 14 days, or **c**, **f** 30 days, followed by 30 min intraluminal MCAO and 24 h reperfusion. Representative Western blots are also shown. Data are median ± interquartile range box-blots with minimum/maximum data as whiskers. *$p < 0.05$ compared with normal diet ($n = 6$ animals/ group)

of oxidative stress markers, indicating a shift from pro-oxidant (Nox-4) to anti-oxidant (Sod-1, Sod-2, Gpx-3, Cat) enzymes, were detected in the liver.

Previous studies in rat and gerbil models of global and focal cerebral ischemia found that protein restriction compromises neurological recovery in motor-coordination tests [5, 7], increases brain inflammation via activation of NF-κb [4], increases neuronal injury [5], and decreases neuronal plasticity, evaluated by the axonal and synaptic proteins growth-associated protein-32, synaptophysin, and synaptosomal-associated protein-25 [6]. These studies have in common that far more severe protein restriction (0.5–2% protein, in all studies casein) was imposed for 3 to 4 weeks. Such severe protein restriction results in a reduction in the total amount of food ingested, since the animals refuse this chow [4–7]. Combined protein-energy malnutrition with a loss of body weight was noted in these animals. A single study so far examined consequences of a more moderate diet containing 7% protein (soybean protein), which, when administered during pregnancy and lactation to mothers, reduced brain injury but augmented sensorimotor deficits and impaired homing behavior of offspring exposed to unilateral cerebral hypoxia-ischemia at 7 days post-birth [8]. The improvement of ischemic injury by protein restriction in this former study goes in line with our study. Unlike this earlier study, we observed an improvement of neurological deficits evaluated with a global and focal behavioral score in adult mice exposed to focal cerebral ischemia. Differences of animal age (adult vs. newborn), ischemia models (intraluminal MCAO vs. unilateral hypoxia-ischemia), diets (casein or soybean protein as protein source), or species (mice vs. rats) may explain the different findings of this previous study [8] and the present one. In this previous study, animals also developed body weight loss [8]. Interestingly, our findings resemble observations following short-term dietary restriction by exposure to a protein-free diet or complete fasting for 6 days in rat models of intraluminal or peripheral MCAO, where similar to the present study reduced infarct volume and decreased motor-coordination deficits were found [21]. Unlike in the present study, body weight was again significantly reduced by both diet protocols [21].

Table 2 Responses of metabolism-related, inflammatory, pro- and anti-oxidant genes in the liver of mice exposed to protein restriction

Liver	*Sirt-1*	*Igf-1*	*Insr*	*Glut-2*	*Nf-κb*	*Nox-4*	*Sod-1*	*Sod-2*	*Gpx-3*	*Cat*
7 days										
Normal diet	0.75 ± 0.20	0.75 ± 0.43	0.40 ± 0.48	0.90 ± 0.48	1.66 ± 0.87	1.08 ± 0.49	0.86 ± 0.23	1.00 ± 0.35	0.89 ± 0.27	0.39 ± 0.18
Protein restriction	1.60 ± 0.80*	0.80 ± 0.75	0.34 ± 0.40	0.99 ± 0.70	1.40 ± 0.93	0.52 ± 0.20*	0.99 ± 0.53	2.05 ± 0.88*	0.97 ± 0.44	2.24 ± 1.40*
14 days										
Normal diet	0.30 ± 0.37	0.60 ± 0.37	0.42 ± 0.40	0.88 ± 0.48	0.75 ± 0.56	0.86 ± 0.35	0.98 ± 0.30	0.99 ± 0.14	2.22 ± 2.06	0.44 ± 0.48
Protein restriction	0.30 ± 0.14	0.76 ± 0.64	0.24 ± 0.40	1.26 ± 1.06	0.68 ± 0.37	0.67 ± 0.21	2.29 ± 1.33*	2.73 ± 1.60*	6.00 ± 3.50*	0.45 ± 0.63
30 days										
Normal diet	0.80 ± 0.18	0.22 ± 0.31	0.24 ± 0.26	1.20 ± 0.31	0.67 ± 0.28	1.17 ± 0.31	1.03 ± 0.08	1.75 ± 1.66	0.76 ± 0.31	0.69 ± 0.54
Protein restriction	1.50 ± 0.64*	1.20 ± 0.40**	0.70 ± 0.63	1.21 ± 0.95	0.74 ± 0.70	1.26 ± 0.34	0.78 ± 0.40	1.60 ± 0.89	1.70 ± 0.71*	0.98 ± 0.97

Data are real-time quantitative polymerase chain reaction (qPCR) results, expressed as mean ± SD values

*$p < 0.05$/**$p < 0.01$ compared with normal diet ($n = 6$ animals/group; analyzed in triplicates, for which mean values were formed)

That in our study protein restriction induced neuroprotection without provoking body weight loss is noteworthy. It indicates that, unlike hypothesized earlier [22], body weight loss is not a necessity for neuroprotection to occur subsequent to dietary restriction.

As potential mechanism, protein restriction in the present study reduced plasma cholesterol, LDL, and triglyceride levels and decreased the infiltration of CD45+ leukocytes into the ischemic brain. At the time-point examined (24 h post-MCAO), leukocyte infiltrates are predominated by polymorphonuclear neutrophil (PMN) granulocytes. Using strategies of antibody-mediated PMN depletion or prevention of PMN brain entry, we have previously shown that PMN contribute to ischemic injury after intraluminal MCAO [23] and that PMN furthermore mediate injury-aggravating effects of hypercholesterolemia induced by a lipid-rich Western diet [24]. In the present study, the reduced brain leukocyte infiltration after protein restriction was associated with decreased iNOS and *Il-1β* mRNA levels, which are strongly expressed by pro-inflammatory M1 microglial cells in the ischemic brain [15]. In our study, the iNOS and *Il-1β* mRNA responses dissociated from patterns of microglial activation, which was reduced by 7 days, but not 14 or 30 days protein restriction. It is conceivable that microglial differentiation shifted towards a neuroprotective M2 phenotype upon protein restriction. The expression of iNOS and Il-1β under inflammatory conditions is tightly controlled by the transcription factor *Nf-κb* [19], which, as we further showed, was not regulated by protein restriction on the mRNA level.

Neuroprotection by protein restriction was associated with increased expression of the NAD-dependent deacetylase Sirt-1 (both on the mRNA and protein level) and increased expression of anti-oxidant *Gpx-3* mRNA in ischemic brain tissue. Independent of the duration of food modification, robust responses of oxidative stress markers (downregulation of pro-oxidant *Nox-4*, upregulation of *Sod-1/2*, *Gpx-3*, and/or *Cat* mRNAs) were also found in the liver. Sirt-1 has a large variety of actions in the healthy and injured brain, stabilizing cellular energy metabolism partly by deacetylating PGC-1α [19], which, besides others, results in the acquisition of ischemic tolerance. Via different downstream targets, mitochondrial energy coupling is promoted, reactive oxygen species formation reduced, and anti-oxidant capacity increased. Our data suggest that Sirt-1 played a role in the regulation of the above pro- and anti-oxidant enzymes. Further studies will be required to elucidate whether elevated Sirt-1 causally contributed to neuroprotection by protein restriction. Interestingly, *Sirt-1*$^{-/-}$ was previously found to exacerbate ischemic injury in mice exposed to intraluminal MCAO but failed to abolish protective effects of calorie restriction [25]. The here presented study complements studies on calorie restriction [26], showing that changes in food composition may similarly induce neuroprotection as changes in the total food or calorie amount. In both types of diet modification, anti-inflammatory and anti-oxidant responses seem to be instrumental for the promotion of stroke outcome.

In view of the important role of food composition for stroke management, several questions still remain to be explored, namely (a) effects of diet modifications initiated after focal cerebral ischemia on neurological recovery and brain remodeling, (b) effects of different protein origins (mammalian, poultry vs vegetarian sources) on ischemic injury and neurological recovery, and (c) effects of diet composition depending on nutrition status and the presence of life style-related risk factors (e.g., hyperlipidemia, diabetes). We need to be aware that nutrition and digestion cannot be transferred one-to-one from rodents to human patients. If thoughtfully addressed, rodent studies might allow us to deduct working hypotheses how modifications in food composition could be used for alleviating stroke consequences.

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Author Contributions TSC and DMH designed the study. TSC performed the animal experiments, assisted by EHSM and MS. TSC, LMNM, ARSM, ED, and MS conducted histochemical and molecular biological studies. DMH and CK provided infrastructural support. TSC, EHSM, LMNM, ARSM, ED, and DMH analyzed the data. TSC and DMH drafted the manuscript. All the authors finalized it.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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6 STUDY 3

Title: Moderate protein restriction promotes post-ischemic neurological recovery, peri-infarct brain remodeling, long-term brain tissue survival and contralesional neuroplasticity by mechanisms involving anti-inflammatory and anti-oxidant actions

Author's contributions:

Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%

Contributed to the acquisition of the data:

Animal experiment: TSC – 100%

Histochemical analysis: TSC – 95% / MS – 5%

Molecular analysis: TSC – 90% / VS – 5% /LMNM, ARSM – 5%

Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 35% / VS, LMNM, ARSM – 5%

Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%

Provided final approval of the version to publication: TSC – 50% / DMH – 50%

Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TSC – 50% / DMH – 50%

Authors' contributions:

Tayana Silva de Carvalho and Dirk M. Hermann designed the study. Tayana Silva de Carvalho performed the animal experiments, assisted by Eduardo H. Sanchez-Mendoza and Maryam Sardari. Tayana Silva de Carvalho, Vikramjeet Singh, Adriana R. Schultz Moreira, Luiza M. Nascentes, and Maryam Sardari conducted histochemical and molecular biological studies. Dirk M. Hermann and Christoph Kleinschnitz provided infrastructural support. Tayana Silva de Carvalho, Eduardo H. Sanchez-Mendoza, Vikramjeet Singh, Adriana R. Schultz Moreira, Luiza M. Nascentes, and Dirk M. Hermann analyzed the data. Tayana Silva de Carvalho and Dirk M. Hermann drafted the manuscript. All authors concluded it.

Currently status: currently in preparation.

Moderate post-ischemic protein restriction promotes sustained neurological recovery, peri-infarct brain tissue survival and remodeling, and contralesional neuroplasticity by mechanisms involving anti-inflammatory and anti-oxidant actions

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Abstract

Background: Moderate food protein restriction confers neuroprotection, when applied prior to ischemic stroke. How moderate protein restriction influences stroke recovery, when administered after stroke, has, although clinically more important, not been assessed.

Methods: Male C57BL6/j mice were exposed to focal cerebral ischemia induced by transient intraluminal middle cerebral artery occlusion. Immediately after the stroke, mice were randomized to two groups receiving moderate protein restriction by providing a normocaloric diet containing 8% protein or normal diet containing 20% protein. Neurological deficits were evaluated by a comprehensive test battery

Results: Besides reduction of urea, an established marker of food protein intake, no metabolic changes were noted in the animals' blood. Notably, protein restriction markedly reduced infarct volume after only 3 days, induced sustained neurological recovery over 6 weeks, increased long-term neuronal survival, reduced microglial activation, reduced whole brain, striatal and corpus callosum atrophy, and increased contralesional pyramidal tract plasticity at the level of the red nucleus. On the molecular level, an elevation of the antioxidant glutathione peroxidase-3 was found in the brain and liver.

Conclusion: Moderate post-ischemic protein restriction potently enhances neurological recovery, long-term brain tissue survival, brain remodeling and plasticity in rodents.

Key-words: Axonal plasticity, ischemic stroke, middle cerebral artery occlusion, neurogenesis, neuroprotection, neuronal plasticity, protein intake**.**

Introduction

Food protein content has pronounced effects on ischemic brain injury. Protein restriction may either exacerbate or attenuate ischemic injury and neurological deficits, depending on the severity of food protein restriction. In rats and gerbils, the delivery of diets containing 0.5-2% protein for 3 to 4 weeks in models of global [1-3] or focal [4] cerebral ischemia aggravated neurological deficits, increased the brain's inflammatory response and reduced neuronal plasticity. Such severe protein restriction results in a reduction in the total amount of food ingested, since the animals refuse this chow [1-4]. In such animals, combined protein-energy malnutrition is noted. Conversely, a more moderate protein restriction using a diet

containing 7 or 8% protein over up to one month improved neurological deficits, reduced ischemic injury and reduced brain inflammation in newborn rats exposed to unilateral cerebral hypoxia-ischemia [5] and adult mice exposed to focal cerebral ischemia [6]. In all studies except one study, which evaluated consequences of combined protein-energy malnutrition in a rat model of global cerebral ischemia [3], dietary modifications were imposed before ischemia. Notably, functional neurological recovery was not evaluated in this study [3]. Hence, the consequences of post-stroke dietary protein modifications for neurological recovery – although clinically more important – have hitherto never been assessed. By exposing mice to intraluminal middle cerebral artery occlusion (MCAO), which thereafter were randomized to a normocaloric diet containing 8% or 20% protein, we now examined this question.

Materials and methods

Legal issues, statistical planning and randomization

Experiments were approved by local government authorities (Bezirksregierung Düsseldorf) in accordance to E.U. guidelines (Directive 2010/63/EU) for the care and use of laboratory animals. Sample size calculations determined that 12 animals per group were required for the neurological examinations and histochemical studies, given that the effect size was 1.167, the alpha error was 5% and the beta error (1– statistical power) was 20%. Experimenters were blinded by a third person not involved in the assessments, randomizing the animals, and providing the food pellets.

Food modifications, animal groups and murinometrics

Adult male C57BL6/j mice (8 weeks, 26-30g; Harlan-Netherlands, Rossdorf, Germany) exposed to 30 min intraluminal MCAO were randomized to two diets: a) normal nutrition (C1000; 3518kcal/kg, 20% protein, 13% lipids; Altromin, Lage, Germany) or b) low protein (C1003 mod.; 3541 kcal/kg, 8% protein, 13% lipids; Altromin) which were administered *ad libitum* over up to 56 days starting with the induction of intraluminal MCAO. Throughout the study, animals were housed in groups of 4 animals in group cages (Green line IVC Sealsafe PLUS Mouse; Tecniplast, Hohenpeißenberg, Germany) in a 12h:12h light/dark cycle. Animals were sacrificed at 3 days (n=12 animals/ group) or 56 days (n=18 animals/ group) post-ischemia (dpi). Animals sacrificed at 3 dpi were used for behavioral analyses and histochemical studies. Animals sacrificed at 56 dpi were divided into two groups used

for (a) behavioral analyses and histochemistry/ immunohistochemistry (n=12 animals/ group) and (b) real-time quantitative polymerase chain reaction (RTqPCR) studies and Western blots (n=6 animals/ group). Food consumption and calorie intake were measured daily. Body (i.e., nose–anus) length was determined before MCAO. Body weight and body mass index (BMI) were determined daily (animals sacrificed at 3 dpi) or weekly (animals sacrificed at 56 dpi) after MCAO [7].

Intraluminal MCAO

Mice were anesthetized with 1.0-1.5% isoflurane (30% O2, remainder N2O). Rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system. Cerebral laser Doppler flow (LDF) was recorded using a flexible probe (Perimed, Järfälla, Sweden) attached to the skull overlying the core of the middle cerebral artery territory. A midline neck incision was made. The left common and external carotid arteries were isolated and ligated, and the internal carotid artery was temporarily clipped. A silicon-coated nylon monofilament (0.21 mm tip diameter; Doccol, Sharon, MA, U.S.A.) was introduced through a small incision of the common carotid artery and advanced to the circle of Willis for MCAO. Reperfusion was initiated 30 minutes thereafter by monofilament removal. Wounds were carefully sutured and anaesthesia was discontinued. After 24 hours, at 2 dpi, 3 dpi, 7 dpi and at weekly intervals thereafter, animals were evaluated using the Clark score [8], which captures general and focal neurological deficits. During the first week after MCAO, animals received daily intraperitoneal injections of the anti-phlogistic carprofen (4 mg/kg; Bayer Vital, Leverkusen, Germany). For analysis of endogenous neurogenesis, BrdU (Sigma-Aldrich, Deisenhofen, Germany; 50 mg/kg) was intraperitoneally administered once daily from 8-18 dpi [9, 10].

Rotarod

The Rotarod is a motor coordination test, which consists of a rotating drum (Ugo Basile, model 47600, Comerio, Italy), in which animals are placed while the drum is accelerating. The time until each animal drops off the drum is measured (maximum testing time 300 s). Animals were trained three times each on three consecutive days before MCAO. Following a baseline evaluation, animals were tested weekly until 42 dpi. The test was performed three times per time point. Means were calculated for each time-point [9, 10].

Tight rope test

The tight rope test consists of a 60 cm long rope that is attached to two opposing platforms. Animals are placed on the middle of the rope and the time until reaching one of the platforms is determined (maximum testing time 60s) [9, 10]. Animals were trained three times on three consecutive days before MCAO. After a baseline examination, animals were tested weekly until 42 dpi. The tight rope test was performed three times per time point. Means were calculated for each time-point.

Open field test

The open field arena is a square platform (52x52x30 cm) subdivided into one center (31.2x31.2 cm), four border (each 10.4x31.2 cm) and four corner (each 10.4x10.4 cm) fields, in which animals are placed near the wall and observed for 300 s for evaluating spontaneous motor behavior. The number of field entries, duration in each field and speed were tracked using VideoMot software (version 7.0.1; TSE Systems, Bad Homburg, Germany) [9, 10]. In addition, circling behavior was analysed. The open field test was performed once at baseline, 7 dpi and 42 dpi.

Zero maze test

The zero maze is an elevated annular platform, which has open and closed segments. During a test session of 300 s, the time spent in the open and closed segment is analysed as measure of anxiety. Animals were tracked using VideoMot software [11]. The zero maze was performed once at baseline, 7 dpi and 42 dpi.

Biotinylated dextran amine (BDA) injection

BDA is an anterograde tract tracer that allows studying efferent projections in the brain [9, 10]. At 42 dpi, animals were anesthetized with 1.5% isoflurane (30% O2, remainder N2O). A cranial bur hole was drilled 0.5 mm rostral and 2.5 mm lateral to the bregma, via which deposits of 10% BDA (MW 10,000; Molecular Probes, Waltham, MA, U.S.A.; diluted in 0.01 M PBS at pH 7.2) were placed into the contralesional motor cortex by means of microsyringe injections. A volume of 2.4 µl tracer was administered to each animal, which was administered in two equal deposits located rostrally and caudally of the bur hole at 1.5 mm depth by inserting the microsyringe needle into the brain at angles of 45°and 135°, respectively.

Plasma measurements and animal sacrifice

Three days after ischemia or fourteen days after the tracer injection, animals were re-anesthetized after 5 hours fasting. Plasma samples were obtained from the animals' hearts by cardiac puncture that were used for analysis of urea nitrogen, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, cholesterol, low-density lipoprotein (LDL), triglycerides and glucose levels (ADVIA® 2400; Siemens, Erlangen, Germany). Healthy animals not exposed to focal cerebral ischemia were used as additional controls. Twelve animals per group were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains were weighted, post-fixed overnight in 4% PFA in 0.1 M PBS and cryoprotected by immersion in 30% sucrose in 0.1 M PBS. Brains were frozen and cut into 20-µm-thick coronal cryostat sections that were used for conventional histochemistry or into 40-µm-thick coronal cryostat sections that were used for tract tracing histochemistry. Additional six animals per group sacrificed at 56 dpi were transcardially perfused with normal saline. These animals were used for Western blots and RTqPCR [12].

Infarct volume and brain atrophy

Coronal 20 µm sections collected at millimeter intervals across the brain were stained with cresyl violet. In animals sacrificed at 3 dpi, infarct volume was measured by subtracting areas of healthy tissue of the ischemic hemisphere from those of the contralesional hemisphere [9, 13]. Likewise, whole brain and striatal atrophy were evaluated in animals sacrificed at 56 dpi by analyzing ipsilesional and contralesional brain areas across the forebrain, of which percent volume ratios were determined [9]. Corpus callosum thickness was evaluated by tracing the corpus callosum area in the ischemic hemisphere from the midline up to one millimeter lateral to the midline. Sections were scanned and quantified using Image J software (National Institute of Health, Bethesda, MD, U.S.A.).

Immunohistochemistry of IgG extravasation

Twenty µm sections obtained from the rostrocaudal level of the midstriatum were rinsed for 20 minutes in 0.3% H_2O_2 in 70% methanol in 0.1 M PBS, immersed in 0.1 M PBS containing 5% bovine serum albumin (BSA) (05470; Sigma-Aldrich, Darmstadt, Germany) and incubated for 1 hour in biotinylated anti-mouse IgG (1:100; Santa Cruz, Heidelberg, Germany), followed by diaminobenzidine tetrahydrochloride

(DAB) (D5905; Sigma-Aldrich, Darmstadt, Germany) staining with an avidin-biotin complex peroxidase kit (Vectastain Elite; Vector Labs, Burlingame, CA, U.S.A.). IgG extravasation was analysed by evaluating the brain area exhibiting IgG extravasation [13].

Immunohistochemistry for neuronal, leukocyte, microglial and astrocytic markers

Adjacent 20 µm sections were immersed in 0.1 M PBS containing 0.1% Triton X-100 (PBS-T) and 5% normal donkey serum (D9663; Sigma-Aldrich). Sections were incubated overnight at 4°C in monoclonal rabbit anti-NeuN (1:400; ab177487; Abcam, Cambridge, U.K.), monoclonal rat anti-CD45 (Set 1; 1:200; 550539; BD Biosciences, Heidelberg, Germany), polyclonal rabbit anti-ionized calcium binding adaptor protein (Iba)-1 (1:500; Wako Chemicals, Neuss, Germany) or monoclonal rat anti-glial fibrillary acidic protein (GFAP) (1:200; 130300; Invitrogen, Dublin, Ireland) antibodies that were detected with Alexa Fluor-488 or Alexa Fluor-594-labeled secondary antibodies (NeuN, Iba-1, GFAP) or biotinylated secondary antibodies followed by DAB staining with an avidin-biotin complex peroxidase kit (Vectastain Elite; Vector Labs, Burlingame, CA, U.S.A.) (CD45). NeuN, Iba-1 and GFAP labelings were counterstained with Hoechst 33342 (H1399; Thermo Fisher Scientific, Waltham, MA, U.S.A). Sections were evaluated under the Zeiss AxioObserver.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning (NeuN, Iba-1, GFAP) by counting the total number of NeuN+ or CD45 in the striatum or analyzing the area covered by microglia (Iba-1) or reactive astrocytes (GFAP). The latter analysis was preferred to cell countings, since individual cells could not always unequivocally be discriminated [9].

Immunohistochemistry for endogenous neurogenesis

Adjacent 20 µm sections were rinsed three times for 5 minutes in 0.1 M PBS and immersed in 0.1 M PBS-T and 10% normal donkey serum for 1 hour at room temperature. Sections were immersed in 1 M HCl at pH 1 for 45min at room temperature, followed by 10 min immersion in 0.1 M sodium borate buffer at pH 8.0. Sections were incubated overnight at 4°C in monoclonal rat anti-BrdU (1:100; ab6326, Abcam), polyclonal goat anti-doublecortin (Dcx; 1:100; sc-8066, Santa Cruz) or polyclonal chicken anti-NeuN (1:400; ab134014, Abcam) antibodies. BrdU was detected by Alexa Fluor-594 or Alexa Fluor-488 conjugated secondary antibody, Dcx

by Alexa Fluor-488 conjugated secondary antibody and NeuN by Alexa Fluor-647 conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342 (H1399; Thermo Fisher Scientific). Sections were evaluated using a motorized Zeiss Axio Observer.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning. For each animal, images covering the ipsilesional subventricular zone (SVZ) and peri-infarct striatum were obtained, in which cells were counted by identifying overlaps of signals in BrdU-labeled, doublecortin-labeled and NeuN-labeled image masks [9].

Immunohistochemistry for BDA and analysis of corticorubral projections

Forty µm sections were rinsed three times for 5 min each in 0.1 M PBS. Stainings were revealed with streptavidin (1:500, S32354; Invitrogen) diluted with 0.3% PBS-T [14]. The location of tracer deposits was checked at the levels of the needle tracks, thus ensuring that the motor cortex had indeed been injected in all animals. To account for variabilities in tracer uptake in different mice, we first evaluated the number of tracer-stained fibers in the pyramidal tract at the level of the parvocellular red nucleus (bregma –3.0 to –3.5 mm). For this purpose, two consecutive sections were analysed, counting the number of fibers crossing the sections in four regions of interest of 2,865 µm² each that had been selected in the dorsolateral, ventrolateral, dorsomedial and ventromedial portion of the pyramidal tract. By measuring the total area of the pyramidal tract using a motorized Zeiss Axio Observer.Z1 inverted epifluorescence microscope equipped with Apotome optical sectioning, we calculated the overall number of labeled pyramidal tract fibers, as described previously [10, 15]. For analysis of corticorubral projections, a 500 µm long intersection line was superimposed on the brain midline. Along that line those fibers crossing into the ipsilesional hemisphere in direction of the red nucleus were quantified. For each animal, the total number of fibers counted was normalized with the total number of labeled fibers in the pyramidal tract and multiplied with 100, resulting in percent values of fibers crossing the midline. Two consecutive sections were analysed for each animal, of which mean values were determined [9].

RTqPCR

From tissue samples harvested from the ischemic middle cerebral artery territory and liver, messenger RNA (mRNA) was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). mRNA was converted to cDNA using the high-capacity RNA-to-

cDNA kit (Thermo Fisher Scientific). RTqPCR was performed using a StepOnePlus real-time PCR instrument (Thermo Fisher Scientific) with primers designed or selected by the PubMed primer BLAST tool (https://blast.ncbi.nlm.nih.gov/) (**Suppl. Table 1**). Melting curves were used to confirm the efficiency of the primers. β-glucuronidase (β-Gluc) was used as housekeeping gene, brain and liver tissue from healthy mice served as control. Results were quantified using the 2-ΔΔCt method [16]. RTqPCR were performed in triplicates, of which mean values were computed for each animal.

Western blots

From the same samples, protein samples were collected after 1-Bromo-3-chloropropane (B9673; Sigma-Aldrich, Darmstadt, Germany) separation. Ethanol was added and samples centrifuged at 12.000g for 5 min. This procedure was repeated twice. The resulting pellet was suspended in 4% sodium dodecyl sulfate (SDS) (436143; Sigma-Aldrich). Protein content was measured using the Bradford method (#500-0113; Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of protein (20 µg) were loaded on 10 % SDS-polyacrylamide gels, submitted to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were blocked by 5% nonfat-dried milk (M7409; Sigma-Aldrich) in 50 mMTris-buffered saline (TBS) containing 0.1% Tween (P9416; Sigma-Aldrich) for 1 hour at room temperature, washed and incubated overnight at 4ºC with monoclonal rabbit anti-sirtuin-1 (Sirt1; 1:2000; ab32441; Abcam), polyclonal rabbit anti-glutathione peroxidase-3 (Gpx3; 1:2000; ab59524; Abcam), polyclonal rabbit anti 4-hydroxy-2-nonenal (4HNE; 1:1000; 393207, Calbiochem) and polyclonal rabbit anti-β-actin (1:10000; 4967; Cell Signaling, Frankfurt, Germany) antibody. The next day, membranes were washed and incubated with secondary donkey anti-rabbit or anti-goat antibody. Blots were revealed using a chemiluminescence kit (RPN2232; ECLTM Prime Western Blotting Detection reagents; Amersham, Vienna, Austria) and scanned using amyECL Imager (Thermo Fisher Scientific). Sirt1, 4HNE and Gpx3 abundance was densitometrically evaluated in three independent experiments. The relative abundance of Sirt1, 4HNE and Gpx3 was normalized to protein loading as determined in β-actin blots [13].

Statistics

Statistical analyses were performed using SPSS for Windows. Murinometric data, nutritional data, LDF recordings and neurological deficits were analysed by repeated measurement ANOVA followed by Bonferroni tests as posthoc tests. Plasma measurements, histochemical data, Western blots and RTqPCR data were analysed by t tests. Plasma measurements, RTqPCR data and data involving repeated measurements are presented as mean ± S.D. values, all other data as median (mean) ± inter quartile ranges (IQR) with minimum and maximum data as whiskers. P values <0.05 were defined to indicate statistical significance.

Results

Protein restriction does not induce any major murinometric changes

Mice exposed to protein restriction did not display any changes in body weight (**Fig. 1A, Suppl. Fig. 1A**) or BMI (**Fig. 1B, Suppl. Fig. 1B**) over up to 56 days, when compared with mice receiving normal diet. In both groups, MCAO was followed by a ~10% drop of body weight and BMI that recovered within 4-5 weeks post-ischemia (**Fig. 1A, B, Suppl. Fig. 1A, B**). The total amount of food ingested (**Fig. 1C**) and calorie intake (**Fig. 1D**) decreased sharply in both groups at 1 dpi (0.9 ± 0.5 vs 1.0 ± 0.7 g/ day and 3.1 ± 1.9 vs. 3.7 ± 1.9 calories/ day in mice exposed to normal diet and protein restriction, respectively) and subsequently recovered to pre-ischemic levels within 3 dpi (**Suppl. Fig. 1C, D**). Plasma urea, a marker of protein consumption, was reduced in mice exposed to protein restriction at 56 dpi (12.5 ± 4.1 and 16.9 ± 6.0 mg/dl at 56 dpi, respectively, $p< 0.05$; **Suppl. Table 2**) but not 3 dpi (**Suppl. Table 3**). Plasma bilirubin, AST, ALT, total protein, albumin, cholesterol, LDL, triglycerides and glucose levels did not differ between ischemic mice exposed to normal diet and protein restriction at any time-point examined (**Suppl. Tables 2, 3**). When compared with non-ischemic, ischemic mice on both diets revealed reduced plasma urea, bilirubin, LDL and glucose levels and increased plasma AST levels at 3 dpi (**Suppl. Table 3**) and increased plasma triglycerides at 56 dpi (**Suppl. Table 2**). No other variables were altered post-ischemia.

Protein restriction promotes post-ischemic motor-coordination recovery and reduces anxiety

Cerebral LDF recordings did not differ between groups during and after MCAO. LDF reproducibly decreased to ~15-20% of baseline values during MCAO, followed by the

restitution to baseline values within 20 minutes after reperfusion (**Figs. 2A** and **3A**). Protein restriction induced an immediate reduction of general (**Figs. 2B** and **3B**) and focal (**Figs. 2C** and **3C**) neurological deficits, which was significant at 3 and 1 dpi, respectively, and persisted until the end of the study. This neurological enhancement was associated with delayed improvements of motor-coordination deficits in the tight rope and Rotarod tests, which became significant at 42 and 35 dpi, respectively (**Fig. 2D and 2E**). The open field test revealed a significant reduction in circling behavior (**Fig. 2F**). Spontaneous motor activity, that is animal speed in the open field test, was not influenced by protein restriction (**Fig. 2G**). The time in the center of the open field test (**Fig. 2H**) and the time in the open segments of the zero maze (**Fig. 2I**) were increased by protein restriction at 42, but not 7 dpi, whereas freezing time in the open field test was reduced (not shown), indicating that protein restriction reduced anxiety.

Protein restriction induces long-term post-ischemic neuroprotection, prevents brain atrophy and reduces microglial activation

Whole brain volume (**Fig. 4A**), striatum volume (**Fig. 4B**) and corpus callosum thickness (**Fig. 4C**) determined by planimetry on cresyl violet stained brain sections were increased by protein restriction at 56 dpi. Likewise, neuronal survival evaluated by NeuN immunohistochemistry in the ischemic striatum was increased by protein restriction at 56 dpi (**Fig. 4D**). These data indicated that the enhanced neurological recovery induced by protein restriction was associated with improved brain tissue survival and remodeling. Indeed, microglial activation assessed by Iba1 immunohistochemistry was reduced by protein restriction at 56 dpi (**Fig. 4E**), whereas reactive astrogliosis examined by GFAP immunohistochemistry was unchanged (**Fig. 4F**)**.**

In view of the rapid improvement of neurological deficits, we also measured brain infarcts on cresyl violet sections at 3 dpi, demonstrating that protein restriction reduced infarct volume (**Fig. 3D**). IgG extravasation, a marker of blood-brain barrier breakdown (**Suppl. Fig. 2A**), microglial activation assessed by Iba1 immunohistochemistry (**Suppl. Fig. 2B**) and brain leukocyte infiltration examined by CD45 immunohistochemistry (**Suppl. Fig. 2C**) were not influenced by protein restriction at 3 dpi.

Protein restriction promotes contralesional pyramidal tract plasticity, but does not influence endogenous neurogenesis

To evaluate whether the motor and coordination recovery induced by protein restriction involved long-distance pyramidal tract plasticity, we next quantified the number of axons labeled with the anterograde tract tracer BDA originating from the contralesional motor cortex as well as the density of axon collaterals branching off the pyramidal tract at midbrain levels in order to cross the midline to innervate the ipsilesional parvocellular red nucleus [10,15]. The density of midline-crossing BDA-labeled fibers (**Fig. 5A, B**), but not the number of BDA-labeled fibers within the contralesional pyramidal tract (**Fig. 5C**) was increased by protein restriction**.** Endogenous cell proliferation and neurogenesis, evaluated by BrdU incorporation analysis and colabeling with the immature neuronal marker doublecortin (Dcx) and mature neuronal marker NeuN, were not influenced by protein restriction, neither in the tissue adjacent to the subventricular zone, nor the ipsilesional striatum (**Suppl. Fig. 3A-F**).

Protein restriction downregulates metabolism-related and inflammatory genes in the brain and elevates glutathione peroxidase-3 in the brain and liver

Real-time qPCR showed that protein restriction downregulated mRNAs for the NAD-dependent deacetylase *Sirt1*, which controls a large set of metabolism-related genes partly by transcriptional regulation [17], its downstream target *insulin-like growth factor-1* (*IGF1*), which has insulin-like properties [18], and the proinflammatory cytokine *interleukin-1* (*IL1β*), which is strongly expressed by activated M1 microglia [19], in the ischemic brain, but did not influence *superoxide dismutase-1* (*Sod1*) mRNA, which encodes a dismutase degrading superoxide anions to hydrogen peroxide [20], and *Gpx3* mRNA, which encodes a peroxidase degrading hydrogen peroxide [20] (**Table 1**). (**Fig.6A**), as was the reactive α, β-unsaturated aldehyde *4HNE* (**Fig. 6B**), a product of phospholipid peroxidation [21], while Gpx3 protein was significantly increased by protein restriction (**Fig. 6C**).

In the liver, no significant changes of mRNAs for *Sirt1* and Sirt1's downstream targets *IGF1*, *nuclear factor-κB* (*NFκB*), *Sod1*, *Sod2* and *Gpx3* were found (**Table 2**). Gpx3 protein, but not Sirt1 protein or 4HNE, was significantly increased by protein restriction in the liver, as revealed by Western blots (**Fig. 6D-F**).

Discussion

We herein show that moderate protein restriction, initiated immediately after intraluminal MCAO, induces sustained post-ischemic neurological recovery that persists up to 8 weeks after stroke and is accompanied by increased long-term neuronal survival in the peri-infarct tissue, reduced microglial activation, reduced whole brain, striatal and corpus callosum atrophy, and increased contralesional pyramidal tract plasticity at the level of the red nucleus. Neurogenesis adjacent to the SVZ and in the ischemic striatum was not influenced by protein restriction. On the molecular level, an elevation of the peroxidase Gpx-3 was found in the brain and liver of mice exposed to protein restriction, whereas *Sirt1* and *IGF1* mRNAs, markers of metabolic demand and adaptation [17, 18], were reduced, as was the mRNA for the pro-inflammatory cytokine *IL1β.* Protein restriction-induced brain tissue protection occurred rapidly. A reduction of infarct volume was noted already 3 days after MCAO. The nutrition status of animals exposed to moderate protein restriction was not compromised. Besides a moderate reduction in plasma urea, an established marker of protein consumption, no plasma changes of metabolic variables were found. Plasma bilirubin, AST, ALT, total protein, albumin, cholesterol, LDL, triglycerides and glucose levels did not differ between mice exposed to normal diet and protein restriction. Total calorie intake, body weight and BMI over 8 weeks were not compromised.

Our data complement findings following moderate protein restriction induced by controlled diets containing 7% soybean protein or 8% casein, which, when administered during pregnancy and lactation to rats, improved cognitive function, evaluated by an inhibitory avoidance task, and reduced brain atrophy of offsprings exposed to unilateral cerebral hypoxia-ischemia [5], and, when administered for 30 days to adult mice, reduced global and focal neurological deficits, infarct volume, brain edema, blood-brain barrier permeability and brain leukocyte infiltration following subsequent focal cerebral ischemia induced by intraluminal MCAO [6], respectively. The previous studies differ from the present study in that nutrition modifications were imposed prior to unilateral global or focal cerebral ischemia. Exceeding the earlier intraluminal MCAO study in mice [6], we herein performed a comprehensive analysis of post-acute motor-coordination recovery, long-term brain tissue survival, endogenous neurogenesis and lesion-remote pyramidal tract connectivity, providing for the first time evidence that protein restriction induces sustained brain tissue

survival and remodeling associated with contralesional motor cortical plasticity which contributes to the neurological recovery we noticed.

Only single studies examined consequences of post-ischemic food protein modification. In a model of global forebrain ischemia induced by two-vessel occlusion in rats, a severe protein restriction induced by a diet containing only 2% protein (also casein), when initiated at 3 dpi, reduced hippocampal CA3 mossy fiber content of the synaptic proteins growth associated protein-43, synaptophysin and synaptosomal-associated protein-25 at 21 dpi [3]. Hippocampal CA1 neuronal survival, microglial activation and astroglial reactivity were not influenced in this study. Neurological recovery was not evaluated. Likewise, a severe protein restriction induced by a diet containing 0.5% protein altered lipid composition and membrane fluidity of neurons in the motor cortex after focal cerebral ischemia induced by photothrombosis at 32 dpi, which was associated with forelimb dysfunction, motor-coordination and posture impairment [22, 23]. The reduction of food protein to only 0.5 or 2%results in a reduction of food ingested, since the animals refuse this chow [1, 2, 4]. In these animals, combined protein-energy malnutrition is noted. Indeed, rats exhibited a pronounced weight reduction of ~35% within 21 dpi and ~40% within 32 dpi in these earlier studies, associated with an atypical acute phase response in the blood characterized by increased α2-macroglobulin, reduced haptoglobin and reduced albumin [3, 23]. Pre-ischemic exposure of rats or gerbils to diets containing 0.5-2% protein for 4 weeks in models of global [1, 2] or focal [4] cerebral ischemia aggravated motor and cognitive deficits in ladder walking, cylinder and open field tasks, increased NFκB activation and increased protein thiol formation that was used as marker of oxidative stress. Neuronal injury was not influenced in these studies.

On the molecular level, animals exposed to moderate protein restriction exhibited metabolism-related, anti-inflammatory and anti-oxidant responses in the brain and liver, which likely contributed to the neurological recovery. Thus, *Sirt1* mRNA, which encodes a NAD-dependent deacetylase that stabilizes mitochondrial function and metabolism partly by deacetylating the transcription regulator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) [17], was reduced in the peri-infarct brain tissue at 56 dpi, as was Sirt1's transcriptional target *IGF1* mRNA, which encodes a growth factor with insulin-like properties [18], and *IL1β* mRNA, which encodes a pro-inflammatory cytokine strongly expressed by activated M1 microglia [19]. Gpx3, a peroxidase degrading hydrogen peroxide [20], was increased in the peri-infarct brain tissue and liver of mice exposed to protein

restriction. Following moderate pre-ischemic protein restriction, reduced *IL1β* mRNA expression and increased Gpx3 abundance have previously already been described in the brains of mice exposed to intraluminal MCAO [6]. In this earlier study, *Sirt1* mRNA and Sirt1 protein were increased in ischemic brain tissue, suggesting that pre-ischemic and post-ischemic protein restriction influences Sirt1 expression in different ways. A neuroprotective role of Gpx3 has previously been shown in $Gpx3^{-/-}$ mice, which exhibited excessive platelet activation and thrombus formation and developed enlarged brain infarcts when exposed to transient intraluminal MCAO [24].

Strengths of our study are the well-controlled nutritional assessments and the use of a broad battery of neurological tests, which we combined with rigid structural volumetry/ planimetry, immunohistochemical and molecular studies. A particularly noteworthy observation in this study was the rapid effect of protein restriction on neurological recovery and brain injury, which already became significant at 1 to 3 dpi. These findings indicate that dietary modifications have profound consequences for brain injury and remodeling, which have been underestimated in the past. Using the same model, that is, intraluminal MCAO in mice, we have previously shown that restorative effects of cell-based therapies [25], growth factors [10, 15], NMDA antagonists [26] and $GABA_A$ antagonists [9] develop much more slowly over periods of up to six weeks, depending on the therapeutic strategy evaluated. We predict that by modifying food composition, we may enhance stroke recovery much more efficiently than using pharmacological therapies. Further mechanistic studies are warranted.

Author contributions

TSC and DMH designed the study. TSC performed the animal experiments, assisted by EHSM and MS. TSC, VS, ARSM, LMNM, ED and MS conducted histochemical and molecular biological studies. DMH and CK provided infrastructural support. TSC, EHSM, VS, ARSM, LMNM, ED, and DMH analysed the data. TSC and DMH drafted the manuscript. All authors finalized it.

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Compliance with ethical standards

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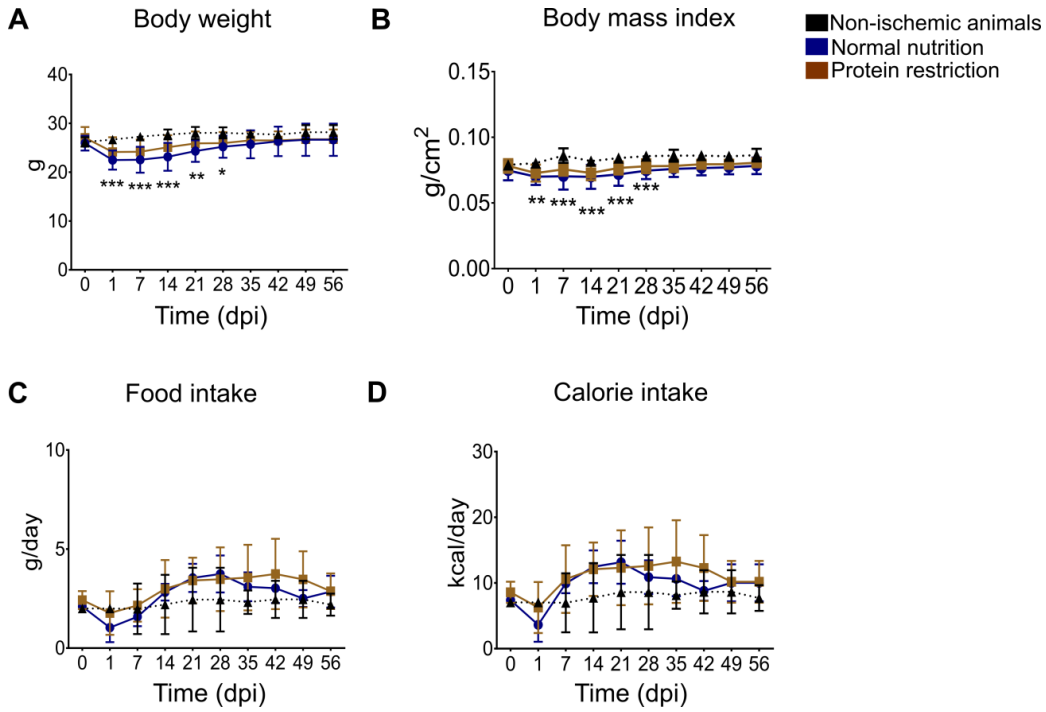

Figure 1. Mild protein restriction does not induce major murinometric changes. **(A)** Body weight, **(B)** body-mass index (BMI), **(C)** daily food intake and **(D)** daily calorie intake in mice exposed to intraluminal middle cerebral artery occlusion (MCAO) fed *ad libitum* with a normal diet (20% protein) or a protein-reduced diet (8% protein) for 56 days starting with the induction of MCAO. Note the reduction of food intake in both groups in the first week after MCAO. No significant group differences were noted. Data are means ± S.D. ***p<0.001/**p<0.01/*p<0.05 compared with pre-ischemic baseline (n=12 mice/ group).

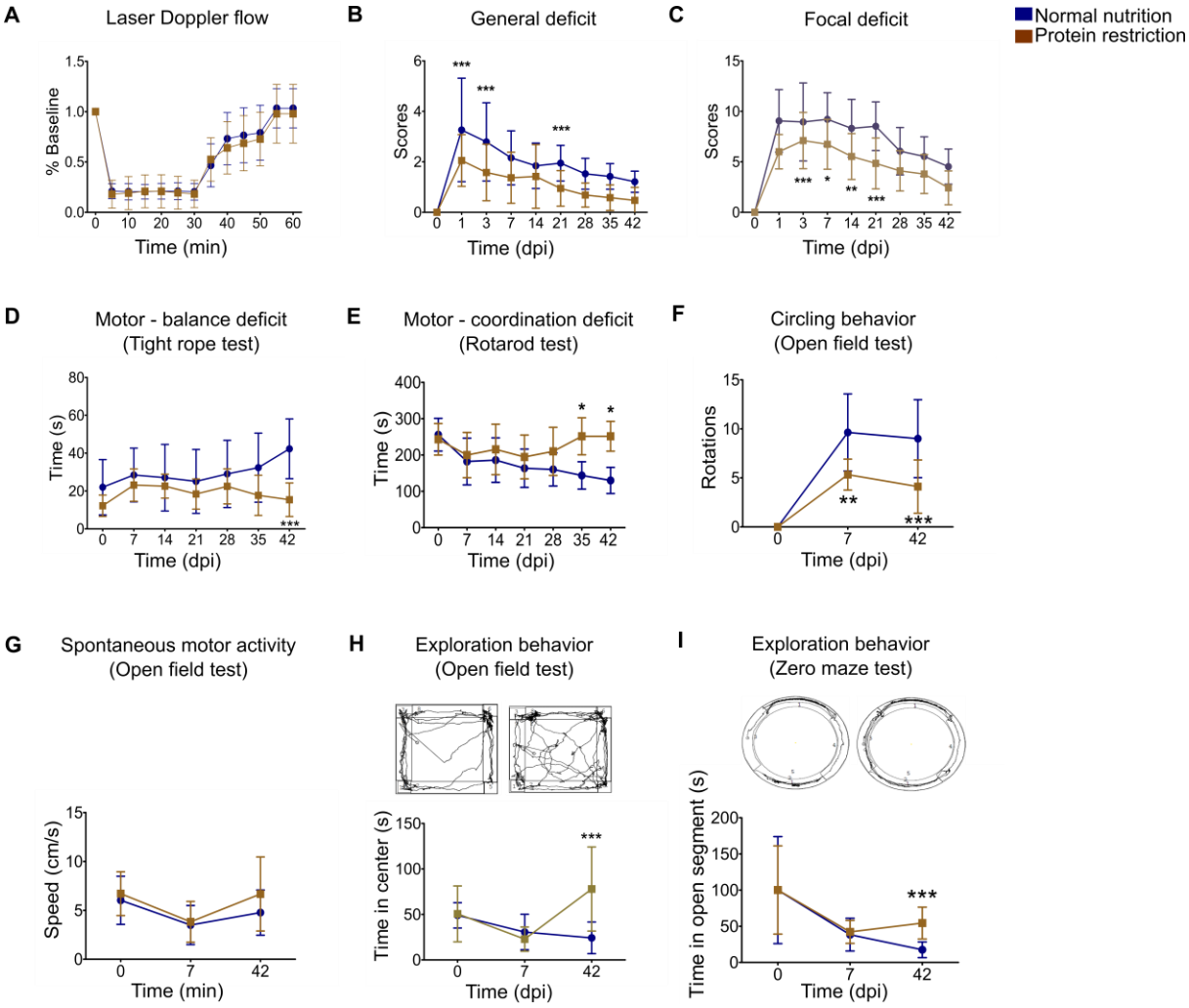

Figure 2. Protein restriction promotes post-ischemic motor-coordination recovery and reduces anxiety. (A) Laser Doppler flow (LDF) recordings above the core of the middle cerebral artery territory, **(B, C)** general and focal neurological deficits examined by the Clark score, **(D, E)** motor-coordination deficits analysed by tight rope and Rotarod tests, **(F)** circling behavior, assessed by open field test, **(G-I)** spontaneous motor activity and exploration behavior, that is, mean speed in the open field test, time in the center of the open field test and time in the open segments of the zero maze (the latter two variables are markers of anxiety),of mice receiving intraluminal middle cerebral artery occlusion (MCAO) followed by exposure to normal nutrition or protein restriction for 56 days. Data are means ± S.D. ***p<0.001/**p<0.01/*p<0.05 compared with normal nutrition (n=12 animals/group).

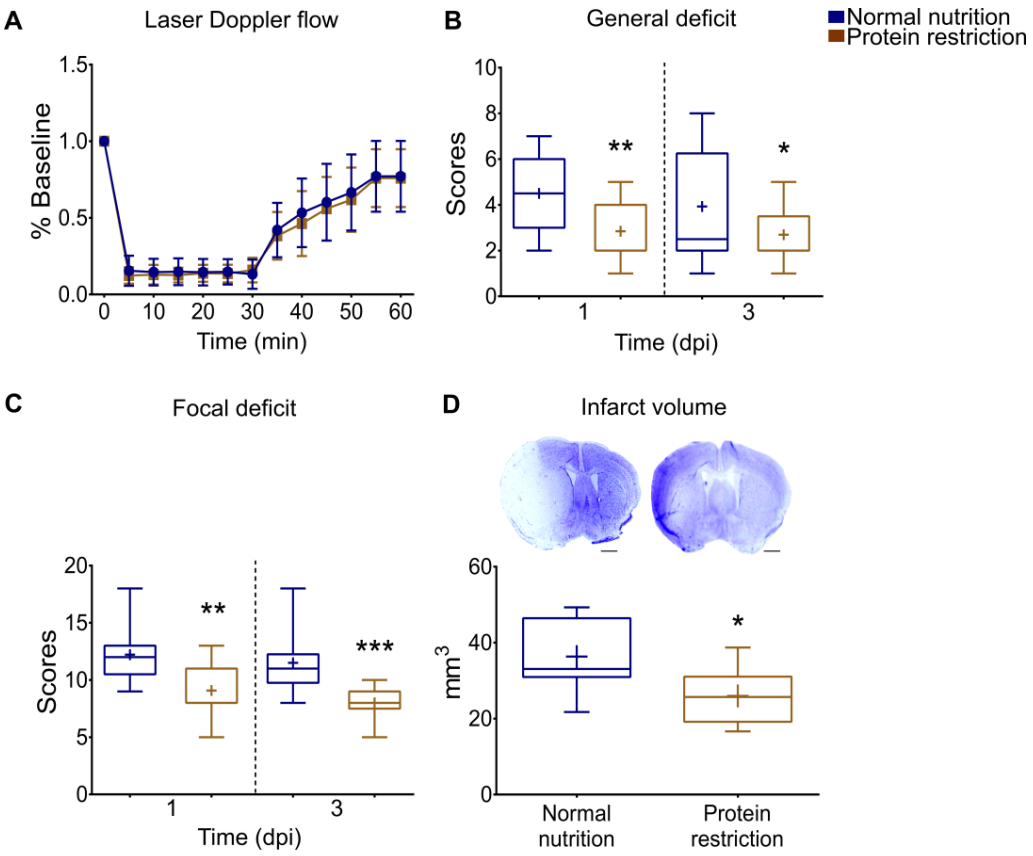

Figure 3. Protein restriction reduces neurological deficits and decreases infarct volume in the acute stroke phase. (A) Laser Doppler flow (LDF) recordings above the core of the middle cerebral artery territory, **(B, C)** general and focal neurological deficits evaluated by the Clark score and **(D)** infarct volume outlined on cresyl violet-stained brain sections of mice receiving intraluminal middle cerebral artery occlusion (MCAO) followed by exposure to normal nutrition or protein restriction for 3 days. Representative microphotographs are shown. Data are means ± S.D. in **(A)** or medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers **(B-D)**. ***p<0.001/ **p<0.01/ *p<0.05 compared with normal nutrition (n=12 animals/ group).

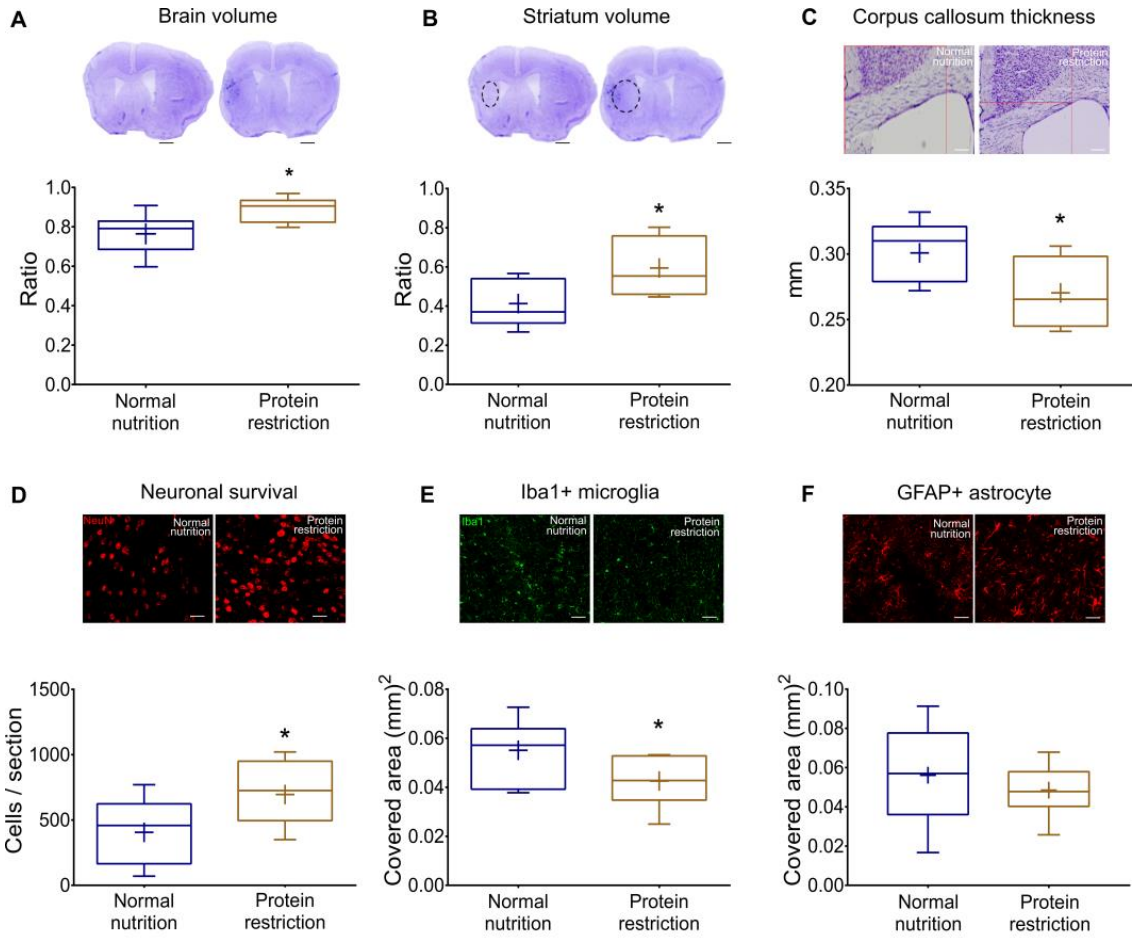

Figure 4. Protein restriction induces long-term neuroprotection, prevents brain atrophy and reduces microglial activation in the post-acute stroke phase. (A) Whole brain volume, **(B)** striatum volume and **(C)** corpus callosum thickness evaluated on cresyl violet stained brain sections, **(D)** neuronal survival in the peri-infarct striatum assessed by NeuN immunohistochemistry, **(E)** microglial activation in the peri-infarct striatum assessed by Iba1 immunohistochemistry and **(F)** astroglial immunoreactivity in the peri-infarct striatum assessed by GFAP immunohistochemistry of mice receiving intraluminal middle cerebral artery occlusion (MCAO) followed by exposure to normal nutrition or protein restriction for 56 days. Representative microphotographs are shown. Data are medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers. **p<0.01/ *p<0.05 compared with normal nutrition (n=12 mice/ group).

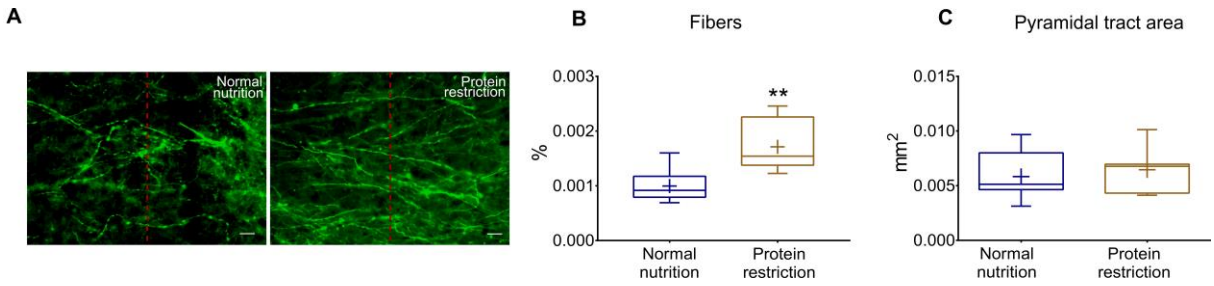

Figure 5. Protein restriction promotes contralesional pyramidal tract plasticity. **(A, B)** Biotinylated dextran amine (BDA)-labeled midline-crossing axon collaterals originating from the contralesionalmotor cortex and **(C)** descending axons inside the contralesional pyramidal tract at the level of the red nucleus of mice receiving intraluminal middle cerebral artery occlusion (MCAO) followed by exposure to normal nutrition or protein restriction for 56 days. Data are medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers. *p<0.05 compared with normal nutrition (n=12 mice/ group).

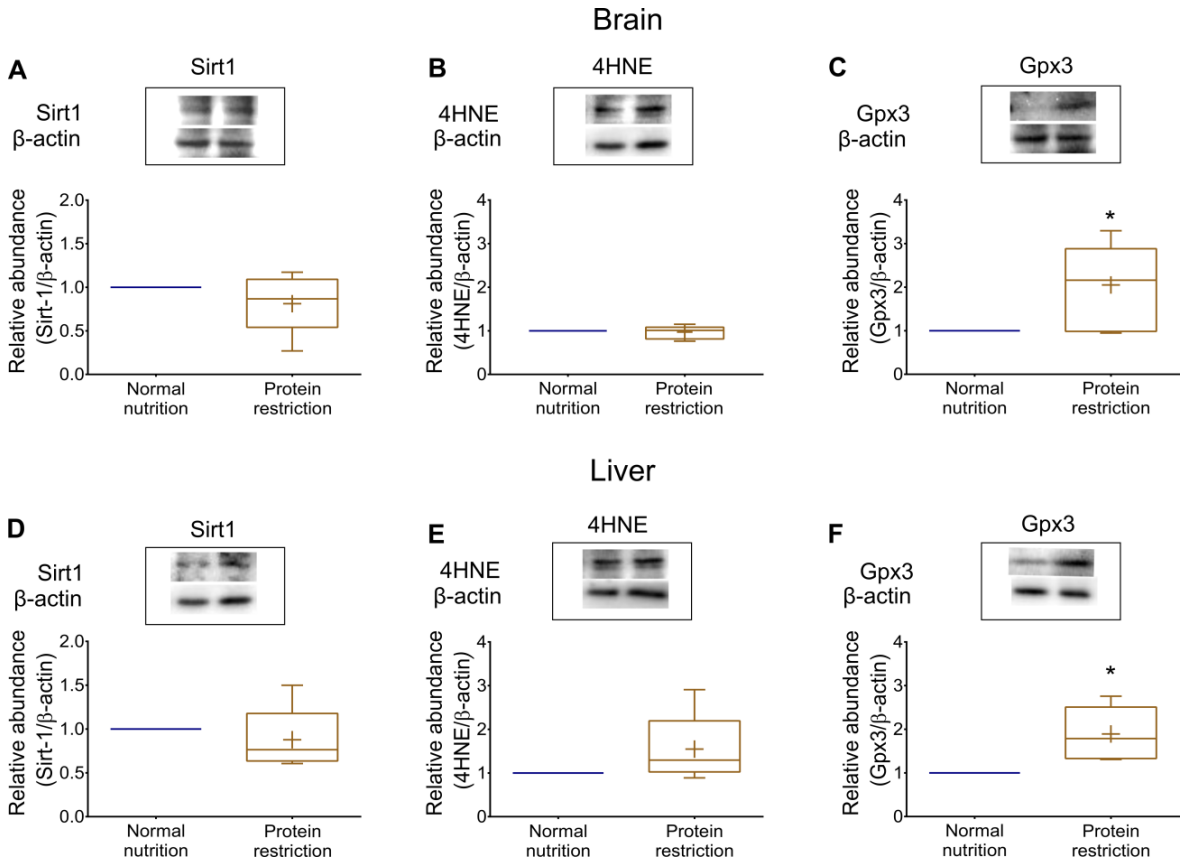

Figure 6. Protein restriction elevates glutathione peroxidase-3 protein in the ischemic brain and liver. Western blot analysis of **(A, D)** sirtuin-1 (Sirt1), **(B, E)** the reactive aldehyde 4-hydroxy-2-nonenal and **(C, F)** glutathione peroxidase-3 (Gpx3) in **(A-C)** the ischemic brain and **(D-F)** the liver of mice exposed to normal nutrition and protein restriction for 56 days. Representative Western blots are also shown. Data are medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers. *p<0.05 compared with normal nutrition (n=6mice/ group).

Table 1. Expression of metabolism-related, inflammatory and anti-oxidant, genes in the brain of mice exposed to protein restriction

Groups	***Sirt1***	***IGF1***	***IL1β***	***Sod1***	***Gpx3***
Normal nutrition	3.42 ± 1.04	1.51 ± 0.50	2.44 ± 0.57	2.00 ± 0.37	3.19 ± 0.41
Protein restriction	1.64 ± 0.52*	0.46 ± 0.23**	1.84 ± 0.68*	1.75 ± 0.44	2.55 ± 1.37

Data are fold changes, expressed as mean ± S.D., evaluated at 56 dpi. **p<0.01/*p<0.05 compared with normal nutrition (n=6 mice/ group).

Table 2. Expression of metabolism-related, inflammatory and anti-oxidant genes in the liver of mice exposed to protein restriction

Groups	***Sirt1***	***IGF1***	***NFκb***	***Sod1***	***Sod2***	***Gpx3***
Normal nutrition	1.51 ± 0.37	1.15 ± 0.24	0.99 ± 0.25	0.89 ± 0.18	1.00 ± 0.10	0.88 ± 0.18
Protein restriction	1.71 ± 0.39	0.97 ± 0.12	0.96 ± 0.23	0.96 ± 0.32	1.03 ± 0.29	1.30 ± 0.60

Data are fold changes, expressed as mean ± S.D., evaluated at 56 dpi. No significant group differences were found (n=6 mice/ group).

7 DISCUSSION AND CONCLUSION

Undernutrition (energy undernutrition and protein-energy undernutrition) can transiently compensate previous excessive calorie intake and metabolism, resulting in neuroprotection in a circumscribed time window after focal cerebral ischemia. However, moderate protein restriction effectively induces longer-lasting post-stroke neuroprotection and brain remodeling, presumably by increasing antioxidant activity. As a common feature in our studies, all pre-ischemic dietary interventions improved neurological deficits, reduced brain injury and BBB permeability, increased neuronal survival and antioxidant activity, and reduced anti-inflammatory responses after stroke. In animals exposed to undernutrition, neuroprotection was dissociated from anti-inflammatory effects. Moderate protein restriction on the other hand induced motor improvements, reduced anxiety, brain remodeling, and neuroplasticity closely associated with its anti-inflammatory effects, but failed to promote endogenous neurogenesis. Mechanistically, the antioxidant activity most closely correlated with the beneficial effects of all 3 diets.

Some experimental studies already reported the efficacy of specific nutrients to repair brain dysfunction, however just a few of them focused on the contribution of nutrition for functional stroke recovery [94]. In the present thesis, we performed a comprehensive analysis of different dietary restriction protocols, evaluating two major conditions: (i) the impact of dietary restriction and energy depletion before stroke and (ii) long-term post-ischemic moderate protein restriction. In the pre-ischemic studies, our analysis showed that dietary restriction can prepare the brain against subsequent cerebral ischemia, but in case of calorie restriction this effect was limited to a short period in which metabolism was still balanced. On the other hand, post-ischemic moderate protein restriction avoided energy depletion and metabolism breakdown and supported neurological recovery after stroke. This recovery promoting effect had previously not been shown.

It is well known that severe undernutrition worsens motor deficits after stroke [21, 38]. This occurs because severe undernutrition itself alters muscle structure and strength during catabolism and this is an important predictor of motor dysfunction after stroke [19]. Interestingly, in our studies, all 3 diets induced motor improvements, but the different interventions showed that (i) a pre-ischemic undernutrition reduces neurological deficits solely in a narrow time window (14 days); (ii) a pre-ischemic and post-ischemic moderate protein restriction has a more persistent effect on neurological recovery and (iii) a post-ischemic moderate protein restriction improved

motor recovery to up to 56 days after stroke. We believe that prolonged undernutrition failed to maintain the neurological deficit improvement due to the advanced exhaustion of energy stores, resulting in a catabolic state associated with cerebral hypoperfusion. Indeed, post-ischemic reperfusion evaluated by laser Doppler flowmetry was compromised in the latter animals. In contrast, we hypothesize that moderate protein restriction can preserve the anabolic state of the brain tissue, protecting the brain´s function, inducing neuronal survival and enhancing brain plasticity. Aquilani et al (2011) suggest that the neuroprotective effect of moderate protein intake is related to the usage of amino acids as a fuel in the Krebs cycle (alternative to glucose), maintaining ATP production and reducing free radical formation in ischemic injury [9]. Moderate protein restriction can also reduce brain edema [23]. On the other hand, severe protein-energy undernutrition has opposite effects, increasing neuronal degeneration and brain injury [11-15, 18, 20-23].

Based on the literature, the effect of post-ischemic dietary restriction on brain injury is still poorly investigated. In our studies, we found that post-ischemic moderate protein restriction mimics its pre-ischemic effects. Up to 56 days, this diet reduced brain, striatum and corpus callosum atrophy, increased neuronal survival and pyramidal tract remodeling, but failed to induce endogenous neurogenesis. The literature suggests that a moderate protein intake increases Brain-derived neurotrophic factor (BNDF) activity and upregulates genes related to dendrite morphogenesis and neuronal repair, explaining the positive effects of this diet on brain injury and neuroplasticity [95, 96]. Recently, some reports have revealed that severe protein restriction has opposite effects, reducing axon terminal markers such as growth associated protein-43 (GAP43), synaptophysin and synaptosome-associated protein-25 (SNAP25) and enhancing the tropomyosin receptor kinase B (TrkB) response [23, 97]. These results of the latte studies could not comprehensively be interpreted, because functional outcome was not measured in these studies. Another major effect of severe undernutrition is altering membrane fluidity and fatty acid profile in mature cortical neurons, thereby decreasing neuronal activity in motor areas [38]. These unfavorable effects may have contributed to motor impairments induced by severe undernutrition.

Recently, proteins with metabolic sensor property such as Sirt1 and IGF1 have been targeted to decrease brain injury, to improve functional performance and to promote neuroplasticity after stroke [17, 55, 98-100]. Previous studies showed that

calorie restriction increases the synthesis of Sirt1 and IGF1 proteins, thus suggesting a possible mechanism via which calorie restriction can protect the brain from focal cerebral ischemia [12, 17]. In our study, moderate protein restriction regulated Sirt1 and IGF1, increased *Gpx3* and reduced *IL1β* in the brain and the liver, but failed to stimulate post-stroke endogenous neurogenesis. Sirt1 and IGF1 have previously been shown to induce axonal growth via protein kinase B (Akt) and glycogen synthase kinase-3 (GSK3), neurite outgrowth via mammalian target of rapamycin (mTOR) and dendritic arborization by way of Rho associated kinase (ROCK), to regulate differentiation between neural stem cells (NSCs) and NPCs and to participate in axonal regeneration and sprouting [55, 78] .Our study for the first time demonstrated increased long-distance axonal plasticity induced by moderate protein restriction, which based on our studies promotes neurological recovery in a particularly potent way.

It is well known that severe undernutrition modifies the brain´s inflammatory response due to changes in acute-phase proteins, serum cortisol, astrogliosis, and NFκB activity [19, 22, 23, 31, 38, 97]. After cerebral ischemia, severe undernutrition increased secondary neuroinflammation, worsening the stroke outcome [19, 21]. In our studies, we demonstrated that pre-ischemic dietary restriction reduced the expression of inducible NO synthase, leukocyte infiltration, microglial activation, brain *IL1β* and *NFκb* mRNA expression. Similarly, post-ischemic moderate protein restriction reduced astrogliosis, urea nitrogen (hemodynamic regulator), and brain *IL1β* mRNA expression over up to 56 days. Interestingly, we showed that pre-ischemic interventions induced short-term (7 days) and long-term (30 days) anti-inflammatory responses. Our data resemble previous studies using mild calorie restriction after cerebral ischemia, in which the authors found less microgliosis and reduced gene expression of *IL1β*, *IL6*, *Tnf-α*, *CXC-motif ligand-1* (*Cxcl1*) and *ICAM1* mRNA in the brain. The beneficial effects of calorie restriction were explained by lower levels of acute phase proteins and by activation of chaperones [22]. We were the first study to systematically describe the time-course of anti-inflammatory markers in ischemic animals exposed to undernutrition.

Following ischemia, increased ROS production and reduced antioxidant activity are noted [101]. If accompanied by undernutrition, catabolic processes can boost oxidative stress, thus challenging stroke recovery [1]. In the present thesis, we systematically investigated oxidative responses in the brain and liver. We observed that even upon progressive metabolic exhaustion, pre-ischemic undernutrition

reregulated pro- and antioxidant markers (*Sod1*, *Sod2*, *Gpx3* and *Nox4*) in both organs. Furthermore, moderate protein restriction boosted the antioxidant Gpx3 activity in the brain and liver. The upregulation of Gpx3 is an important finding, because this antioxidant inhibits platelet activation and thrombus formation and reduces brain infarction, being a potential target for stroke therapies [102]. In global ischemia, intermittent fasting stimulated Sod2, catalase and HO1 in ischemic brain areas, but beneficial effect disappeared when more severe undernutrition was imposed [15, 18, 103]. The above studies lacked information about oxidative responses in the liver. Changes in antioxidant activity are a very plausible mechanism via which dietary restriction enhance stroke recovery.

The clear strength of this study is the use of a well-controlled and systematic experimental design supported by functional, histological and molecular data. We aimed to understand the effects of both pre- and post-ischemic dietary restriction on acute brain injury, functional recovery, brain remodeling and plasticity. Each of the 3 studies was adequately powered to detect protective effects with 80% statistical power (1 - β-error) and 5% α-error. Our studies point out the great potential of nutritional interventions for promoting clinical stroke recovery. We predict that by modify food intake, we might enhance stroke recovery more efficiently than by pharmacological tools [76, 104-106]. Further mechanistic studies connecting nutrition and stroke recovery are needed.

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infarct tissue remodeling, and contralesional brain plasticity. *J Cereb Blood Flow Metab*. 2017;37:980-993

9 Supplementary material

9.1 Supplementary material (Study 1)

Running title: Neuroprotection by energy and protein-energy undernutrition

Author's contributions:

Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%
Contributed to the acquisition of the data:
Animal experiment: TSC – 100%
Histochemical analysis: TSC – 90% / MS, ED – 10%
Molecular analysis: TSC – 70% / EHSM, LMNM, ARSM – 30%
Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 30% / EHSM, LMNM, ARSM, ED – 10%
Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%
Provided final approval of the version to publication: TSC – 50% / DMH – 50%
Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TSC – 50% / DMH – 50%
Authors' contributions:
Tayana Silva de Carvalho and Dirk M. Hermann designed the study. Tayana Silva de Carvalho performed the animal experiments, assisted by Eduardo H. Sanchez-Mendoza and Maryam Sardari. Tayana Silva de Carvalho, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Maryam Sardari conducted histochemical and molecular biological studies. Dirk M. Hermann and Christoph Kleinschnitz provided infrastructural support. Tayana Silva de Carvalho, Eduardo H. Sanchez-Mendoza, Luiza M. Nascentes, Adriana R. Schultz Moreira, Egor Dzyubenko and Dirk M. Hermann analyzed the data. Tayana Silva de Carvalho and Dirk M. Hermann drafted the manuscript. All authors concluded it.
Journal: Translational Stroke Research
Impact factor: 8.266 (2018)

Neuroprotection induced by energy and protein-energy undernutrition is phase-dependent after focal cerebral ischemia in mice

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Running title: Neuroprotection by energy and protein-energy undernutrition

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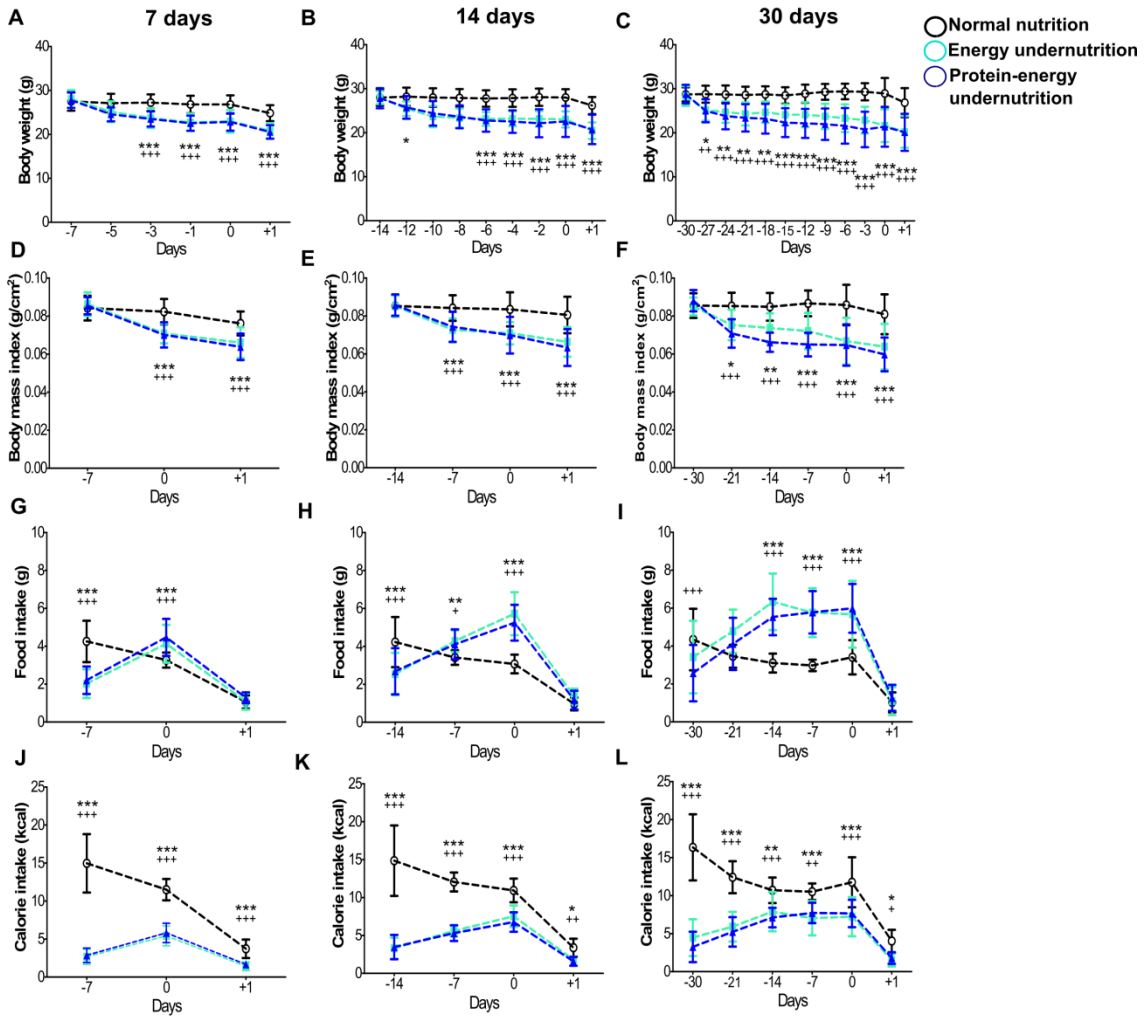
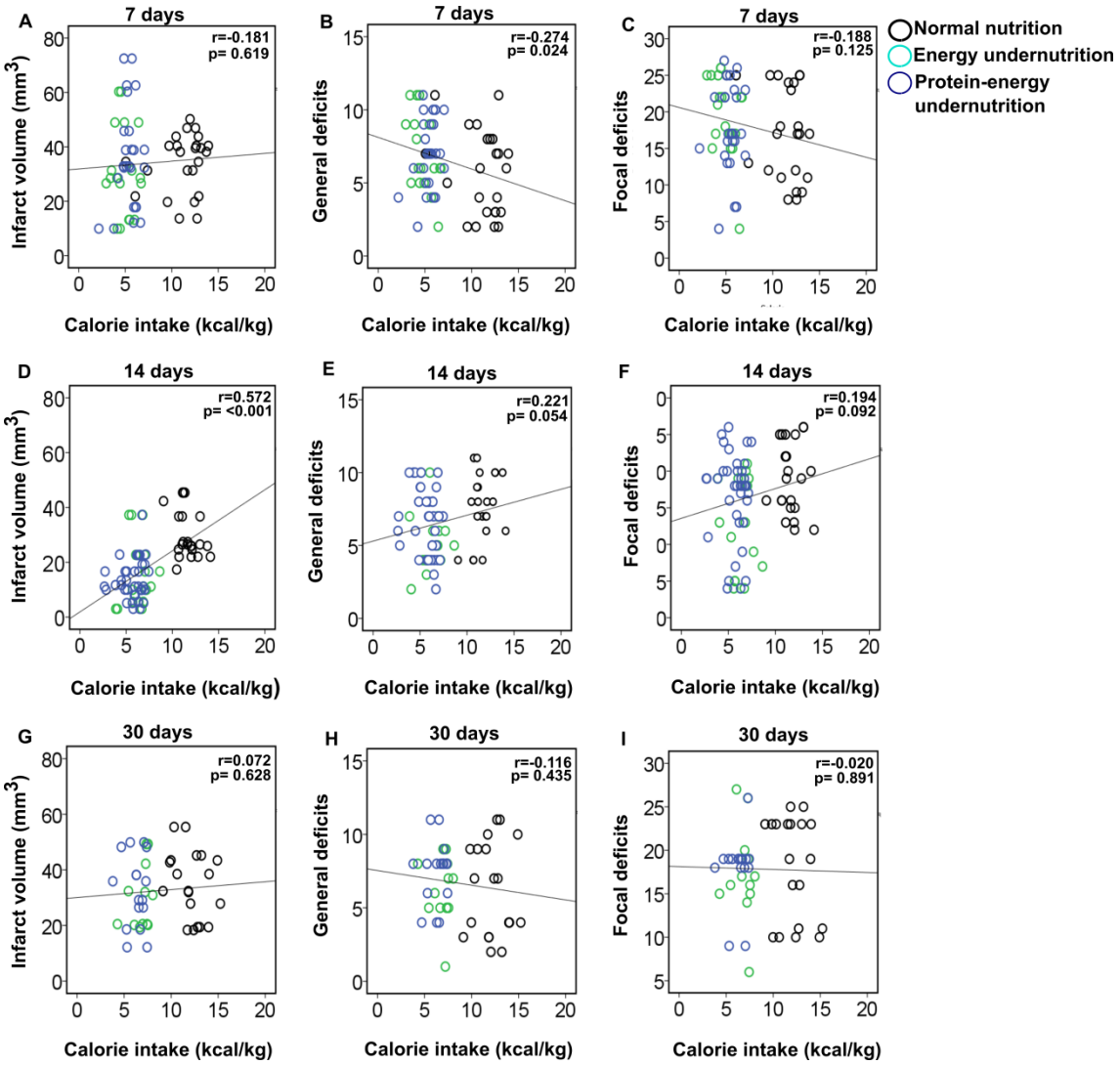
Supplemental Figures:



Supplemental Figure 1. Energy and protein-energy undernutrition induce weight loss despite increased food intake. (A-C) Body weight, **(D-F)** body-mass index (BMI), **(G-I)** daily food intake, **(J-L)** daily calorie intake in mice receiving normal nutrition, energy-reduced nutrition or protein-energy-reduced nutrition for 7 days **(A, D, G, J)**, 14 days **(B, E, H, K)**or 30 days **(C, F, I, L)**, followed by 30 minutes intraluminal MCAO and 24 hours reperfusion. The x-axis reflects the time-point with respect to tMCAO (=day 0). ***p<0.001 for energy undernutrition compared with normal nutrition/ $^{+++}$p<0.001 for protein-energy undernutrition compared with normal nutrition (n=12 animals/ group).

Supplemental Figure 2. Energy and protein-energy undernutrition induce stool changes indicative of malabsorption syndrome. Representative stool samples in animals exposed to **(A, D, G)** normal nutrition, **(B, E, H)** energy **under**nutrition and **(C, F, I)** protein-energy **under**nutrition over 7 days **(A-C)**, 14 days **(D-F)** or 30 days **(G-I)**. Stool samples were collected on the last day prior to MCAO.



Supplemental Figure 3. Correlations of daily calorie intake with infarct volume and neurological deficits. Pearson's correlations of daily calorie intake with **(A, D, G)** infarct volume, **(B, E, H)** general neurological deficits and **(C, F, I)** focal neurological deficits in animals exposed to normal nutrition, energy **under**nutrition and protein-energy **under**nutrition for 7 days **(A-C)**, 14 days **(D-F)** or 30 days **(G-I)**, followed by 30 minutes intraluminal MCAO and 24 hours reperfusion. Correlation coefficients (r), p values and univariate regression lines are shown. Note the negative correlation of calorie intake with general neurological deficits 7 days after food manipulation and the positive correlation of calorie intake with infarct volume 14 days after food manipulation.

Supplemental Tables:

Supplemental Table 1. List of PCR primers

Primer	Sequence (5'->3')		Tm	G-C (%)	Gene bank number
Sirt1	Forward	GATGACAGAACGTCACACGC	59.56	55.00	NM_019812.3
	Reverse	ATTGTTCGAGGATCGGTGCC	60.46	55.00	
IGF1	Forward	GACTCAGAAGTCCCCGTCCC	61.61	65.00	NM_010512.5
	Reverse	GCATTTTCTGCTCCGTGGG	59.49	57.89	
Insr	Forward	ACCTTCTCTGATGAACGGCG	60.11	55.00	NC_000074.6
	Reverse	CTGATATGGGATCCAGGGGG	58.71	60.00	
Glut1	Forward	GTTAATCGCTTTGGCAGGCGG	62.78	57.14	NM_011400.3
	Reverse	AGCATCTCAAAGGACTTGCCC	60.62	52.38	
Glut2	Forward	GTGCTGCTGGATAAATTCGCC	60.27	52.38	NM_031197.2
	Reverse	TCAGCAACCATGAACCAAGGG	60.82	60.82	
IL1β	Forward	TCTTTGAAGTTGACGGACCCC	60.20	52.38	NC_000068.7
	Reverse	CTTGTTGATGTGCTGCTGCG	60.73	55.00	
NFκb	Forward	TTTCGACTACGCAGTGACGG	60.39	55.00	NM_008689.2
	Reverse	GCTAAGTGTAAGACACTGTCCC	58.41	50.00	
Nox4	Forward	CCTGCTCATTTGGCTGTCCC	59.96	50.00	NM_015760.5
	Reverse	GCTTAAACACAATCCTAGGCCC	59.97	55.00	
Sod1	Forward	CATCCACTTCGAGCAGAAGGC	61.34	57.14	NM_011434.1
	Reverse	GGTACAGCCTTGTGTATTGTCCC	61.18	52.17	
Sod2	Forward	GAACAACAGGCCTTATTCCGC	61.32	60.00	NM_013671.3
	Reverse	GTGTATCTTTCAGTAACATTCTCCC	59.31	50.00	
Gpx3	Forward	GCACTACAAGAAGAACTTGGGC	59.77	50.00	NM_001329860.1
	Reverse	TCGAACATACTTGAGACTGGGG	59.50	50.00	
Cat	Forward	TGGTATAAGACGCATCAGAAGCC	60.49	47.83	NC_000071.6
	Reverse	GGTACTCCTCACTGAACATGCG	60.99	54.55	
βGluc	Forward	TGGTATAAGACGCATCAGAAGCC	60.49	47.83	NC_000071.6
	Reverse	GGTACTCCTCACTGAACATGCG	60.99	54.55	

Supplemental Table 2. General observations in mice exposed to energy and protein-energy undernutrition

Clinical observations	Stool size (mm)	Stool color loss (%)	Stool blood beddings (%)	Mild hypoactivity (%)
7 days				
Normal nutrition	3.9±1.1	0	0	0
Energy undernutrition	7.0±0.8***	100	0	7.1
Protein-energy undernutrition	6.6±0.9***	100	7.1	21.4
14 days				
Normal nutrition	2.9±0.3	0	0	0
Energy undernutrition	7.4±0.5***	100	5.6	22
Protein-energy undernutrition	8.9±2.0***	100	5.6	5.6
30 days				
Normal nutrition	3.4±0.6	0	0	0
Energy undernutrition	9.1±1.2***	100	15.4	38.5
Protein-energy undernutrition	14.3±1.3***	100	30.8	54

***p<0.001 compared with corresponding normal nutrition.

Supplemental Table 3. Plasma lipid and glucose levels in mice exposed to energy and protein-energy undernutrition

	Cholesterol (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)	Glucose (mg/dl)
7 days				
Normal nutrition	244.0±94.0	30.4±20.0	216.0±142.0	89.4±51.0
Energy undernutrition	253.0±108.0	25.3±18.0	224.0±113.0	84.4±40.3
Protein-energy undernutrition	219.4±53.0	11.6±3.3*****	197.0±69.0	77.1±25.7
14 days				
Normal nutrition	244.5±130.3	22.5±23.0	225.0±155.9	115.0±53.7
Energy undernutrition	208.0±99.1	20.0±21.5	244.0±126.1	97.5±52.0
Protein-energy undernutrition	270.0±110.1	25.5±22.5	256.0±107.7	100.0±27.7
30 days				
Normal nutrition	252.1±120.0	32.7±29.7	259.3±147.2	97.7±46.0
Energy undernutrition	317.8±101.0	30.5±17.3	333.3±113.7	73.6±50.0
Protein-energy undernutrition	218.6±94.8	20.7±13.0	198.6±130.4	85.0±23.5

*$p<0.05$ compared with corresponding normal nutrition.

9.2 Supplementary material (Study 2)

Running title: Protein restriction protects against focal cerebral ischemia

Author's contributions:

Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%

Contributed to the acquisition of the data:

Animal experiment: TSC – 100%

Histochemical analysis: TSC – 90% / MS, ED – 10%

Molecular analysis: TSC – 70% / EHSM, LMNM, ARSM – 30%

Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 30% / EHSM, LMNM, ARSM, ED – 10%

Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%

Provided final approval of the version to publication: TSC – 50% / DMH – 50%

Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TSC – 50% / DMH – 50%

Authors' contributions:

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Moderate protein restriction protects against focal cerebral ischemia in mice by mechanisms involving anti-inflammatory and anti-oxidant responses

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Running title: Protein restriction protects against focal cerebral ischemia

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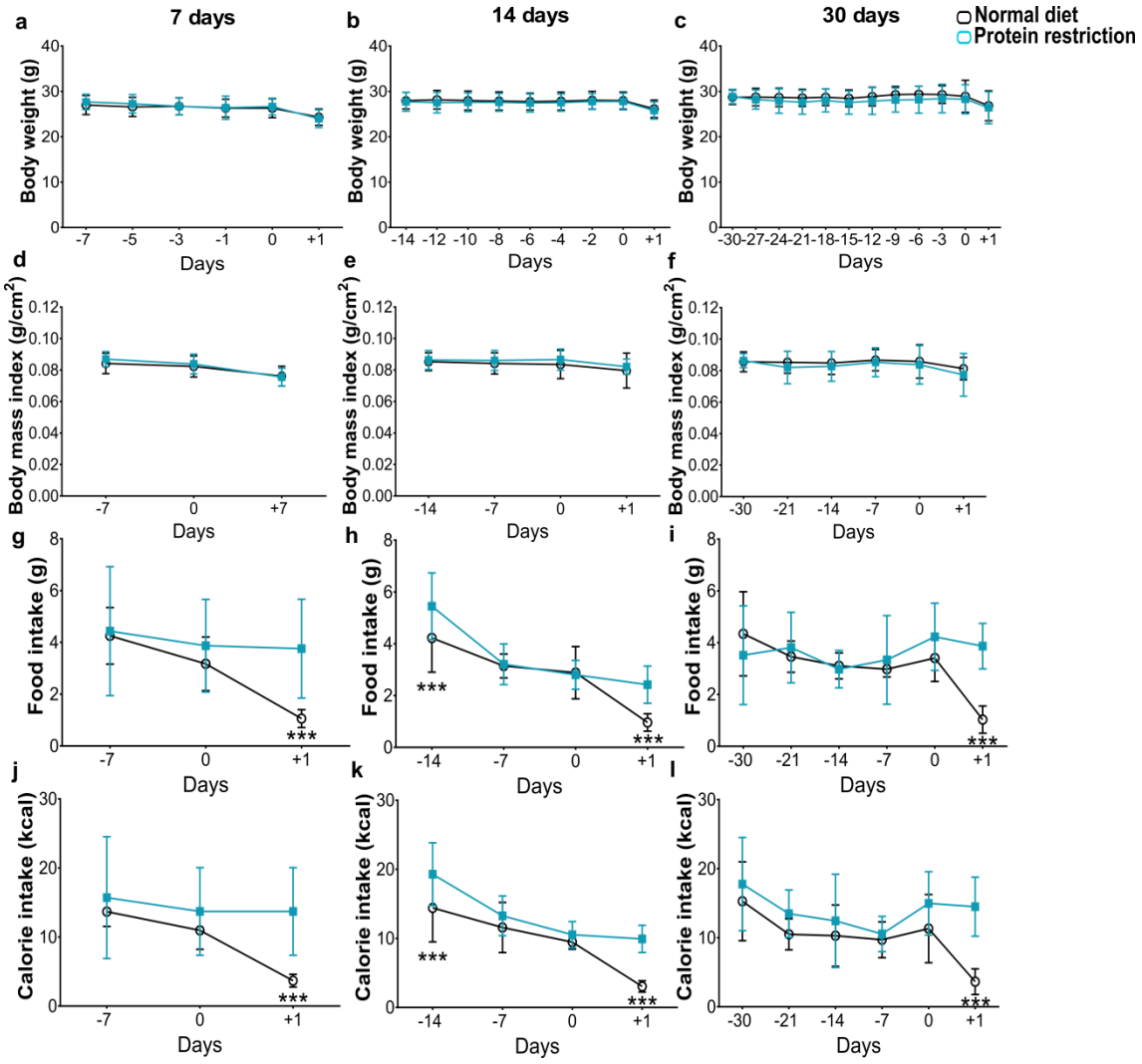
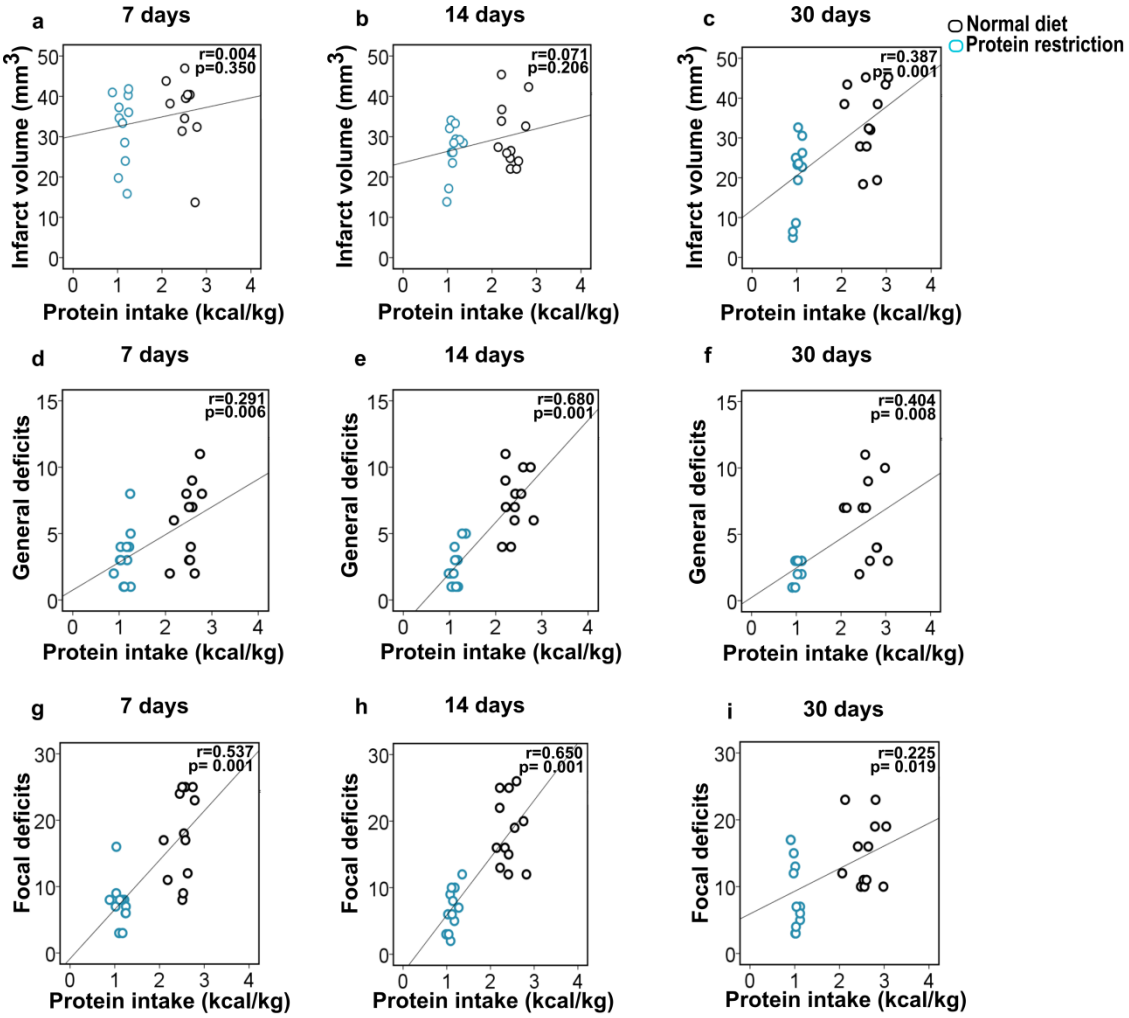
Supplemental Figures and Tables:

Supplemental Figures:



Suppl. Figure 1. Protein restriction does not alter major murinometric and nutritional variables prior to ischemia. (a-c) Body weight, **(d-f)** body-mass index (BMI), **(g-i)** daily food intake, **(j-l)** daily calorie intake in mice receiving normal or protein-reduced diet for 7 days **(a, d, g, j)**, 14 days **(b, e, h, k)** or 30 days **(c, f, i, l)**, followed by 30 minutes intraluminal MCAO and 24 hours reperfusion. The x-axis depicts the time-point with respect to MCAO (=day 0). Note the elevated food (**g-i**) and calorie (**j-l**) intake during the 24 hours after MCAO (day +1) in animals receiving protein-reduced compared with normal diet. Hence, food and calorie intake post-MCAO in mice exposed to protein restriction was very similar to pre-stroke food and calorie intake. ***p<0.001 for protein restriction compared with normal diet (n=12 animals/ group).



Suppl. Figure 2. Correlation of daily protein intake with infarct volume and neurological deficits. Pearson's correlations of daily protein intake with **(a-c)** infarct volume, **(d-f)** general neurological deficits and **(g-i)** focal neurological deficits in animals exposed to normal or protein-reduced diet for 7 days **(a, d, g)**, 14 days **(b, e, h)** or 30 days **(c, f, i)**, followed by 30 minutes intraluminal MCAO and 24 hours reperfusion. Correlation coefficients (r), p values and univariate regression lines are shown. Note the positive correlation of protein intake with infarct volume in mice exposed to food manipulation for 30 days, but not 7 or 14 days. Protein intake was positively correlated with general and focal neurological deficits in all three experimental conditions (food manipulation for 7, 14 and 30 days).

Supplemental Tables:

Supplemental Table 1. List of PCR primers.

Primer	Sequence (5'->3')		Tm	G-C (%)	Gene bank number
Sirt1	Forward	GATGACAGAACGTCACACGC	59.56	55.00	NM_019812.3
	Reverse	ATTGTTCGAGGATCGGTGCC	60.46	55.00	
IGF1	Forward	GACTCAGAAGTCCCCGTCCC	61.61	65.00	NM_010512.5
	Reverse	GCATTTTCTGCTCCGTGGG	59.49	57.89	
Insr	Forward	ACCTTCTCTGATGAACGGCG	60.11	55.00	NC_000074.6
	Reverse	CTGATATGGGATCCAGGGGG	58.71	60.00	
Glut1	Forward	GTTAATCGCTTTGGCAGGCGG	62.78	57.14	NM_011400.3
	Reverse	AGCATCTCAAAGGACTTGCCC	60.62	52.38	
Glut2	Forward	GTGCTGCTGGATAAATTCGCC	60.27	52.38	NM_031197.2
	Reverse	TCAGCAACCATGAACCAAGGG	60.82	60.82	
IL1β	Forward	TCTTTGAAGTTGACGGACCCC	60.20	52.38	NC_000068.7
	Reverse	CTTGTTGATGTGCTGCTGCG	60.73	55.00	
NFκb	Forward	TTTCGACTACGCAGTGACGG	60.39	55.00	NM_008689.2
	Reverse	GCTAAGTGTAAGACACTGTCCC	58.41	50.00	
Nox4	Forward	CCTGCTCATTTGGCTGTCCC	59.96	50.00	NM_015760.5
	Reverse	GCTTAAACACAATCCTAGGCCC	59.97	55.00	
Sod1	Forward	CATCCACTTCGAGCAGAAGGC	61.34	57.14	NM_011434.1
	Reverse	GGTACAGCCTTGTGTATTGTCCC	61.18	52.17	
Sod2	Forward	GAACAACAGGCCTTATTCCGC	61.32	60.00	NM_013671.3
	Reverse	GTGTATCTTTCAGTAACATTCTCCC	59.31	50.00	
Gpx3	Forward	GCACTACAAGAAGAACTTGGGC	59.77	50.00	NM_001329860.1
	Reverse	TCGAACATACTTGAGACTGGGG	59.50	50.00	
Cat	Forward	TGGTATAAGACGCATCAGAAGCC	60.49	47.83	NC_000071.6
	Reverse	GGTACTCCTCACTGAACATGCG	60.99	54.55	
βGluc	Forward	TGGTATAAGACGCATCAGAAGCC	60.49	47.83	NC_000071.6
	Reverse	GGTACTCCTCACTGAACATGCG	60.99	54.55	

Supplemental Table 2. Plasma lipid and glucose levels in mice exposed to protein restriction.

	Cholesterol (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)	Glucose (mg/dl)
7 days				
Normal diet	244.0±94.0	30.4±20.0	216.0±142.0	89.4±51.0
Protein restriction	182.7±43.0	15.2±10.0	217.3±128.4	72.0±46.33
14 days				
Normal diet	244.5±130.3	22.5±23.0	225.0±155.9	115.0±53.7
Protein restriction	252.2±86.2	25.8±12.5	255.5±111.3	95.5±40.0
30 days				
Normal diet	252.1±120.0	32.7±29.7	259.3±147.2	97.7±46.0
Protein restriction	158.3±19.5*	13.6±14.1*	177.1±74.6*	70.4±35.7

*$p<0.05$ compared with corresponding normal diet.

9.3 Supplementary material (Study 3)

Running title: Moderate protein restriction promotes stroke recovery

Author's contributions:

Contributed substantially to the conception and design of the study: TSC – 40% / DMH – 40% / EHSM – 20%

Contributed to the acquisition of the data:

Animal experiment: TSC – 100%

Histochemical analysis: TSC – 95% / MS – 5%

Molecular analysis: TSC – 90% / VS – 5% /LMNM, ARSM – 5%

Contributed to analysis and interpretation of the data: TSC – 60% / DMH – 35% / VS, LMNM, ARSM – 5%

Drafted or provided critical revision of the article: TSC – 50% / DMH – 50%

Provided final approval of the version to publication: TSC – 50% / DMH – 50%

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Authors' contributions:

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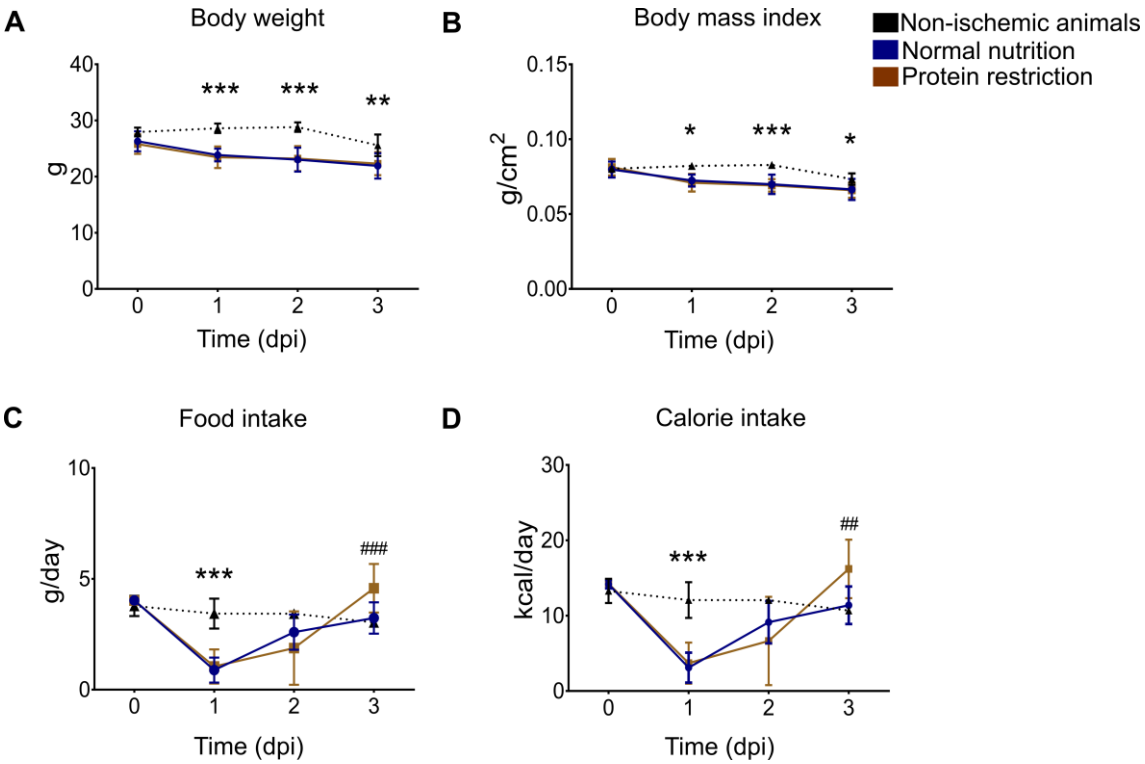
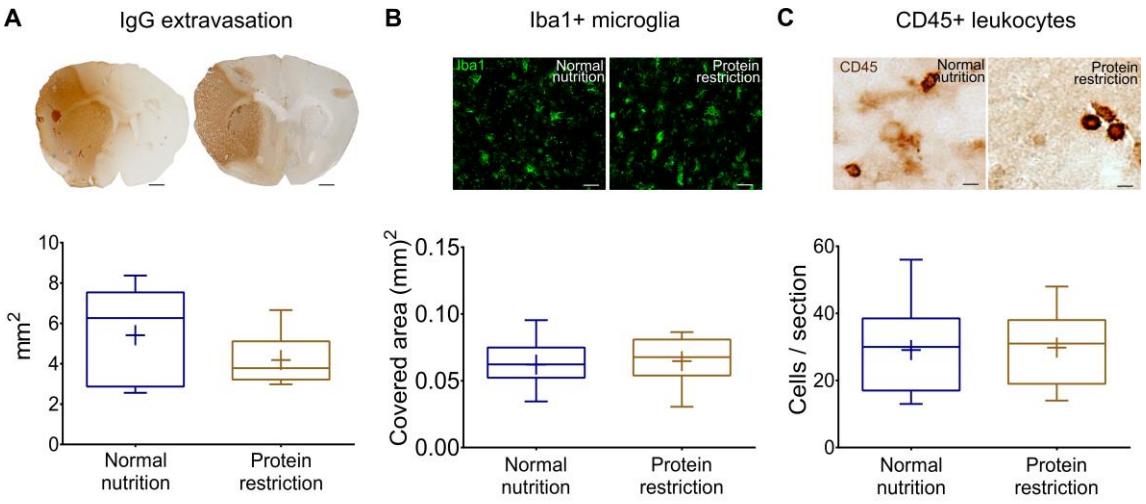
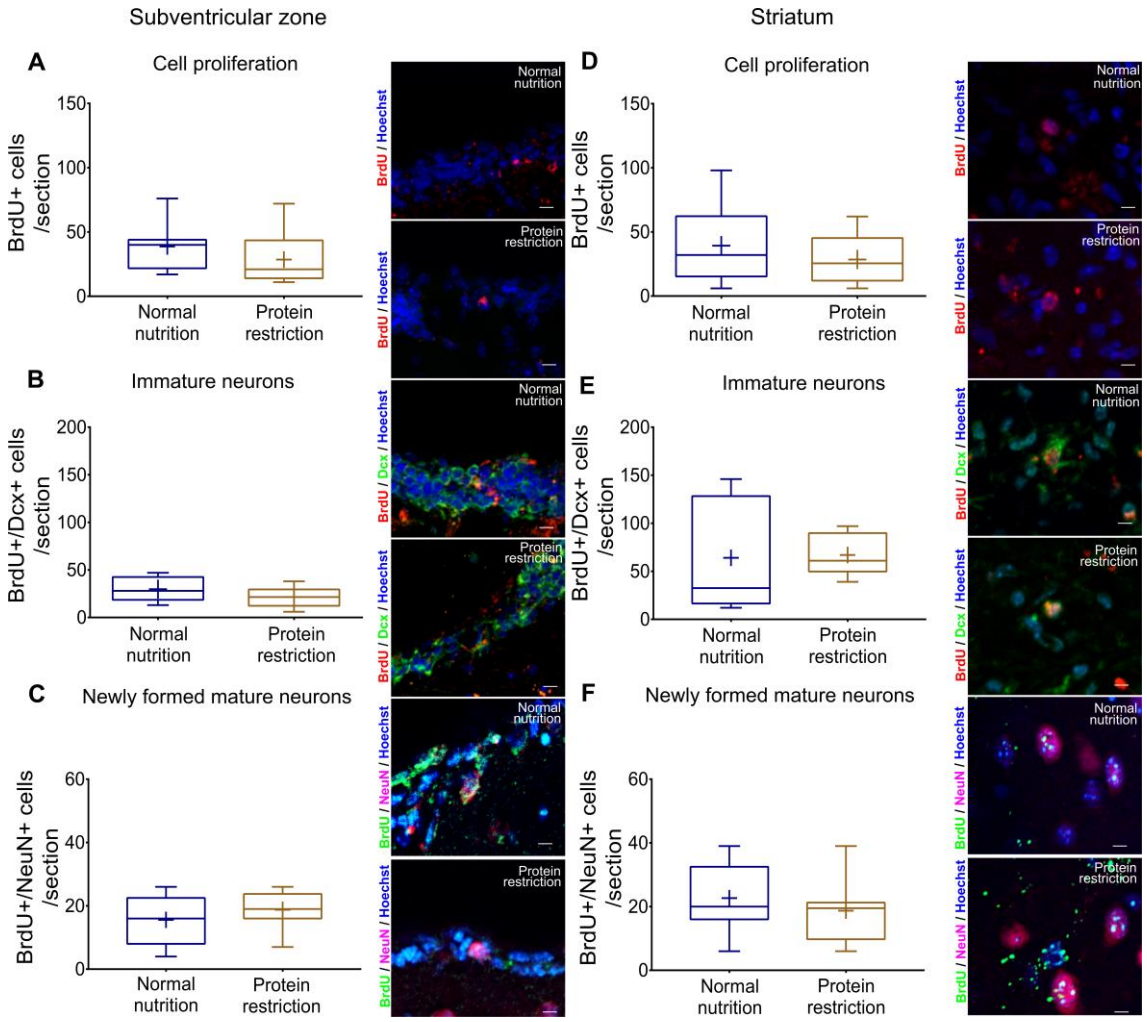
Currently status: Paper under submission.



Supplemental Figure 1. Mild protein restriction does not induce major murinometric changes. (A) Body weight, **(B)** body-mass index (BMI), **(C)** daily food intake and **(D)** daily calorie intake of mice exposed to intraluminal middle cerebral artery occlusion (MCAO) fed *ad libitum* with a normal diet (20% protein) or a protein-reduced diet (8% protein) over 56 days starting with the induction of MCAO. Note the reduction of food intake at 1 and 2 dpi. A modest, but statistically significant elevation of food and calorie intake was noted at 3 dpi in mice receiving protein restriction compared with mice on normal diet. Data are means ± S.D.***p<0.001/*p<0.05 compared with pre-ischemic baseline/ $^{\#\#}$p< 0.01/$^{\#}$p<0.05 compared with normal nutrition (n=12 mice/ group).



Supplemental Figure 2. Protein restriction does not influence blood-brain barrier breakdown or brain inflammation in the acute stroke phase. (A) IgG extravasation evaluated by immunohistochemistry, **(B)** microglial activation, examined by Iba1 immunohistochemistry and **(C)** brain leukocyte infiltration, analysed by CD45 immunohistochemistry of mice receiving intraluminal MCAO followed by exposure to normal nutrition or protein restriction for 56 days. Representative microphotographs are shown. Data are medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers. No significant group differences were found (n=12 mice/ group).



Supplemental Figure 3. Protein restriction does not influence endogenous neurogenesis. Endogenous cell proliferation and neurogenesis, evaluated by **(A, D)** BrdU incorporation analysis and colabeling with **(B, E)** the immature neuronal marker doublecortin (Dcx) and **(C, F)** the mature neuronal marker NeuN, which were evaluated **(A-C)** adjacent to the subventricular zone (SVZ) or **(D-F)** in the peri-infarct striatum, of mice receiving intraluminal middle cerebral artery occlusion (MCAO) followed by exposure to normal nutrition or protein restriction for 56 days. Data are medians (lines inside boxes)/ means (crosses inside boxes) ± interquartile ranges (IQR; boxes) with minimum/ maximum values as whiskers. No significant group differences were found (n=12 mice/ group).

Supplemental Tables:

Supplemental Table 1. List of PCR primers

Primer	Sequence (5'->3')		Tm	G-C (%)	Gene bank number
Sirt1	Forward	GATGACAGAACGTCACACGC	59.56	55.00	NM_019812.3
	Reverse	ATTGTTCGAGGATCGGTGCC	60.46	55.00	
IGF1	Forward	GACTCAGAAGTCCCCGTCCC	61.61	65.00	NM_010512.5
	Reverse	GCATTTTCTGCTCCGTGGG	59.49	57.89	
IL1β	Forward	TCTTTGAAGTTGACGGACCCC	60.20	52.38	NC_000068.7
	Reverse	CTTGTTGATGTGCTGCTGCG	60.73	55.00	
NFκb	Forward	TTTCGACTACGCAGTGACGG	60.39	55.00	NM_008689.2
	Reverse	GCTAAGTGTAAGACACTGTCCC	58.41	50.00	
Sod1	Forward	CATCCACTTCGAGCAGAAGGC	61.34	57.14	NM_011434.1
	Reverse	GGTACAGCCTTGTGTATTGTCCC	61.18	52.17	
Sod2	Forward	GAACAACAGGCCTTATTCCGC	61.32	60.00	NM_013671.3
	Reverse	GTGTATCTTTCAGTAACATTCTCCC	59.31	50.00	
Gpx3	Forward	GCACTACAAGAAGAACTTGGGC	59.77	50.00	NM_001329860.1
	Reverse	TCGAACATACTTGAGACTGGGG	59.50	50.00	
βGluc	Forward	TGGTATAAGACGCATCAGAAGCC	60.49	47.83	NC_000071.6
	Reverse	GGTACTCCTCACTGAACATGCG	60.99	54.55	

Supplemental Table 2. Plasma measurements in mice exposed to long-term protein restriction

Groups	Urea/ mg/dl	Bilirubin/ mg/dl	AST/ U/l	ALT/ U/l	Total protein/ g/dl	Albumin/ g/dl	Cholesterol/ mg/dl	LDL/ mg/dl	Triglycerides/ mg/dl	Glucose/ mg/dl
Non-ischemic	13.3 ± 3.1	1.3 ± 0.5	247.1 ± 97.2	77.2 ± 44.8	3.9 ± 0.6	2.8 ± 0.5	124.6 ± 23.7	8.4 ± 2.9	60.4 ± 13.3	188.0 ± 36.9
Ischemic/ normal nutrition	16.9 ± 6.0*****	1.6± 1.0	390.8 ± 239.8	82.7 ± 36.2	4.4 ± 1.3	3.2 ± 1.6	132.1 ± 44.9	11.7 ± 5.9	85.3 ± 49.6 ******	187.7 ± 40.0
Ischemic/ protein restriction	12.5 ± 4.1#	1.3 ± 0.7	228.1 ± 119.1	65.9 ± 19.1	3.9± 0.9	2.5 ± 0.7	121.0 ± 25.1	9.7 ± 0.7	72.6 ± 18.7*	179.7 ± 33.9

Data are means ± S.D., evaluated at 56 dpi.**p<0.01/ *p<0.05 compared with non-ischemic mice and #p<0.05 compared with normal nutrition (n=12 mice/ group).

Supplemental Table 3. Plasma measurements in mice exposed to post-ischemia protein restriction for 3 days.

Groups	Urea-N mg/dl	Billirubin mg/dl	AST U/l	ALT U/l	Total protein g/dl	Albumin g/dl	Cholesterol mg/dl	LDL mg/dl	Triglicerydes mg/dl	Glucose mg/dl
Non-ischemic	24.2 ± 4.5	0.4 ± 0.0	121.2 ± 44.0	36.0 ± 8.7	4.7 ± 0.1	3.1 ± 0.0	123.8 ± 9.7	7.4 ± 1.2	81.8 ± 16.8	203.6 ± 34.1
Ischemic/ normal nutrition	18.9 ± 6.7	0.8 ± 0.2*	206.3 ± 73.5*	48.3 ± 32.4	4.5 ± 0.5	3.0 ± 0.3	129.6 ± 23.2	12.7 ± 4.2*	63.3 ± 30.9	148.3 ± 32.1
Ischemic/ protein restriction	14.3 ± 4.6*	0.7 ± 0.3	176.9 ± 61.1	34.3 ± 8.8	4.6 ± 0.6	3.1 ± 0.7	116.6 ± 33.1	12.5 ± 4.5*	63.8 ± 22.6	161.1 ± 28.6*

Data are means ± S.D., evaluated at 3 dpi. *p<0.05 compared with non-ischemic mice (n=12 mice/ group).

10 PERMISSION AND IMAGES SOURCE

Introduction, material and methods, results and discussion are based on 2 published papers and on one unpublished paper.

Figures:

- Figure 1. Ischemia/reperfusion presents numerous opportunities for formation of reactive oxygen/nitrogen species and resultant tissue injury (with permission).
- Figure 2. Schematic overview on the complex pathophysiology of systemic metabolic changes and weight loss in patients with stroke (adapted from the original picture).
- Study 1 - Neuroprotection induced by energy and protein-energy malnutrition is stage-dependent after focal cerebral ischemia in mice (open access)

- Study 2 - Moderate protein restriction protects against focal cerebral ischemia in mice by mechanisms involving anti-inflammatory and anti-oxidant responses (open access)

11 CURRICULUM VITAE

"The curriculum vitae is not included in the online version for data protection reasons."

12 LIST OF PUBLICATIONS

- **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Luiza M. Nascentes Melo; Adriana R. Schultz-Moreira; Maryam Sardari; Egor Dzyubenko; Christoph Kleinschnitz; Dirk Hermann. Neuroprotection induced by energy and protein-energy undernutrition is phase-dependent after focal cerebral ischemia in mice. Transl Stroke Res. 2019;11:135–146
- **de Carvalho TS,** Sanchez-Mendoza EH, Nascentes LM, Schultz Moreira AR, Sardari M, Dzyubenko E, Christoph Kleinschnitz; Dirk Hermann. Moderate protein restriction protects against focal cerebral ischemia in mice by mechanisms involving anti-inflammatory and anti-oxidant responses. Molecular Neurobiology. 2019;56:8477–8488
- **de Carvalho TS,** Cardoso PB, Santos-Silva M, Lima-Bastos S, Luz WL, Assad N, Kauffmann N, Passos A, Brasil, A, Bahia CP, Moraes S, Gouveia A, de Jesus EOB, Oliveira KRMH, Herculano, AM. Oxidative stress mediates anxiety-like behavior induced by high caffeine intake in zebrafish: Protective effect of alpha-tocopherol. Oxidative Medicine and Cellular Longevity. 2019; 2019:1-9.
- Manrique-Castano D, Sardari M, **Silva de Carvalho T**, Doeppner TR, Popa-Wagner A, Kleinschnitz C, Chan A, Hermann, DM. Deactivation of atp-binding cassette transporters abcb1 and abcc1 does not influence post-ischemic neurological deficits, secondary neurodegeneration and neurogenesis, but induces subtle microglial morphological changes. Front Cell Neurosci. 2019; 13:412.
- Wang YC, Dzyubenko E, Sanchez-Mendoza EH, Sardari M, **Silva de Carvalho T**, Doeppner TR, Kaltwasser B, Machado P, Kleinschnitz C, Bassetti, CL, Hermann, DM. Postacute delivery of gabaa alpha5 antagonist promotes postischemic neurological recovery and peri-infarct brain remodeling. Stroke. 2018; 49:2495-2503.
- Brasil, A.; Rocha, F. A. F.; Gomes, B. D.; **Carvalho, T. S**.; Batista, E. J. O.; Silveira, L. C. L.; Kremers, J.; Oliveira, K. R.; Herculano, A. M. O. S. Diet enriched with the Amazon fruit açaí (Euterpe oleracea) prevents electrophysiological deficits and oxidative stress induced by methyl-mercury in the rat retina' to Nutritional Neuroscience. Nutritional Neuroscience, v. 10, p. 1-8, 2016.
- Sanchez-Mendonza, E. H.; **Carvalho, T. S**; Hermann, D. M. Methods for the analysis of neuronal plasticity and brain connectivity during neurological recovery. Neural Regeneration Research., v. 11, p. 1701-1703, 2016.

- Maximino, C.; Silva, R. X. C.; Silva, S. N. S.; Rodrigues, L. S. S.; Barbosa, H.; **Carvalho, T. S**.; Leão, L. K. R.; Lima, M. G.; Oliveira, K. R. M.; Herculano, A. M.. Non-mammalian models in behavioral neuroscience: consequences for biological psychiatry. Frontiers in Behavioral Neuroscience, v. 9, p. 1- 21, 2015.

13 POSTER PRESENTATIONS

- 92. Kongress der Deutschen Gesellschaft für Neurologie, Stuttgart (09/19)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Luiza M. Nascentes Melo; Adriana R. Schultz-Moreira; Maryam Sardari; Egor Dzyubenko; Christoph Kleinschnitz; Dirk Hermann. Neuroprotection induced by energy and protein-energy undernutrition is phase-dependent after focal cerebral ischemia in mice.

- 92. Kongress der Deutschen Gesellschaft für Neurologie, Stuttgart (09/19)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Luiza M. Nascentes Melo; Adriana R. Schultz-Moreira; Maryam Sardari; Egor Dzyubenko; Christoph Kleinschnitz; Dirk Hermann. Moderate protein restriction protects against focal cerebral ischemia in mice by mechanisms involving anti-inflammatory and anti-oxidant responses. Molecular Neurobiology, 2019.

- Federation of European Neuroscience Societies congress (FENS), Berlin (07/18)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Adriana R. Schultz-Moreira; Maryam Sardari; Daniel Castaño; Britta Kaltwasser; Christoph Kleinschnitz; Dirk Hermann. Long-term of protein restriction diet improves neurological recovery and brain injury after transient focal cerebral ischemia in mice.

- Tag der Forschung, Essen (11/17)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Adriana R. Schultz-Moreira; Maryam Sardari; Daniel Castaño; Britta Kaltwasser; Christoph Kleinschnitz; Dirk Hermann. Effects of hypocaloric and hypoprotein malnutrition on focal cerebral ischemic brain injury.

- The European Neuroscience Conference by Doctoral Students (ENCODS), Alicante (05/17)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Adriana R. Schultz-Moreira; Maryam Sardari; Daniel Castaño; Britta Kaltwasser; Christoph Kleinschnitz; Dirk Hermann. Effects of hypocaloric and hypoprotein malnutrition on focal cerebral ischemic brain injury.

- Neurovisionen 11 & 3rd Neuroinflammation Symposium, Münster (11/16)

Poster: **Tayana Silva de Carvalho**; Amauri Gouveia Jr; Waldo Lucas Luz da Silva, Nadyme Assad Holanda da Silva, Tatiana Alves do Nascimento; Alódia Brasil; Caio Maximino; Karen Renata Matos Oliveira; Evander de Jesus Oliveira Batista;

Domingos Luis Wanderley Picanço Diniz; Anderson Manoel Herculano. Caffeine-induced anxiety-like behavior in danio rerio (zebrafish) is prevented by treatment with alpha-tocopherol and L-NAME

- Society of Environmental Toxicology and Chemistry Europe German Language Branch e.V. (SETAC GLB), Tübingen (09/16)

Poster: Tatiana Alves do Nascimento; **Tayana Silva de Carvalho**; Waldo Lucas Luz da Silva; Nadyme Assad Holanda da Silva; Caio Maximino; Evander de Jesus Oliveira Batista; Alódia Brasil; Karen Renata Matos Oliveira; Amauri Gouveia; Anderson Manoel Herculano. Anxiety-like behavior and neural activity in zebrafish are potential biomarkers of water contaminated with methylmercury.

- European Neuroscience Conference by Doctoral Students (ENCODS), Helsingor (06/16)

Poster: **Tayana Silva de Carvalho**; Amauri Gouveia Jr; Waldo Lucas Luz da Silva, Nadyme Assad Holanda da Silva, Tatiana Alves do Nascimento; Alódia Brasil; Caio Maximino; Karen Renata Matos Oliveira; Evander de Jesus Oliveira Batista; Domingos Luis Wanderley Picanço Diniz; Anderson Manoel Herculano. Caffeine-induced anxiety-like behavior in Danio rerio (zebrafish) is prevented by treatment with α-tocopherol.

- DAAD-Stipendiatentreffen, Bonn (10/15)

Poster: **Tayana Silva de Carvalho**; Eduardo H. Sanchez-Mendoza; Dirk Hermann. Effects of nutrition deprivation on stroke injury and recovery in rodents: Consequences for brain remodeling and plasticity.