Effects of PGC1a-induced exercise adaptations in muscle on plasticity and recovery mechanisms in the CNS

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Gothenburg 2019

Cover illustration: Running induces birth of new cells in the adult mouse dentate gyrus

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ISBN 978-91-7833-406-3 (PRINT)
ISBN 978-91-7833-407-0 (PDF)

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

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ABSTRACT

In this thesis, we sought to determine if muscle-derived exercise-induced signaling via PGC-1α muscle activation influences neuroplasticity under physiological or pathophysiological conditions. For this purpose, transgenic mice with muscle-specific overexpression of PGC-1α that display an endurance exercise muscle phenotype were evaluated in models of cranial irradiation and photothrombotic stroke, as well as in aging and in a voluntary running paradigm. We also measured the response on proliferation and differentiation of NSPCs from treatment with either serum from exercised and transgenic mice, or conditioned media from PGC-1α-transfected myocytes.
In **paper I**, we found that muscular PGC-1α overexpression in mice did not ameliorate irradiation-induced reduction of neurogenesis and rather resulted in increased infarct size without any differences in inflammatory response. In **paper II** and **paper III**, animals of both sexes displayed robust age-related reductions, and exercise-induced increases, in hippocampal neurogenesis. No differences were detected in these measurements between the genotypes. Further, transgenic animals had increased levels of myokines and reduced levels of pro-inflammatory cytokines. In **paper IV**, mouse sera from exercised or transgenic animals had no effect on proliferation of NSPCs, while conditioned medium from PGC-1α-overexpressing myocytes slightly increased proliferation. No differences existed in differentiation from treatment with different mouse sera or conditioned media.

We conclude that artificial chronic muscle activation through the PGC-1α pathway, despite potent systemic changes, does not translate into exercise-induced effects on hippocampal neurogenesis, and is not sufficient to mimic exercise-induced effects on recovery after cranial irradiation or stroke, or prevent age-related reduction in neurogenesis. Likewise, circulating factors in serum from exercised animals, or from animals with muscle-specific PGC-1α overexpression, are not sufficient to directly induce changes in proliferation or differentiation of NSPCs *in vitro*.
Despite evidence indicating that exercise-induced factors from muscle and other tissues are capable of influencing brain function, our results highlight the difficulty in mimicking sustained effects of exercise on the brain. The study of PGC-1α and related molecular pathways, in muscle and other tissue, contributes to our understanding of mechanisms behind exercise-related benefits on the brain.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mekanismerna bakom konditionsträningens enastående effekter på hjärnan är fortfarande inte helt klarlagda. Inte minst gäller detta den avgörande rollen av cirkulerande faktorer i blodet för träningsinducerad neurogenes, dvs. nybildningen av nervceller i hjärnan. Transkriptionsfaktorn PGC-1α anses vara en av de centrala faktorer som förmedlar träningens fördelar i skelettmuskulatur, inklusive frisättningen av faktorer i cirkulationen som kan påverka hjärnfunktion. I denna avhandling studerade vi hur ett specifikt överuttryck av PGC-1α i skelettmuskel bidrar till träningsinducerade effekter på hjärnan under fysiologiska och patofysiologiska förhållanden och om faktorer i blodet bidrar till dessa effekter. Vi använde en transgen musmodell med muskelspecifikt överuttryck av PGC-1α och fann att muskulärt PGC-1α överuttryck inte lindrade strålningsinducerad reduktion av neurogenes och istället resulterade i större infarkter utan påverkan på inflammatoriskt svar. Transgena och vildtyp djur av båda kön uppvisade en tydlig åldersrelaterad reduktion, och träningsinducerad ökning, av neurogenes i hippocampus. Ingen skillnad i dessa parametrar observerades dock för genotyp. Transgena möss hade ökade serumnivåer av myokiner och reducerade nivåer av pro-inflammatoriska cytokiner. Vi fann även att musserum från tränade eller transgena djur inte hade någon effekt på proliferation hos neurala stam- och progenitor celler (NSPC) medan konditionerat medium från PGC-1α-överuttryckande myocyter gav en lätt ökning i proliferation. Ingen skillnad fanns i differentiering mellan olika musserum eller konditionerade medier.

Vi drar slutsatsen att artificiell kronisk muskelaktivering genom PGC-1α signalering, trots potenta systemiska effekter, inte översätts till träningsinducerade effekter på återhämtning efter kraniell strålning eller stroke, och inte heller skydd mot reducerad neurogenes vid åldrande. Vi drar även slutsatsen att cirkulerande faktorer i serum från tränade eller transgena möss inte är tillräckligt för att ge direkta effekter på proliferation eller differentiering av NSPC *in vitro*. Trots att studier visat att träningsinducerade faktorer från muskel och andra organ kan påverka hjärnfunktionen, understryker dock våra resultat svårigheten att efterlikna hållbara träningsinducerade effekter på hjärnan. Studier på PGC-1α och relaterade signaleringsvägar i muskel och andra vävnader bidrar till vår förståelse om mekanismerna bakom träningsrelaterade effekter på hjärnan.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Karlsson L.**, González-Alvarado M.N., Larrosa-Flor M., Osman A., Börjesson M., Blomgren K., Kuhn H.G. "Constitutive PGC-1α overexpression in skeletal muscle does not improve morphological outcome in mouse models of brain irradiation or cortical stroke". Neuroscience. 2018 Aug 384:314-328.

II. **Karlsson L**, González-Alvarado M.N., Motalleb R., Blomgren K., Börjesson M., Kuhn H.G. "Constitutive PGC-1α overexpression in skeletal muscle does not protect from age-dependent decline in neurogenesis". *Submitted.*

III. **Karlsson L.**, González-Alvarado M.N., Motalleb R., Blomgren K., Börjesson M., Kuhn H.G. "Constitutive PGC-1α overexpression in skeletal muscle does not contribute in exercise-induced neurogenesis". *Manuscript.*

IV. **Karlsson L.**, Savvidi P., Onyeanwu C., Kumar Malipatlolla D., Vidal A., Motalleb R., Kuhn H.G. "Effects of exercise and muscle-specific PGC-1α overexpression on neural stem cell responses in vitro". *Manuscript.*

Related papers not included in this thesis.

i. Michaëlsson H., Andersson M., Svensson J., **Karlsson L.**, Ehn J., Culley G., Engström A., Bergström N., Savvidi P., Kuhn H.G., Hanse E., Seth H. “The novel antidepressant ketamine enhances dentate gyrus proliferation with no effects on synaptic plasticity or hippocampal function in depressive-like rats”. Acta Physiol (Oxf). 2019 Apr 225(4):e13211.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BAIBA	Beta-amino-isobutyric acid
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BHA	Beta-hydroxy acid
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
CaMK	Calcium/calmodulin-dependent protein kinase
CNS	Central nervous system
DCX	Doublecortin
DG	Dentate gyrus
ERRα	Estrogen-related receptor alpha
FAT	Fatty acid translocase
FDR	False discovery rate
FGF	Fibroblast growth factor
FNDC5	Fibronectin type III domain-containing protein 5
GABA	Gamma-aminobutyric acid
GCL	Granular cell layer
GFP	Green fluorescent protein
GW	GW501516, a PPARδ-agonist
IGF	Insulin-like growth factor
IL	Interleukin
IR	Ionizing radiation
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor-2

ML	Molecular layer
NAD	Nicotinamide adenine dinucleotide
NFkB	Nuclear factor kappa-light-chair-enhancer of activated B cells
NRF	Nuclear respiratory factor
NSPC	Neural stem and progenitor cell
PCR	Polymerase chain reaction
PGC-1α	PPAR-gamma co-activator 1-alpha
PPAR	Perixosome proliferator-activated receptor
PRC	PGC-1-related co-activator
RM-ANOVA	Repeated measures ANOVA
RNS	Reactive nitrogen species
ROS	Radical oxygen species
SGZ	Subgranular zone
SVZ	Subventricular zone
TG	Transgenic
TrkB	Tropomysin receptor kinase B
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WT	Wildtype

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INTRODUCTION

Physical exercise, particularly aerobic exercise, has remarkable effects on the brain. It is considered a powerful treatment strategy to improve general brain health, brain plasticity, and cognition. The molecular mechanisms underlying these effects are still largely unknown, especially the essential role of systemic factors in the circulation. Recently, muscle been uncovered as a secretory organ, with many of the exercise-induced effects of endurance training in skeletal muscle orchestrated by the transcription factor PGC-1α (perixosome proliferator-activated receptor gamma co-activator 1-alpha), including the release of downstream factors with neurotrophic properties. In this thesis, we sought to investigate if muscle-specific PGC-1α activation could contribute to exercise-induced effects on the central nervous system.

One of the most prominent and reproducible features of exercise in the rodent brain is increased hippocampal neurogenesis, i.e. the generation in new neurons in the adult hippocampus. The role of systemic factors in the circulation capable of influencing exercise-induced neurogenesis has just recently begun to be explored, with several circulating factors found to be essential for exercise-induced neurogenesis (1). However, mechanisms underlying exercise-induced release of such signals are still unclear. Muscle releases factors with potent systemic effects during exercise, some which also influence the brain. With the identification of exercise-induced transcriptional regulators in skeletal muscle, such as PGC-1α, AMPK, and PPARδ, the possibility of genetic manipulations and pharmacological targeting has enabled ways to study the influence of muscle activation pathways on the body and brain. In 2012, activation of the exercise-induced transcription factor PGC-1α in muscle was discovered to secrete systemic factors with health-promoting effects on other organs (2). With regards to the CNS, FNDC5, or irisin, was found to stimulate the expression of the important neurotrophic growth factor BNDF in the hippocampus, a brain region of central importance for learning and memory (3). Likewise, pharmacological activation of muscle activation pathways, such as AMPK and PPARδ, have yielded positive effects on hippocampal neurogenesis and spatial memory (4). Taken together, this implicates the involvement of muscle activation pathways in exercise-induced effects on the CNS.

This thesis aimed at studying the effect of muscle-derived exercise-related signals on stem cell activation and brain plasticity as a means to improve recovery and protect from neurological conditions such as irradiation-injury to the brain, stroke, and age-related cognitive decline. In order to determine the effect of muscle-derived factors, we overexpressed the transcription factor PGC-1α, considered to be responsible for many of the exercise-related benefits in muscle, including the

release of downstream factors into the circulation. This was done through the use of a transgenic mouse, in which the transcriptional co-activator PGC-1α is overexpressed under a muscle-specific promoter. This yields a chronic activation of skeletal muscle cells with an endurance exercise phenotype that can be used to study molecular mechanism underlying exercise-induced effects in skeletal muscle. The same gene was also overexpressed in a cell culture system of myoblasts to study how factors released from muscle cells into the medium influence neural stem cell behavior. These experiments give insights into how exercise-induced signaling influences neuroplasticity under physiological and pathophysiological conditions.

Due to physical or mental constraints, whether induced by diseases or genetic makeup, many patients are unable to get the full benefits of exercise. By studies of cellular and molecular mechanisms, we are able to advance our knowledge about the interplay underlying the complex effects of exercise on the body. This knowledge would potentially aid the clinical implementation of exercise as a treatment option in health care. Identification of possible systemic signals that mediate some parts of exercise-induced effects could enable development of novel pharmacological strategies. Such signal molecules could be useful as therapeutics, either alone or as adjuvants to lifestyle changes, for treating disorders that otherwise would improve with exercise. A better understanding of molecular mechanisms could also enable identification of novel biomarkers for monitoring health status and optimization of physical therapy based on genetics and molecular responses.

In this chapter, the effects and mechanisms behind exercise-induced effects on the body and brain are described, including the influence of exercise on adult neurogenesis. After this, the role of neurotrophic factors in the CNS and circulation are described, including the role of muscle and the PGC-1α pathway in exercise-induced signaling. Finally, we will provide a background on the experimental animal models employed in this thesis, including the transgenic mouse model, models of brain injury, and aging.

Benefits of aerobic exercise

Physical exercise undoubtedly has astonishing effects for the body and brain, and is a central life style factor for maintaining general health and wellbeing throughout life (5). Here, we focus on the effects of aerobic exercise, which is what primarily has been documented to improve health and function on the body and brain, while the health promoting effects of resistance exercise are less well established and outside the scope of this thesis. In this thesis, we use the terms ‘physical activity’ and ‘physical exercise’ as defined by Caspersen, Powell, and Christenson (6), with ‘physical activity’ defined as “any bodily movement produced by skeletal muscles that results in energy expenditure” and ‘physical

exercise' defined as "a subset of physical activity that is planned, structured, and repetitive, and has as a final, or an intermediate objective, the improvement or maintenance of physical fitness". The minimum recommended levels of aerobic physical activity for maintaining health in adults determined by the World Health Organization (WHO) corresponds to 150 minutes per week of moderate-intensity activity, or alternatively, 75 minute per week of high-intensity activity. For children, WHO recommends at least 60 minutes of moderate-intensity physical activity daily. Moderate-intensity physical activity is defined as an activity level corresponding to at least 3 times the energy expenditure of rest (also known as metabolic equivalent; 1 MET), or an oxygen consumption rate (VO_2) of over 45% of an individual's VO_2max (7).

A very active life style, greatly surpassing the minimum recommendations of physical activity by the WHO, has been the norm throughout human evolution (8). From an evolutionary point of view, humans and other animals are built to run, due to the fact that movement has been a necessity for finding food and shelter, hunting, as well as escaping danger (9). In the animal kingdom, humans are poor sprinters compared to other species, but perform well at endurance running (10). Since humans evolved into running through simultaneous adaptations in metabolism, musculoskeletal system, and central nervous system, the human brain is likely dependent on exercise to function properly. Therefore, being active is an important factor of who we are as a species and health-promoting effects from endurance exercise should be regarded as the normal state, while physical inactivity should be regarded as the abnormal state.

Technological advancements have led to a sedentary behavior in the global population, with physical inactivity being a major risk factor for chronic disease estimated as the fourth leading cause of death worldwide (11). One-third of adults worldwide, and half in the US (12), fail to meet WHO minimum recommendations, which can be translated into an enormous health economic impact for society. It should be noted that studies using accelerometers have demonstrated that self-reported data overestimates the levels of physical activity, suggesting that physical inactivity is an even more widespread problem than previously thought (13). Arem and colleagues pooled data from population-based prospective cohort studies in the US and Europe with self-reported physical activity levels for over 600,000 individuals, and from this data generated multivariable-adjusted hazard ratios over a mean follow-up period of 14 years (14). The study found that the WHO minimum recommended levels of physical activity corresponded to a ~30% lower risk of death, with maximum benefits for longevity, of ~40% lower risk, occurring at around 3 to 5-fold the recommended minimum. Physical inactivity is associated with increased risk for a range of chronic diseases, with low exercise capacity (VO_2max) being an independent predictor of all-cause mortality and morbidity (15). VO2max is a measurement of exercise capacity, and corresponds to the highest energy demand that can be met

aerobically while exercising. It reflects many parameters simultaneously such as mitochondrial oxidative phosphorylation potential, cardiovascular and cardiopulmonary capacity, as well as neuromuscular function. Exercise intensity is relative to the individual and typically expressed as a percentage of an individual's VO2max, in low- (<45%), moderate- (45-75%), and high-intensity (>75%). Interestingly, genes that govern physical activity levels, cardiorespiratory fitness, and risk of death have been found to be the same (16).

Aerobic exercise offers many health benefits to the individual. It reduces all-cause mortality and has been reported to prevent and treat numerous chronic conditions, including metabolic, cardiovascular and pulmonary diseases, cancer, musculoskeletal and autoimmune disorders (17). Exercise leads to a long list of physiological improvements in the body including, but not limited to, improvements in musculoskeletal system, cardiorespiratory fitness, cardiac function, blood pressure, blood flow, vascularity, blood hemodynamics, glucose control, lipid profile, visceral adiposity, and immune function (18, 19). Exercise mediates a part of its effect by reducing systemic low grade inflammation which is associated to aging and many chronic diseases (20).

Effects of exercise in the human brain

In the brain, aerobic exercise reduces risk and represents a treatment strategy for neurodegenerative, cerebrovascular, and psychiatric illnesses, with physical inactivity being a risk factor for depression, dementia, and stroke (17, 21).

In humans, physical exercise improves cognition with positive effects on learning, memory, attention, processing speed, and executive functions (22). Levels of physical activity and exercise capacity are also positively associated with academic achievement in children (23), intelligence in adolescents (24), as well as education and income (25). In addition, exercise improves several basic physiological functions governed by the CNS such as sleep (26), appetite (27), and mood (28). Structurally, exercise improves functional connectivity between different brain regions, thus improving the performance of important brain networks such as the central-executive and default-mode networks that recently have been discovered to be responsible for higher cognitive functioning (29). Exercise training also leads to increased hippocampal volume, and can prevent gray and white matter loss in prefrontal, parietal, and temporal cortex of older adults (30). Exercise may exert these behavioral and functional effects by correlated improvements in cerebral blood flow and brain oxygenation (18), anti-inflammatory actions, or increases in release of growth factors (31).

In rodents, one of the most reproducible effects of exercise on the brain is an increase in neurogenesis. Before detailing the mechanisms of exercise-induced neurogenesis and other exercise-related effects on the brain in rodents, we will shortly describe neurogenesis in the adult brain.

Adult neurogenesis

In 1965, Joseph Altman serendipitously discovered that new neurons form in the adult rodent brain (32). However, this finding did not receive much attention until the 1980s when Nottebohm found that adult neurogenesis also occurs in songbirds, which later led to controversies regarding the existence of neurogenesis in mammals, and primates in particular (33). Since then, the field has come a long way and accumulating evidence supports the existence of adult neurogenesis in the human brain (34). The existence of human neurogenesis was first demonstrated by administering bromodeoxyuridine (BrdU) to terminal cancer patients for analysis of post-mortem brain tissue, proving that new neurons are generated in the human hippocampus even late in life (35). Human neurogenesis has also been confirmed to exist using an elegant method in the form of carbon-14 dating, showing that neuronal turnover continuously occurs in the hippocampus of humans (36). The study reported that, one-third of the hippocampal neurons are subject to turnover, with 700 new neurons added in each hippocampus every day in adult humans, corresponding to an annual neuronal turnover of 1.75% with only a modest decline in turnover during aging. Since there is an obvious ethical and practical dilemma with trying to study neurogenesis in humans, rodent models have allowed us to better study this process. Even though studies in humans has correlated exercise to improved learning and memory, increased hippocampal volumes, and increased hippocampal blood flow (30, 37), it is still not known whether exercise-induced neurogenesis also occurs in humans.

In mammals, neurogenesis takes place mainly during embryonic and early post-natal developmental, but also to a lesser extent throughout adult life, i.e. adult neurogenesis. The two main neurogenic regions of the brain are the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation (35, 38) and the subventricular zone (SVZ) of the lateral ventricle walls (39).

The hippocampal formation belongs to the limbic system located in the medial temporal lobe of the human brain. This region is considered to play a key role in learning, declarative, and spatial memory (40). The hippocampus formation is considered archicortex, which is a form of allocortex, i.e. non-neocortex. The archicortex, meaning ‘ancient cortex’, is the phylogenetically oldest region of the brain’s cortex, having three cortical layers instead of six layers as for the neocortex. The layers of the hippocampal formation are the dentate gyrus, the hippocampus proper, and the subiculum. Dentate gyrus consists of three different anatomical layers: the molecular layer (ML), the granular cell layer (GCL), and the hilus (polymorphic layer), see Figure 1. SGZ is a subregion of the GCL, outlining the border between the GCL and hilus. The middle layer of the hippocampus proper is called the pyramidal layer, or cornu ammonis (CA), and is divided into three regions (CA1-3).

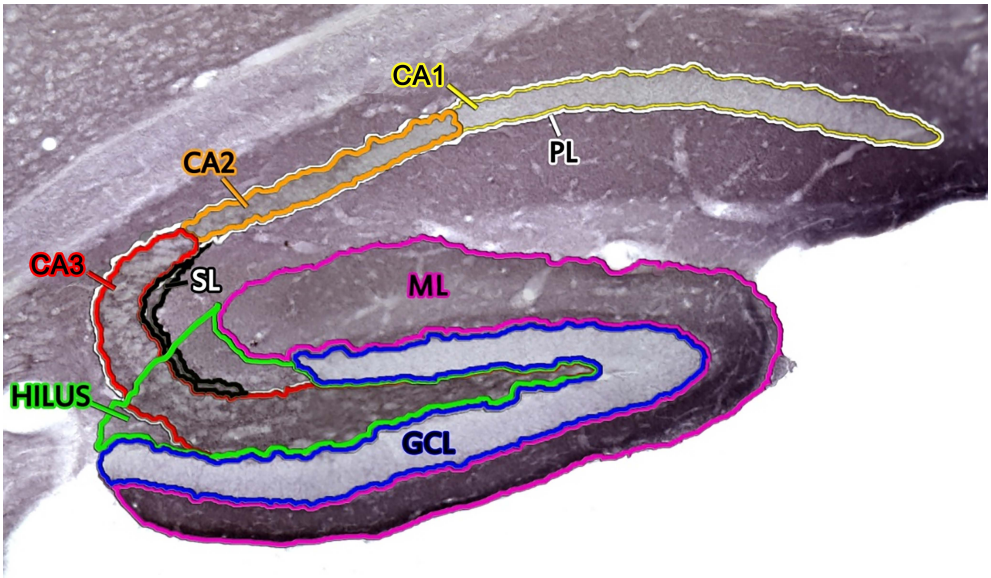

Figure 1. Anatomical description of the different hippocampal subregions in mouse. The SGZ outlines the border between the GCL and hilus. The pyramidal layer (PL) is divided into three subareas (CA1-3). The stratum lucidum (SL) is a thin layer adjacent to the PL where the mossy fibers from the DG are located. *GCL (blue), ML (magenta), hilus (green), PL (white), CA1 (yellow), CA2 (orange), and CA3 (red).*

Information flows through the hippocampus by three separate pathways, known as the trisynaptic circuitry (41), see Figure 2. The perforant pathway inputs information from other brain regions via the entorhinal cortex to the granular neurons of the GCL. The mossy fiber pathway consists of axonal projections from the granular neurons to the pyramidal neurons in the CA3. Finally, the Schaffer collateral pathway involves axonal projections from pyramidal neurons in the CA3 area to the CA1 area, for final output of processed information from the hippocampus to other brain regions.

As for the other neurogenic region of the brain, the SVZ, newly generated neuronal progenitors migrate along the rostral migratory stream towards the rodent olfactory bulb where they integrate as interneurons (42), see Figure 3. For humans, however, SVZ neuroblasts migrate to the striatum, a region of importance for motor and reward systems (43).

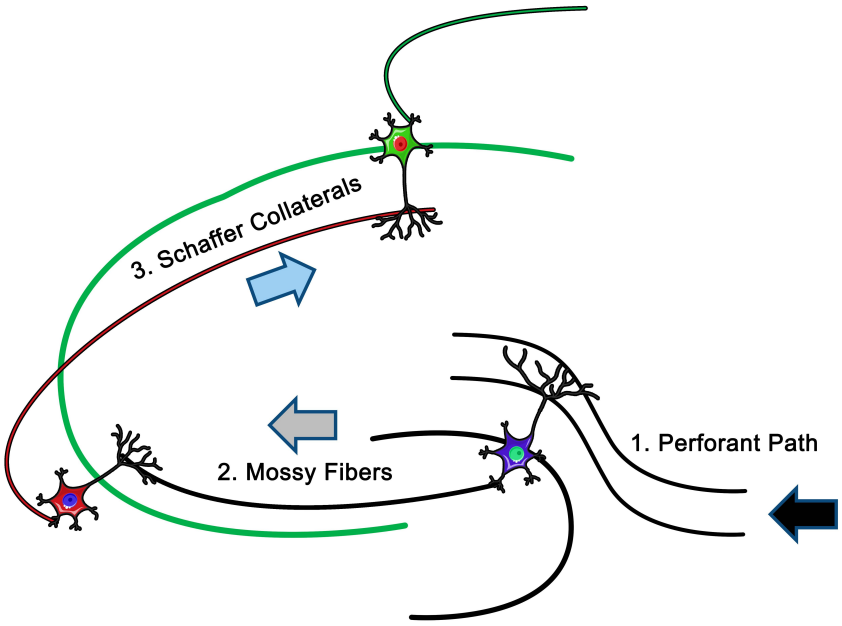

Figure 2. Schematic illustration of the hippocampal trisynaptic circuitry. The circuitry consists of granular neurons in the GCL and pyramidal neurons in the CA3 and CA1 areas.

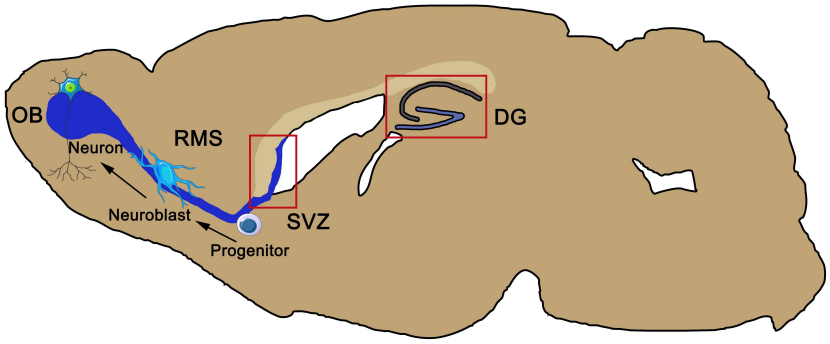

Figure 3. The neurogenic niches of the rodent brain and the rostral migratory stream. The blue area reaching from the SVZ to the OB represents the RMS, along which neuroblast migrate to the olfactory bulb. *RMS, rostral migratory stream. OB, olfactory bulb.*

The process of adult hippocampal neurogenesis

Adult hippocampal neurogenesis can be described to occur in four general phases: precursor phase, early survival phase, maturation phase, and late survival phase (44), see Figure 4. The process begins in the precursor cell phase with a highly proliferative bipotent radial glia-like (type-1) stem cell within the SGZ. These cells have astrocytic properties with a process attached to the basal membrane of blood vessels, thus having close interaction with endothelial cells and circulation. Type-1 cells undergo asymmetric division and generate transiently amplifying progenitor cells (type-2a/b) that rapidly divide and begin to migrate as they differentiate into neurons. Type-3 cells are migrating neuroblasts that have lost their proliferative capability. The early survival phase begins with neural progenitors exiting the cell cycle. The minority of early neuronal cells that survive begin to establish functional connections by extending their processes, axons into the hilus, and dendrites through the GCL into the ML. During the maturation phase, inhibitory GABAergic input from interneurons residing in the hilus promotes the maturation process until the excitatory glutamatergic input from the entorhinal cortex via the perforant pathway reaches the surviving maturing granular neurons. After cell cycle exit, a majority of newborn neurons undergo apoptosis within 10-12 days by default, however, survival of immature neurons can be increased by glutamatergic stimulation such as by cognitive stimuli in the form of new experiences and learning. In the late survival phase, the neuronal maturation process continues concomitant with increased synaptic activity and reduced threshold for long-term potentiation (LTP). Newborn neurons in the rodent brain becomes indistinguishable from mature neurons after approximately 7-8 weeks.

Newly born neurons have a vital role in memory formation (45), with hippocampal neurogenesis being important for spatial memory (46) and pattern separation (47), i.e. the ability to discriminate between similar experiences. The dentate gyrus is also polarized in its functionality, with the dorsal hippocampus being associated with spatial learning and memory, and the ventral hippocampus being associated with emotional response (45).

Neurogenesis has also been implicated in pathological conditions, as it is induced after ischemic stroke, traumatic brain injury and epileptic seizures (48-50). This pathological induction leads to neurons with ectopic integration, resulting in reduced memory function (51, 52). In Alzheimer's disease, there is an initial increase in neurogenesis, theorized as the brains attempt to replace degenerating neurons (53), however, this initial increase is followed by a decrease at later stages of the disease (54).

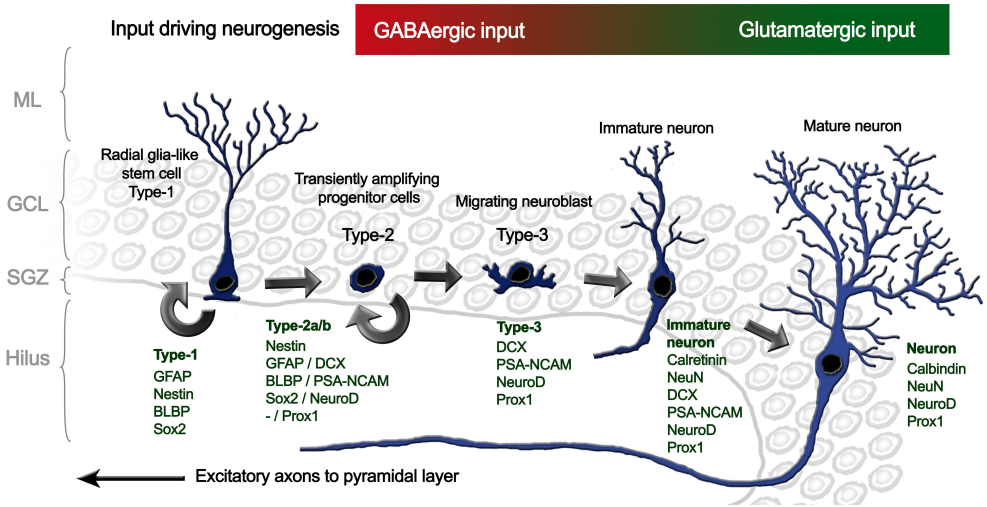

Figure 4. Developmental stages and corresponding protein markers in hippocampal neurogenesis. The figure illustrates neural stem and progenitors migrating from the SGZ into the GCL as they differentiate and integrate into the hippocampal circuitry.

Exercise-induced effects in the rodent brain

In rodents, exercise leads to improvements in learning and memory, correlated with increased adult hippocampal neurogenesis, as well as neuronal and synaptic plasticity (55, 56).

Exercise-induced adult hippocampal neurogenesis

Exercise increases proliferation, survival, differentiation, and integration of newly born hippocampal neurons (55), resulting in increased volume of the GCL (56). The neurogenic response to running results in a ~2-3 fold increase in newborn neurons, depending on genetic background (57), age (58), labeling method used (59), and distance run (60).

Running has a strong and acute effect on cell proliferation that occurs within 24 hours from start of running, peaking at four to 10 days and levels out after four weeks (61, 62). This proliferative stimulus affects primarily type-2 progenitor cells in the hippocampus, likely by increasing survival of neuronal precursor cells rather than shortening their cell cycle (63). In addition to the hippocampus, exercise has also been found to increase adult neurogenesis in the SVZ (64) and hypothalamus (65), suggesting that the neurogenic effect of exercise may occur throughout the brain. Prolonged (66), but not short-term (67), voluntary wheel running may influence neurogenesis in the SVZ. Cell proliferation in non-neurogenic regions are also regulated by exercise, but the patterns are complex and not yet fully understood (68). Apart from inducing proliferation, running also

increases survival and integration of newborn neurons (69). Even after proliferation returns to baseline, the population of immature doublecortin (DCX^+)-neurons continues to increase (62). Exercise-induced increase in number of DCX^+-cells in the dentate gyrus has been correlated with running distance (70). Related to this is the fact that forced running on a treadmill does not seem to increase hippocampal neurogenesis as much as voluntary running, which leads to considerably longer running distances (57). However, rewarding mice for running increased running but did not further enhance neurogenesis, suggesting a ceiling effect (71). Also, neither high-intensity interval training or pure anaerobic resistance training have an effect on neurogenesis in rats (57). Even though increased neurogenesis is one of the most reproducible findings from exercise in laboratory animals, mice caught in the wild do not show an increase in adult neurogenesis after exposure to a running wheel (72), suggesting that an impoverished cage environment may cause a decrease in neurogenesis under standard rodent housing conditions which can be reversed by exercise.

Exercise-induced neuronal and synaptic plasticity

Exercise also induces potent changes in neuronal and synaptic plasticity. Running accelerates maturation of adult-born DG neurons, and induces changes in neuronal morphology and connectivity, such as increased dendritic length, complexity, and spine density of granular neurons (73). Running also increases afferent connections onto newborn neurons by recruiting presynaptic inputs from the entorhinal cortex, mammillary nuclei, and medial septum, regions important for relaying content and context of experiences, spatial-temporal information processing, and short-term memory (74). The increase in adult hippocampal neurogenesis is accompanied by an increase in LTP in the DG, a phenomenon driving the strengthening of synapses and considered as one of the major cellular mechanisms in learning and memory. Exercise lowers the LTP threshold level in the DG, as well as potentiates LTP-induced synaptic response (56). These changes in synaptic plasticity are likely mediated by newly born neurons that exhibit increased expression of glutamate receptors. At the same time, running increases expression of proteins involved in inhibitory neurotransmission in the form of GABA receptor subunits in hippocampus and pre-frontal cortex, with pre-synaptic GABAergic inhibition considered to be of importance in the maintenance of memory (75).

Exercise-induced increase in blood flow and vascularization

Aerobic exercise increases blood flow in the dentate gyrus both in humans and mice (37). The increase in blood flow likely increases both metabolic and trophic support to the neurogenic niche. Many factors that stimulate neurogenesis are also implicated in angiogenesis and maintenance of the vasculature, such as erythropoietin, Notch ligands, Sonic Hedgehog, fibroblast growth factor-2 (FGF-

2), and vascular endothelial growth factor (VEGF). Exercise leads to an increase in blood flow with enhanced vascularity through vasorelaxation and angiogenesis in the hippocampus, striatum and motor cortex, known to be mediated by VEGF (76-78).

Exercise-induce improvement in mitochondrial function and oxidative stress

Mitochondrial function is a central aspect in survival and differentiation of neural progenitor cells (73), with neuronal mitochondrial density and oxidative capacity being positively influenced by exercise training. Oxidative stress results from incapacity to eliminate reactive molecules as a response to increased metabolism that attack and degrade proteins, nucleic acids and lipids. Even though there is a higher oxidative capacity in the mitochondria, exercise actually reduces oxidative stress and improves resistance to radical oxygen species (ROS) in mitochondria (79). This occurs through an increase in endogenous antioxidants, such as nitric oxide (77, 80), and detoxifying enzymes.

Exercise-induced anti-inflammatory effects

Inflammation can reduce electron-transport-chain enzyme activity, induce oxidative stress, and induce mitochondrial dysfunction, which in turn inhibits neurogenesis (81). Regular exercise has been reported to reduce low-level inflammation (82). While acute exercise appears to induce pro-inflammatory cytokines, exercise training is linked to an anti-inflammatory cytokine profile.

Running intensity

Studies have shown that wild mice spontaneously run in running-wheels that are placed out in nature (83), supporting the notion that the act of running occurs due to a reward-seeking behavior in rodents (84). Endocannabinoids have been involved in voluntary running behavior and regulates running performance (84).

Rodents are nocturnal animals being most active during the night. Voluntary running in laboratory rodents occurs almost exclusively during the active, dark, phase of the animal, initially to increase in intensity for the first days until it peaks and stabilizes at a lower level (85). Voluntary running activity occurs in bursts in a periodical pattern likely influenced by the need for recovery (86). In mice, running distance varies across strains and gender from 3 to 12 km per day, and C57BL6 mice are in the top one-third segment of mouse strains (87).

Since wheel-running triggers reward in rodents, they can keep on running for longer than what is good for them. Beneficial effects of exercise on the brain may be optimal at moderate intensity (88, 89), where more extreme levels of exercise are considered harmful. Excessive exercise leads to a high generation of ROS (90) with oxidative damage on DNA, RNA, proteins and lipids in the brain. This can

lead to disturbances in cellular, metabolic, and hormonal homeostasis, causing negative effects on learning and memory. Also, the compulsive behavior of running, despite being voluntary, might be experienced as stressful, with the release of adrenocorticotropic hormone (ACTH) and cortisol in relation to intensity and duration of exercise (91).

Modulators of adult hippocampal neurogenesis

Apart from exercise, hippocampal neurogenesis can be influenced by a many internal and external elements, such as environmental enrichment (92), stress (93), and blood-borne signals (45).

An enriched environment is a multi-faceted challenge of senses and abilities. In rodents, this can be created by a spacious home cage, ability to socialize with other animals, adding toys and objects for the animals to climb on and investigate, and, importantly, giving them access to a running-wheel (92). The novel experiences appear to increase the activity-dependent survival of new adult-born hippocampal neurons (94). Enriched environment combines the proliferative effects of physical activity with the survival-promoting effect of a learning-based environment, leading to increased survival of newborn neurons than from with a running-wheel alone (95).

The release of ACTH from the pituitary gland in response to stress, regulates the release of cortisol, an endogenous glucocorticoid, from the adrenal gland. Cortisol is important for learning and memory with glucocorticoid-receptors expressed throughout the brain including the hippocampus, prefrontal cortex, and amygdala (93). However, cortisol is a negative regulator of hippocampal neurogenesis, and reduces dendritic size and complexity of hippocampal neurons, as well as disrupts dendritic structure in the prefrontal cortex. Antidepressants, in contrast to stress, increase neurogenesis via cell proliferation, thereby associating increased neurogenesis with improvement in mood (96).

The neurogenic niches in the SGZ and SVZ interact closely with the vasculature, to allow response to changes occurring in other parts of the body via the circulation (97, 98). Radial glial-like stem cells in the SGZ, and stem and progenitor cells in the SVZ, also have direct contact with endothelial cells, thus participating in the formation of the blood-brain barrier (99, 100). Signals from vasculature have been found to regulate cell proliferation and differentiation of neural stem and progenitor cells (NSPCs). Endothelial cells can respond to circulating factors in the blood stream, mediating communication via the circulation and NSPCs. Some blood-borne factors capable of influencing neurogenesis have also been found to cross the blood-brain-barrier to exert their effects directly in the neurogenic niche (45). Further, the beneficial effects of exercise, especially on endothelial cells, could be associated with blood flow-induced shear stress, since vascular gene expression is influenced by the pattern

and magnitude of the shear stress (101), which enables endothelial cells to respond to mechanical signals from exercise-induced blood flow.

Neurotrophic factors

The plasticity-inducing effects of aerobic exercise on the CNS are mediated at least in part by neurotrophic growth factors, particularly brain-derived neurotrophic factor (BDNF) (31), but also circulating signals such as VEGF and insulin-like growth factor-1 (IGF-1).

While many growth factors have effects on proliferation, differentiation, and survival in the CNS, neurotrophins are the ones most widely expressed in the CNS (102). BDNF is the best known neurotrophin and binds to (tropomysin receptor kinase B (TrkB) expressed in neurons, whereby the complex is internalized for activation of intracellular signaling pathways. BDNF has been found to be essential for hippocampal neurogenesis, neuronal and synaptic plasticity, as well as learning and memory. Further, BDNF also has been reported to induce angiogenesis in the hippocampus (103). BDNF is increased after a single bout of exercise in different brain areas including the hippocampus (31, 104). BDNF has been found to be essential for environmental enrichment-induced and antidepressant-induced increase in neuronal survival (105).

BDNF, VEGF, and IGF-1 levels, are upregulated in the hippocampus from acute exercise, but return to basal levels within 2 days after ending chronic exercise, indicating that local upregulation only occurs from acute exercise bouts (88). Exercise-induced transcription of neurotrophic factors, such as BDNF and VEGF, has been shown to be greater in animals with lower exercise intensity than in those with higher intensity (106), supporting the idea that the dose-response relationship is not linear. Moderate and sustained aerobic exercise is therefore required for an adequate induction of neurotrophic growth factor response necessary for exercise-induced hippocampal neurogenesis (57).

Neurotrophic exercise factors

Vasculature in direct vicinity can influence hippocampal neural stem cell proliferation and differentiation through signals from both endothelial cells and the circulation (97, 99), implicating that hippocampal NSPCs can readily respond to changes in oxygen, nutrients, hormones and other factors in the blood. Exercise factors, is a term describing circulating factors regulated by exercise. Skeletal muscle, liver and adipose tissue all release a variety of molecules and vesicles into the circulation upon exercise with potent systemic effects, of which some have neurotrophic influence on the CNS (1).

BDNF, VEGF, and IGF-1, are essential for exercise-induced neurogenesis, all being upregulated in the circulation from exercise and capable of crossing the

blood-brain barrier (31, 107, 108). Additional factors have been reported to mediate effects on neurogenesis, such as cathepsin B (109), irisin (3), β-hydroxybutyrate (110), beta-endorphin (111), adiponectin (112), and angiotensin II (113).

Exercise-inducible myokines

In 1961, Goldstein conducted cross-transfusion experiments between resting dogs and dogs in which muscle contraction was induced by electrical stimulation (114). He found that muscular work triggered humeral factors that could enhance glucose utilization in resting dogs. Decades later, interleukin-6 (IL-6) was discovered to be secreted from muscle stimulation, having potent effects on glucose and lipid metabolism (115). This designated skeletal muscle as an endocrine organ and IL-6 as the first myokine.

Myokines are factors released by muscle with autocrine, paracrine and/or endocrine effects (115). Myokines mediate signaling within the muscle and crosstalk with the liver, gut, pancreas, adipose tissue, bone, vascular bed and skin (1, 116), and are involved in the effects of exercise on metabolic and cardiovascular health.

More than a decade ago, Bortoluzzi and colleagues identified ~300 myokines in human muscle (117), of which almost one quarter had not been previously characterized. Since then, many hundreds of other potential myokines have been identified, most of which are not uniquely released from muscle, but also from other organs such as adipose tissue (adipokines), liver (hepatokines) and immune cells. Exercise is known to elevate several myokines in the circulation, i.e. exercise factors, with endocrine effects, including IL-6, BDNF, VEGF, IGF-1, FNDC5/irisin, cathepsin B, FGF-21, musclin, decorin, GDF-15, IL-15, meteorin-like, myonectin, SPARC, CCL2, ANGPTL4, and BAIBA (118). Exercise-inducible myokines also have been reported to exert systemic effects on the CNS by improving spatial memory and stimulating BDNF expression in the hippocampus (3, 109, 119), see Figure 5.

Myokines regulated by PGC-1α

The mouse model overexpressing the transcription factor PGC-1α in skeletal muscle, mediates many of the cellular adaptations to endurance exercise in skeletal muscle. It led to the discovery of exercise-induced myokines with potential health-promoting effects (2). The PGC-1α pathway is known to regulate several exercise-induced myokines, including irisin, VEGF, cathepsin B, meteorin-like, BAIBA, 3-HIB, IL-15, and musclin (2, 118, 120), with some being reported to influence neuroplasticity, such as irisin, cathepsin B, and VEGF (1, 31).

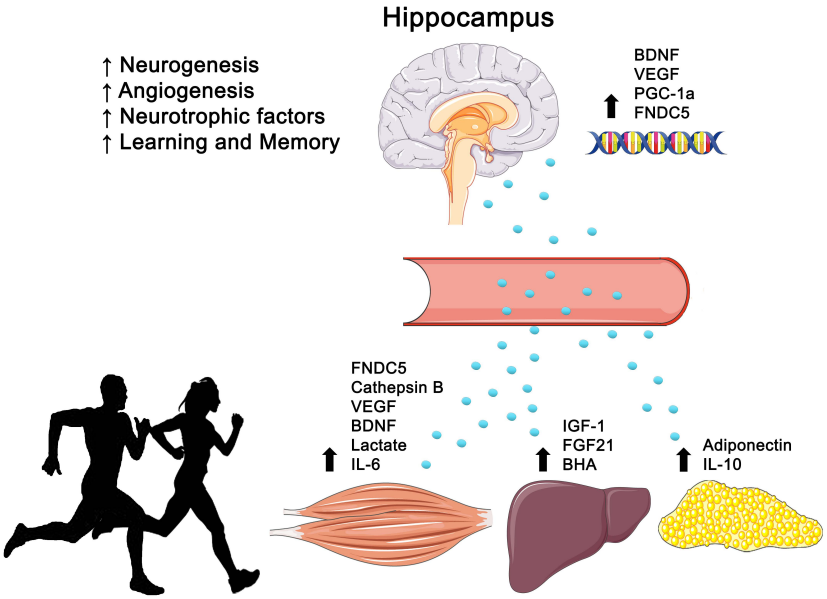

Figure 5. Neurotrophic exercise factors. Systemic factors are released from peripheral organs such as muscle, liver, and adipose tissue into the blood stream during exercise, which are capable of upregulating BDNF, VEGF, PGC-1α, and FNDC5, in the hippocampus, thereby resulting in increased neurogenesis, angiogenesis, neurotrophic factor expression, and learning and memory.

FNDC5/irisin

In 2002, a novel peroxisomal and membrane-bound protein named PeP was discovered, which later was renamed fibronectin type III domain-containing protein 5 (FNDC5). In 2012, Boström and colleagues discovered FNDC5 to be a PGC-1α-inducible protein that is cleaved and secreted into the circulation as the myokine irisin upon exercise (2). Irisin can induce browning of white adipocytes by increasing lipid metabolism, thermogenesis, and energy expenditure. From experiments in muscle and liver cell cultures, irisin also promotes intracellular uptake and storage of glucose and lipid, and in muscle cells promotes the shift from carbohydrate to fat metabolism (121). Irisin has also been reported to improve mitochondrial function in kidney tubule cells and being able to protect from kidney damage and fibrosis (122).

Peripheral overexpression of FNDC5 protein in the liver by an adenoviral vector upregulated BDNF levels specifically in the hippocampus, but not in the forebrain (3). It is unclear if FNDC5 exert its effects directly by passing the blood-brain barrier (BBB), or indirectly through endothelial cells or other peripheral factors.

Further, intravenous administration of an FNDC5-containing adenoviral construct lead to overexpression of the protein in both brain and circulation, resulting in ameliorated memory impairment and improved synaptic plasticity in a model of Alzheimer's disease (123). In the same mouse model, blockade of peripheral FNDC5 attenuated neuroprotective effects of exercise on memory and synaptic plasticity. Apart from being expressed in skeletal muscle and heart, FNDC5 has also been found to be highly expressed in the brain, including cerebellar Purkinje neurons and hypothalamus (124). FNDC5 has been found to be important for neuronal development and to be capable of inducing neuronal differentiation in embryonic neural stem cells (125). In a study by Wrann and colleagues, exercise upregulated *Fndc5* mRNA in the hippocampus in a PGC-1α/ERRα-dependent manner (3). Overexpression of FNDC5 in cortical neuronal cultures increased BDNF gene expression, while knock-down of FNDC5 inhibited BDNF transcription. BDNF treatment of neuronal cultures led to downregulation of FNDC5 gene expression, indicative of a negative feedback mechanism. Even though FNDC5 is involved in neuronal differentiation, irisin did not induce neuronal differentiation in a murine neural stem cell line, but increased proliferation at 10 times the physiological concentration (126). Irisin protected against neuronal cell injury due to oxygen and glucose depravation (127), as well as improved morphological and functional outcome in a mouse model of middle cerebral artery occlusion (128). Further, blockade of irisin with an intravenously administered antibody attenuated the neuroprotective effects of physical exercise against cerebral ischemia.

Whether exercise leads to increased levels of irisin in the bloodstream of humans has been controversial (129), which we will return to in the Discussion. Through the use of tandem mass spectrometry, circulating irisin was demonstrated to be upregulated with exercise training in humans at concentrations comparable to essential metabolic hormones, such as insulin and leptin (130). However, the functional relevance of irisin in humans remains to be determined.

VEGF

Neurogenesis and angiogenesis in the hippocampal neurogenic niche are closely co-regulated, with many of the factors that influence angiogenesis also influencing neurogenesis and maintenance of the vasculature. VEGF is one of the most important pro-angiogenic factors in most tissues and mediates its effect by binding to the tyrosine kinase receptor on endothelial cells (131). Intracerebral infusion of VEGF enhances angiogenesis, hippocampal neurogenesis, and cognition (131), with exercise alleviating anxiety and depression in a VEGF-dependent manner (132).

Exercise induces VEGF expression specifically in the cortex, hippocampus, muscle and lung (133). VEGF is rapidly expressed in skeletal muscle cells after

an acute exercise bout (133, 134), where it has an essential role in neovascularization and endurance capacity. VEGF is also acutely and transiently upregulated in the circulation with exercise, but has a short half-life in blood (135) and does not appear to be upregulated in the circulation during the resting period in regular exercise training (88).

Peripheral blockade of VEGF inhibits exercise-induced neurogenesis (107), but also neurogenic effects of enriched environment and anti-depressants (131, 136). Interestingly, selective ablation of VEGF in skeletal muscle is sufficient to inhibit exercise-induced effect on neurogenesis (137). It is still unclear if VEGF can cross the BBB, or if the effects on CNS are mediated through endothelial cells or indirect signaling to other peripheral organs.

Cathepsin B

Proteomic and biochemical analyses from treatment of L6 myotubes with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), led to the identification of cathepsin B in conditioned medium. This protein, capable of crossing the BBB, was upregulated in skeletal muscle and increased in muscle, hippocampus and plasma of mice, rhesus macaques and humans following 4 months of treadmill training (109). Treatment of hippocampal NSPCs with recombinant cathepsin B increased levels of *Bdnf* mRNA, BDNF protein and levels of DCX (109), but did not affect cell proliferation.

Kynurenic acid

The essential amino acid tryptophan is metabolized to kynurenine, a substance which upon accumulation in the CNS can lead to neuroinflammation, depression and stress (138). Kynurenine aminotransferases convert kynurenine to kynurenic acid, which is not able to cross the blood–brain barrier (138). Exercise activates the PGC1α–PPARα–PPARδ pathway in skeletal muscle, which stimulates the expression of kynurenine aminotransferase, reducing plasma levels of kynurenine in rodents and humans (139). Transgenic mice with muscle-specific PGC1α overexpression are protected from neuroinflammation and depression induced by chronic stress (138).

BDNF

Even though BDNF is a crucial neurotrophic growth factor in the brain, the role of circulating BDNF in regulating exercise-induced plasticity is unclear. In human studies, BDNF is upregulated in a dose-dependent manner in the circulation correlated with improved spatial and verbal memory, enhanced pattern recognition, as well as alleviation of chronic stress and cognitive impairment (140, 141). With between 70-80% of circulating BDNF considered to be released from the brain, changes in circulating BDNF following exercise may reflect altered

BDNF expression in the CNS (104). A smaller portion of BDNF is also produced peripherally by skeletal muscle, endothelial cells, and immune cells, such as T-, B-cells and monocytes (141). In muscle, BDNF is being used at the neuromuscular junctions, where it acts to induce protein synthesis and lipid metabolism. Even though BDNF is an exercise-inducible myokine, it is unclear to what extent the protein is released from muscle into the bloodstream during exercise (142). BNDF can pass through the BBB by passive diffusion (141), with levels of BDNF in the brain being directly influenced by levels in the blood, and vice versa. In blood, 90% of BDNF is stored in platelets and released during clotting, thus, serum includes both freely and stored BDNF, with plasma only including the free fraction.

Metabolites

Lactate, a source of energy for CNS neurons, may have a role in exercise-induced changes in the brain, with blood-born lactate released from contracting muscles mediating VEGF-dependent vascularization in the CNS (143). Further, BAIBA is an example of a PGC-1α regulated metabolite released from muscle cells that induces adaptive thermogenesis in adipocytes, beta-oxidation in hepatocytes, and improves glucose homeostasis (144). However, its effects on the CNS are unknown.

Inflammatory mediators

Exercise induces muscle damage and release of inflammatory molecules after exercise. Immune cells control inflammatory reactions and support regeneration of muscle tissue following exercise.

One example of an inflammatory mediator is IL-6, a cytokine with complex effects in the body, with effects varying depending on its mode of release. The cytokine was originally classified as a pro-inflammatory cytokine, and chronically elevated levels of IL-6 in the circulation was associated to inflammation and metabolic disease (116). However, IL-6 is also released from muscle upon exercise with acutely elevated levels in the circulation associated with anti-inflammatory and beneficial metabolic effects. The cytokine also has a vital role in regulation of glucose homeostasis and lipid metabolism through effects on skeletal muscle, liver, adipose tissue, and pancreatic cells (115). IL-6 can reach up to 100-fold concentrations in the blood after intense exercise and is capable of crossing the BBB (116, 145). IL-6 is also directly upregulated in the hippocampus following exercise, and has been found to regulate both cognition and neurogenesis (146).

Other examples of inflammatory mediators serving as neuromodulatory exercise factors are CXCL12, a chemokine implicated in learning and memory, IL-8, a

cytokine involved in regulating neurotransmission and synaptic plasticity, and IL-15, a cytokine with an important role in adult neurogenesis (147).

Adiponectin

More than two decades ago adipose tissue was discovered to be an endocrine organ, with many hundreds of adipokines being released into the circulation capable of affecting a range of physiological processes in the body (1). Adiponectin is an example of an adipokine, which has insulin-sensitizing, anti-inflammatory, anti-atherogenic properties and neuroprotective effects (112). Adiponectin is increased in the circulation from exercise and can pass through the blood–brain barrier to induce cell proliferation and anti-depressive effects, suggesting that adiponectin is involved in mediating the effect of exercise on hippocampal neurogenesis and depression (112).

Hepatokines

Liver also releases hepatokines with endocrine effects that regulate glucose and lipid homeostasis (148). Several hepatokines are known to be involved in organ cross-talk. For example, hepatokines such as IGF-1 and FGF-21 may mediate crosstalk between the liver and brain in response to exercise.

IGF-1 is an important metabolic regulator, primarily with insulin-sensitizing effects. It is produced in the liver, muscle, and the brain (148). IGF-1 crosses the BBB to mediate neuroplasticity and neuroprotection. IGF-1 upregulates BDNF expression in the hippocampus, increases the number of newborn neurons in the DG, and restores abilities to perform hippocampus-dependent tasks (149, 150). Blockade of circulating IGF-1 using antiserum inhibits the exercise-induced increase in adult hippocampal neurogenesis (108, 149), indicating that exercise-induced effects on the brain rely on an increased uptake of IGF-1 from blood into the brain.

Systemic FGF-21 is released from the liver during exercise, having a role in regulation of metabolism mediated in part by acting on the CNS (1). FGF-21 is induced through the PPARα and PGC-1α pathways, and can cross the BBB. FGF-21 has also been shown to prevent cognitive decline in obese insulin-resistant rats by improving hippocampal synaptic plasticity and brain mitochondrial function (151).

Ketone bodies

The formation of ketone bodies has recently been shown to play an important role in the effects of exercise on the brain. Ketone bodies are markedly increased in the circulation and brain after fasting, dieting and intense exercise (1). Under conditions of reduced glucose levels, such as prolonged exercise, excessive acetyl-CoA is redirected into formation of ketone bodies, such as acetoacetate and β-

hydroxy acid (BHA), to serve as an energy source in the body. BHA crosses the blood-brain barrier, accumulates in the hippocampus, which increases expression of BDNF through HDAC inhibition (110), and can act as a neuroprotectant in experimental models of Huntington and Parkinson disease (1).

Exercise-induced changes in muscle and PGC-1α

Physical exercise impacts the entire body, but the organ that is activated most strongly by the energy demanding mechanical work is the skeletal muscle system. Muscle is a regulator of whole-body energy metabolism and an endocrine organ producing and releasing factors that have vital roles in communication with other organs (152). After an acute bout of exercise, muscle cells respond with a robust upregulation of metabolism-related genes. Skeletal muscle undergoes adaptations from regular aerobic exercise that lead to improved energy metabolism, mitochondrial density, oxidative capacity, fatty acid oxidation, glucose uptake, angiogenesis, and muscle fiber-type switching (2, 153).

Skeletal muscle is a heterogeneous mixture of different types of myofibers classified on the basis of specific myosin heavy-chain isoform expression. Type I myofibrils are slow-twitch fibers, due to their slow contraction time to peak tension, and type II fibers fast-twitch with quicker contraction but rapid fatigue profile (154). Of the main fiber types in rodents, type I and IIa exhibit high oxidative potential and capillary supply, while IIb are primarily glycolytic. Exercise activates adaptations in skeletal muscle dependent on the type of exercise performed. While aerobic/endurance exercise promotes increased mitochondrial biogenesis, oxidative capacity, and glycolytic-to-oxidative fiber-type switching, anaerobic/resistance exercise promotes hypertrophy and oxidative-to-glycolytic fiber-type switching (19). Endurance exercise mainly leads to a switch in fiber type from fast-twitching, glycolytic type IIb fibers to more oxidative, slow-twitching type I fibers.

Muscle contractions leads to many intracellular signals (e.g. increased sarcoplasmic calcium, increased AMP/ATP ratio, increased ROS levels, and increased NAD^+/NADH), activating several signaling pathways such as calcium-calmodulin-dependent kinases, calcineurin, mitogen-activated protein kinases (p38 MAPK, ERK1/2), PGC-1α, and PPARα/γ/δ (19, 154), see Figure 6. Sarcolemma Ca^{2+}-signaling through calcium/calmodulin-dependent protein kinases (CaMKII and CaMKIV) and calcineurin A (CnA) activation lead to phosphorylation and activation of p38 MAPK, MEF2, and ATF2, which interact with binding sites of PGC-1α promoter to upregulate PGC-1α transcription. Another activation pathway occurs through the generation of AMP by the hydration of ATP and ADP, sensed by AMPK, which in turn induces and phosphorylates PGC-1α. Yet another activation pathway occurs through modulation of NAD levels, which activate SIRT1 through deacetylation and

regulate important post-translational modifications of PGC-1α. Further, accumulation of free radicals in the skeletal muscle from exercise, sensed by via ROS/RNS ratio, signals that the antioxidant system should be turned on by activating PGC-1α.

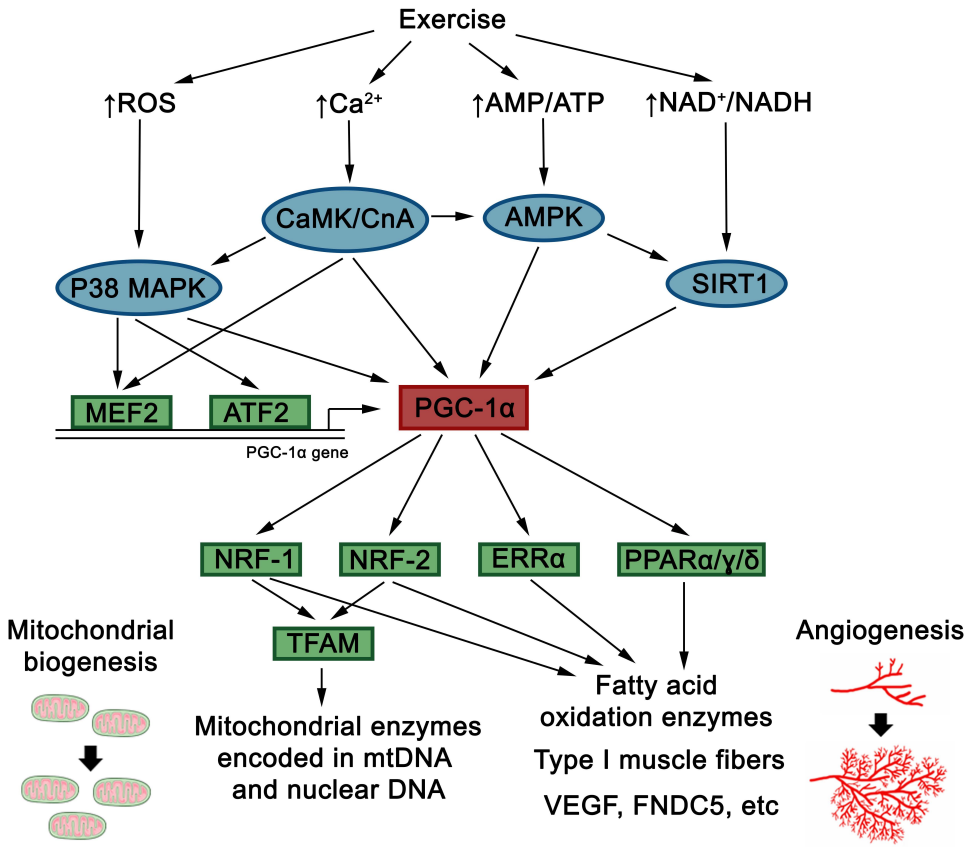

Figure 6. Signaling pathways from exercise in skeletal muscle with downstream effects such as mitochondrial biogenesis and angiogenesis. PGC-1α is activated by p38 MAPK, CaMK/CnA, AMPK, and SIRT1. P38 MAPK and CaMK/CaN also induces transcription of PGC-1α through activation of nuclear factors such as MEF2 and ATF2. PGC-1α mediates myocellular adaptations to exercise by interacting with NRF-1/2, ERRα, and PPARs.

The transcription factor PGC-1α is a master regulator of mitochondrial biogenesis that mediates many exercise adaptations in skeletal muscle including enhanced oxidative capacity, antioxidant factors, fatty acid oxidation, glucose uptake, angiogenesis, and muscle fiber-type switching (2, 153), as well as the release of neurotrophic factors into the blood stream, such as growth factors, hormones,

cytokines and metabolites (2, 144, 155). In loss of function studies, PGC-1α knock-out mice have a lower ratio of oxidative to glycolytic muscle fibers, lower number of mitochondria, reduced oxidative capacity, and reduced endurance capacity (154). Even though PGC-1α is not essential for exercise-induced mitochondrial biogenesis or fiber type change in skeletal muscle (156), it seems to be essential for exercise-induced angiogenesis (157).

Exercise induces PGC-1α expression also in various areas of the brain with PGC-1α being involved in neuronal differentiation and function, through the formation and maintenance of neuronal dendritic spines (1, 3). PGC-1α has also been reported to mediate neuroprotection in animal models of neurodegenerative disease and cerebral ischemia (128).

Exercise, muscle regeneration and metabolism are all linked to inflammatory mechanisms. Overexpression of PGC-1α has an inhibiting effect on NFkB (158), resulting in an anti-inflammatory pattern of cytokine expression and upregulated anti-oxidant defense.

MCK-PGC-1α

Transgenic mice with skeletal muscle-specific PGC-1α overexpression under the muscle creatinine kinase promoter (MCK-PGC-1α) display a constitutive endurance exercise phenotype, with increased mitochondrial density, oxidative capacity, and vO2max, as well as improved lipid oxidation, glycolytic-to-oxidative fiber-type switching, resistance to muscle fatigue, and increased endurance exercise performance (153, 155, 159-161). While PGC-1α expression is induced in skeletal muscle and brain of exercising mice (3, 162), MCK-PGC-1α transgenic mice have chronic overexpression of PGC-1α in skeletal muscle, but no upregulation in the brain (163). Transcripts for several myokines are upregulated in skeletal muscle of MCK-PGC-1α animals, including *Fndc5*, *Vegf*, *Bdnf*, *Ctsb* (cathepsin B), and *Il15* (2, 120, 122). Interestingly, irisin, as well as BDNF, and IL-15, circulates at 2-fold higher levels in MCK-PGC-1a mice compared to wildtype (122).

In skeletal muscle, MCK-PGC-1α mice show protection from denervation- and fasting-induced muscle atrophy (164, 165), as well as Duchenne muscular atrophy (166, 167). PGC-1α expression in skeletal muscle has been reported to decrease with age in both rodents and humans (168, 169), with MCK-PGC-1α mice having improved muscle mitochondrial function in aging, and a muscle gene expression profile similar to that of young mice (170, 171). MCK-PGC-1α animals are protected from age-related motor dysfunction (163, 171) and have a slightly increased life span (170). Further, PGC-1α mediates mitochondria-dependent recovery in a mouse model of accelerated aging (172, 173), in which muscle-specific overexpression of PGC-1α was able to ameliorate both age-related mitochondrial dysfunction in muscle, as well as age-related signs of anemia (174).

Systemically, MCK-PGC-1a mice have improved kidney energy metabolism with protection against kidney damage and fibrosis, an effect that was found to be mediated by circulating irisin (122). In the brain, MCK-PGC-1α mice show protection from stress-induced neuroinflammation associated with depression (138). Due to anabolic effects in the form of accumulation of lipid and glucose, along with inhibited glycolysis, sedentary MCK-PGC-1α mice are more susceptible to fat-induced insulin resistance (160, 175). However, when exercising MCK-PGC-1α mice are subjected to a high-fat diet, this instead enhances weight loss and whole-body glucose homeostasis (176). In the same manner, both skeletal muscle-specific overexpression of related transcription factors, PPARδ (177) and ERRγ (178) have also failed to protect against diet-induced metabolic derangement.

AMPK and PPARδ skeletal muscle activation

AMPK is a key regulator of glucose metabolism and exercise-induced adaptations in muscle (19). AMPK is activated by low AMP to ATP ratio, which occurs during exercise in humans, triggering PGC-1α-dependent muscular adaptations to aerobic exercise such as glycolytic-to-oxidative muscle fiber-type switching and improved mitochondrial energy capacity. AICAR, an activator of AMPK, and voluntary exercise have similar effects on muscle metabolism, despite no energy being expended, with both treatments reliably upregulating PGC-1α expression (179). However, AICAR does not mimic all the exercise-induced effects in the body as it failed to increase VO_2max in rats, casting doubt on the exercise-mimicking claims of the AICAR model (180). Kobilo and colleagues studied the effects of hippocampal neurogenesis and hippocampus-dependent learning by intravenous injections of AICAR and GW501516 (GW) (119). Both treatments, but more so AICAR, had an effect on spatial memory and hippocampal neurogenesis at 1 week. AICAR treatment-induced enhancement of memory function was precluded by muscle-specific AMPK α2-subunit deficiency (181), indicating that AICAR-induced effects on the brain are primarily due to crosstalk between muscle and brain. In an experiment comparing the exercise effect and AICAR, Guerrieri and van Praag found that the action of both treatments resulted in increased proliferation in the DG and BDNF expression in the hippocampus (Guerrieri and van Praag 2015). However, the CNS effect of AICAR was lost at after 2 weeks of treatment and genes related to oxidative stress and inflammation were upregulated in skeletal muscle at this time point. This indicates that AMPK-activation likely can have positive short-term effects, but no or even negative long-term effects (182). There are off-target effects due to AMPK receptors in other tissue types and the brain could be particularly sensitive to extended activation of AMPK (183).

A physiological effect of endurance exercise is to improve fatty acid oxidation (19) to delay the depletion of glucose as the primary energy source. PPARδ is a

key regulator of substrate utilization, also regulated by AMPK, which increases fatty acid oxidation and represses glycolytic genes in muscle to slow glucose consumption and maintain glucose levels during exercise. Pharmacological PPARδ activation by the agonist GW yielded animals with improved exercise capacity, increased insulin sensitivity, and resistance to diet-induced obesity (179). When administering a higher dose of GW for longer duration, animals were able to run for longer periods due to higher availability of glucose in the circulation (184). However, GW treatment did not induce changes in muscle usually occurring with exercise such as increased mitochondrial density, improved vascularity, or fiber-type switching. GW treatment has been found to improve hippocampal neurogenesis and spatial memory, but not as strongly as AICAR (119). Since GW is unable to cross the BBB, it likely exerts its effects indirectly through muscle-brain cross-talk (109, 119, 179).

Finally, it should be noted that studies using transgenic animals and pharmacological manipulation have shown that many regulators involved in the muscle activation cascade are sufficient for upregulating mitochondrial biogenesis, substrate utilization, and fiber-type transformation, even though many are not essential for exercise-induced muscular adaptations (154). This indicates that there are numerous redundancies in the signaling network for exercise-induced skeletal muscle adaptations, which is likely of evolutionary importance.

Cranial irradiation

Cranial radiation therapy is an important treatment modality for brain tumors and other forms of cancer. However, the treatment often results in long-term irreversible cognitive deficits, especially for the juvenile brain, which in part is caused by irradiation-induced reduction in hippocampal neurogenesis (185). Cranial irradiation is effective at eradicating cells with prominent proliferative capacity, such as tumor cells, but stem and progenitor cells in the hippocampus are also severely affected (186). Irradiation also induces vascular abnormalities, demyelination, and white matter necrosis (187). Irradiation causes DNA strand breaks, interferes with cell-cycle regulatory proteins and leads to apoptosis (188). Furthermore, irradiation creates a hostile microenvironment in the brain tissue with neuroinflammation, microglia activation, pro-inflammatory cytokine release, oxidative stress, and reduction of neurotrophic factor (185, 189). Physical exercise can counteract irradiation-induced changes by attenuating neuroinflammation (82), upregulating trophic support (190), restoring neurogenesis, and improving learning and memory (191).

Cortical stroke

Ischemic stroke is a major cause of mortality and disability worldwide (192). Aerobic exercise reduces the risk for ischemic stroke and can improve functional and morphological recovery after stroke (21, 193).

Ischemic stroke starts with necrosis, apoptosis and inflammatory reactions, leading to disruption of the BBB, neuroinflammation and oxidative stress, which aggravates the primary ischemic tissue damage (194). Also, peri-infarct depolarization, excitotoxicity, and apoptosis, are important causes of cell death following ischemia (195). Early central events include the production of ROS, proteolytic enzymes, pro-inflammatory cytokines, chemokines, and vascular adhesion molecules (194). Exercise can prevent secondary ischemic damage by inhibiting pro-inflammatory cytokine release, activation of microglia and astrocytes, and reduction of leukocyte recruitment into the brain parenchyma (194).

Even though astrocytes and immune cells contribute to the secondary tissue damage, they are also important for limiting ischemic injury and promoting regeneration. Astrocytes are important for maintaining brain homeostasis and preserving the BBB (194). Astrocytes contribute to recovery by inducing angiogenesis, neurogenesis and secretion of trophic factors. Microglia and blood-borne macrophages are rapidly recruited and activated. They acquire either a classic pro-inflammatory or an alternative anti-inflammatory profile, by scavenging of cell debris and releasing neurotrophic factors, which promote neuronal survival and plasticity (194).

BDNF is released in the brain tissue after stroke (196) which improves mitochondrial metabolism and plays a critical role in repair processes (194), with exercise being able to promote the release of neurotrophic factors, including BDNF, after brain ischemia (194).

Importantly, exercise also ameliorates ischemia-dependent reduction of mitochondrial biogenesis (197). These PGC-1α mediated exercise-inducible changes may have an impact on oxidative stress and mitochondrial biogenesis-dependent recovery after CNS insults, such as ischemic stroke (198).

Cerebral blood flow is influenced after stroke due to impairments in vasomotor reactivity (77). To ensure a proper delivery of oxygen and nutrients the vasomotor capacity needs to be restored. Exercise leads to vascular remodeling, vasorelaxation and increased blood flow in the brain parenchyma, which lead to improved tissue regeneration, as well as short- and long-term functional recovery after stroke (80, 196). Exercise reduces infarct sizes and neurological sequel in middle cerebral artery occlusion (77), due to VEGF-mediated increase in cerebral blood flow (80).

Aging

Adult hippocampal neurogenesis gradually diminishes with age, a deterioration process considered to contribute to age-dependent cognitive decline (199, 200). Mechanisms behind this age-related effect include low-level systemic inflammation, neuroinflammation with microglial activation, mitochondrial dysfunction, oxidative damage, telomere dysfunction, genomic instability, dysfunction of the BBB, reduced cerebral blood flow, abnormal protein accumulation and phagolysosomal function, reduced trophic support, depletion of quiescent neural stem cells, shift from neuronal to astroglial commitment of neural stem cells, and decreased neuroplasticity (201-203). All of the above mentioned changes have been reported to be directly or indirectly ameliorated by exercise (204). In animal models, exercise has been observed to prevent age-related decline in neurogenesis (62), as well as to enhance angiogenesis, synaptogenesis, and to upregulate several neurotrophic factors (31). Exercise attenuates age-dependent decline in neurogenesis (58) by maintaining the hippocampal stem cell pool during aging and promoting neuronal lineage commitment of NSPCs (205). The PGC-1α pathway plays a part in exercise-induced effects in the brain by regulating mitochondrial biogenesis, oxidative capacity, and ROS production in the CNS (3, 162, 206), and as a consequence, mitochondrial biogenesis-dependent recovery may impact age-dependent decline in hippocampal neurogenesis (207). Similar to exercise, PGC-1α overexpression is also known to modulate many of the processes of aging, such as inflammatory cytokine profile, telomere dysfunction, mitochondrial dysfunction, oxidative stress, insulin resistance, and genomic instability (208). Further, aging reduces exercise-induced adaptations in the muscle, with diminished induction of PGC-1α, NRF-1, and cytochrome c (209), to levels comparable to that of PGC-1α knock-out mice. The age-related changes in muscle can be ameliorated by overexpression of PGC-1α in muscle, which appears to rejuvenate aging tissue and enhance a subset of young-like molecular patterns (170).

The regenerative potential of tissue-specific stem cells diminishes with age (199, 210), and loss of regenerative capability contributes to loss of tissue function (211). Thus, by improving regeneration, tissue function could be improved. Enhancement of aged stem cell functions can be accomplished either by restoration of stem cell-intrinsic characteristics or restoration of the microenvironment (210, 211). Transplanting muscle cells from aged animals into young animals restores their regenerative capacity, but transplanting muscle cells from young animals into aged animals reduces their regenerative capacity (212), leading to the notion that systemic factors could influence the microenvironment of the stem cell niches (211). By reducing the inflammatory environment, neural stem cells are thought to be revitalized (201). Therefore, targeting the systemic

environment could be an effective approach to improve stem cell regeneration and tissue function.

Exercise is one such strategy that works to improve stem cell functions by an increase in autophagy, protection from metabolic stress, increase in telomere lengths, counteraction of telomere shortening, and promoting neuronal lineage differentiation (204, 205). Another treatment strategy is 'young blood' that has recently been reported to rejuvenate many organs including the brain, with improved hippocampal function at a molecular, cellular, structural, and behavioral level. Transfusion of blood or plasma from young mice, or umbilical cord, into aged mice increases neurogenesis, reduces microglial reactivity, and improves learning and memory (201, 213). Several anti-aging factors have been identified in young blood, such as TIMP2 and GDF11, which, through systemic administration, have been found to improve synaptic plasticity, hippocampal neurogenesis, and cognitive functions in aging (201, 213). Likewise, the disruption of the vascular structure and cerebral blood flow has been reported to be able to recover in aged parabionts exposed to young factors (213). Several studies have also observed positive effects in various tissue and injury models through local and systemic administration of mesenchymal stem cells (214). It is possible that factors in blood regulated by aging also are regulated by exercise.

AIMS

In this thesis we sought to determine if muscle-derived exercise-induced signaling via PGC-1α muscle activation influences neuroplasticity under physiological or pathophysiological conditions and if factors are released into the circulation that contribute to exercise-induced effects on the CNS.

These specific aims were addressed:

- To determine if muscle-specific PGC-1α overexpression can ameliorate irradiation-induced decline in neurogenesis or improve morphological outcome in cortical stroke (**paper I**).
- To determine if muscle-specific PGC-1α overexpression contributes to exercise-induced neurogenesis in aging, and if this contribution is sex-dependent or enhanced in a running wheel paradigm (**paper II** and **paper III**).
- To determine if serum-induced changes from exercise and muscle-specific PGC-1α overexpression, or conditioned medium from PGC-1α-overexpressing muscle cells, can influence the behavior of neural stem cells (**paper IV**).

METHODOLOGY

In this chapter the rationale for the methodology used in this thesis is outlined. See the Methods sections in the corresponding thesis papers for detailed descriptions of the methods.

Animal models

Transgenic animals and genotyping

Transgenic MCK-PGC-1α animals on C57BL/6J background have been previously described (153). Female C57BL/6J mice were used for breeding purposes. Transgenic animals were bred as hemizygous mice and wild type (WT) C57BL/6J) littermates were used as controls. Animals were housed at a constant temperature (24°C) with 50-60% relative humidity. A 12-h light/dark cycle was maintained with lights from 07:00 to 19:00 and with *ad libitum* access to food and water. All experiments were approved by the Gothenburg ethical committee on animal research (#317-2012 and #181-2015) and performed in accordance with relevant guidelines and regulations. Genotyping was performed as described by the donating investigator (Bruce Spiegelman, Harvard University, Boston) in the Jackson Laboratory database (Stock no. 008231).

Comments:

Muscle-specific overexpression of PGC-1α in a transgenic mouse model yields a chronic activation of skeletal muscle cells with an improved muscle function and an endurance exercise phenotype. We used both young (3-month-old) and older (11-month-old) animals of both sexes for the projects. Young male animals were used for irradiation and stroke (**paper I**). Young female animals were used for the running experiment (**paper III**). Older male and female animals were used for the aging and running experiments (**paper II** and **paper III**).

MCK-PGC-1α animals were originally created by injection of transgenic construct containing the PGC-1α gene under the control of muscle creatine kinase promoter into fertilized C57BL6 mouse oocyte in Bruce Spiegelman's lab and bred as hemizygotes (153). Mice that are hemizygous for the transgene are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. Animals display a clear redness of skeletal muscle that allowed easy identification of the phenotype, see Figure 7.

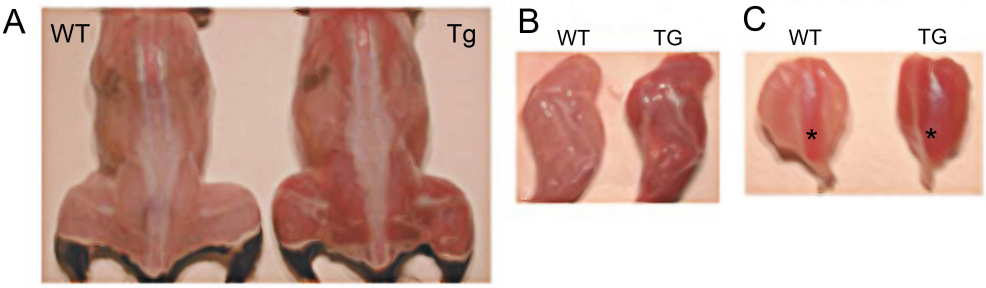

Figure 7. Oxidative muscle phenotype of transgenic MCK-PGC-1α mice. Image shows **(A)** a dorsal view of WT and transgenic MCK-PGC-1α (TG) animals, morphology of **(B)** hindlimb and **(C)** gastrocnemius/soleus (asterisk) muscle. The more intense red coloring of the transgenic muscles is due to the higher level of oxygen transporter protein myoglobin, associated with higher mitochondrial density. Image reprinted from (153) with permission from Springer Nature.

Genotyping was performed preferably before weaning. Pups were sedated by isoflurane anesthesia and earmarked. DNA extracted from earclippings were used in PCR with primers for detection of both an internal C57BL6 control gene (384 bp) and for the transgene insert (168 bp). Animals that had the transgene insert were identified as transgenic animals and the animals that did not have the transgene insert were identified as wildtype, see Figure 8.

Comments on aging:

Mice have been used extensively in experimental research as a mammalian model for humans. Mice and humans reach reproductive age at 35-50 days and 13 years, with maximum life span being 4 and 120 years, respectively (215). At an age of 30 months, 50% of C57BL/6 have died, which would correspond to an age of approximately 70 years in humans. The ages of young adult mice at 3 months and older animals at 11 months used in **paper II** and **paper III**, would correspond to teens and fifties, respectively (216). Although senescent changes begin in middle age in mice (10-15 months) markers of aging are not present (216). The age difference used in our aging studies was sufficient to observe significant decline of neurogenesis with age, even though the difference would have been greater at an even higher age.

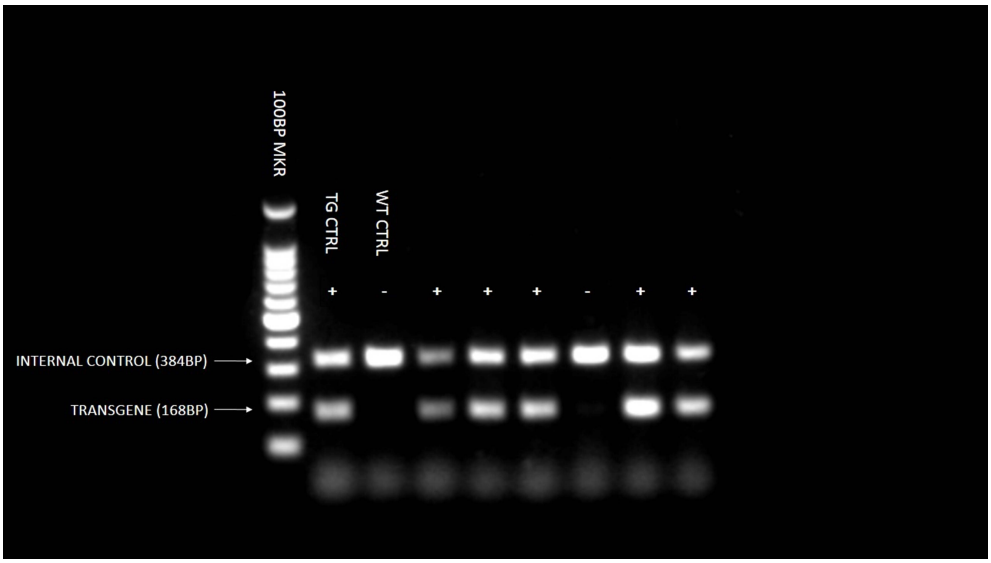

Figure 8. Electrophoresis gel from genotyping of MCK-PGC-1α mice.

Irradiation procedure

In **paper I**, male 4-month-old mice were anesthetized with an intraperitoneal injection of tribromoethanol. The head was covered with a 1 cm tissue-equivalent material to ensure an even irradiation dose into the underlying tissue. Whole-brain irradiation was administered as a single dose of 4 Gy using a linear accelerator. The sham-irradiated control mice were anesthetized but were not subjected to irradiation. After irradiation, the animals were returned to their home cages. Animals received daily intraperitoneal injections with BrdU from day 3 to 8 post-irradiation, and were euthanized and perfused 28 days after irradiation.

Comments:

A linear accelerator produce megavoltage x-ray beams by the deceleration of electrons in a tungsten alloy (217). Ionizing radiation affects all atoms and molecules in the cell, but DNA injury is considered to be the most important factor for the induction of cell death. Damage to DNA occurs by either breakage in both DNA strands due to a single ionization event, or by two single-strand breaks happening on the same strand. Cell damage can also take place by the formation of free radicals, by the ionization of other molecules that indirectly exerts toxic effects on DNA. The most radiation-sensitive cells are those that are undifferentiated and quickly dividing, such as stem cells, progenitors, and cancer cells. The absorption of energy to tissue is measured in Gray (Gy) units, where 1 Gy corresponds to 1 Joule/kg.

Photothrombotic stroke procedure

In **paper I**, male 3-month-old mice were subjected to a photothrombotic stroke in the sensory-motor cortex area. The lesion was induced by peritoneal injection of Bengal rose and an infrared laser beam aimed at the exposed skull. After the procedure animals were returned to their cages. Animals were given daily intraperitoneal injections with BrdU from post-lesion day 7 to 10, to be euthanized and perfused 28 days after stroke.

Comments:

The photothrombotic stroke model has both advantages and disadvantages. It is a minimally invasive method, with low mortality and highly reproducible cortical lesions (218). The model induces an end-capillary thrombosis that leads to a well-defined lesion and a marginal penumbral zone. This clear border can facilitate the study of cellular responses within the ischemic or intact cortical area. Similar to artery occlusion in naturally occurring thromboembolic stroke, platelet aggregation and clot formation interrupt blood flow in the laser-illuminated area. However, thrombosis in human stroke is caused by interruption of blood flow in a single artery, whereas photothrombosis produces simultaneous clotting in a large number of blood vessels within the illuminated area. Thus, collateral blood supply, which normally is capable of preventing necrotic cell death in the penumbra, is hindered in photothrombosis. The penumbra is the main target of post-ischemia neuroprotective agents, which makes the photothrombotic stroke less ideal for studying these agents. Also, photothrombotic stroke does not lead to reperfusion-injury as can occur in human stroke, and to study this type of injury a transient occlusion model is more appropriate. Further, photothrombotic stroke may not be adequate to study anti-thrombotic agents due to the fact that photothrombotic infarction occurs despite blocking platelet aggregation and inhibition of the intrinsic coagulation pathway (219).

Voluntary running

In **paper III**, female and male wildtype and MCK-PGC-1α mice at 2 and 10 months of age were single-housed in cages with free access to locked low-profile running wheels. In **paper IV**, male and female mice at ages of 7-10 months, were acclimatized with locked low-profile running wheels in groups of 2-4 animals per cage (single-housed for males) for animals whose serum was used in proliferation and differentiation assays, and single-housed for females used in RT-qPCR experiment. After 5 days, running wheels were unlocked for half of the animals, and the running activity was wirelessly monitored for each cage throughout the experiment. Wildtype and transgenic animals were randomly assigned to voluntary running or to the sedentary control group. All animals were euthanized

and perfused 28 days after the first day of irradiation, stroke, or start of BrdU injection.

Comments:

Forced exercise models such as swimming and treadmill running results in a higher degree of standardization in an exercise paradigm compared to voluntary running. However, forced exercise results in anxiety-like behavior and higher cortisol levels compared the voluntary running (220). Voluntary, or 'natural', running is characterized by fast-paced running during shorter periods, whereas forced running involves a shorter pace of running for longer periods. In voluntary running, it is important to use locked running-wheels as a control, since the presence of a locked running wheel can be sufficient to induce proliferation in the SGZ (221).

BrdU labeling

Animals were injected with BrdU in order to investigate survival of newborn neurons, as well as determining the ratio of newborn neurons to glial cells.

In **paper I**, we injected animals intraperitoneally with BrdU (50mg/kg) between post-irradiation day 3 and 8, as well as between post-stroke day 7 and 10. In **paper II**, group-housed male and female WT and transgenic (TG) mice were given daily intraperitoneal injections of BrdU for 5 consecutive days at 2 or 10 months of age. In **paper III**, after 5 days of acclimatization, half of the running wheels were unlocked and animals were given daily intraperitoneal injections of BrdU for 5 consecutive days.

Comments:

BrdU is a synthetic thymidine analog that gets incorporated into the DNA of mitotic cells during the S-phase over a period of 2 hours (222). The molecule is administered to date birth of cells and report their fate through co-labeling with other markers. BrdU does not appear to be significantly incorporated during DNA repair and is not taken up by dying neurons (223), and therefore provides a good measure of proliferation or survival based on the experimental design.

Phenotyping

In order to validate the phenotype of MKC-PGC-1α transgenic animals gene expression analysis was determined through RT-qPCR, and ratio of nuclear to mitochondrial DNA was determined through qPCR, on gastrocnemius muscle tissue.

In **paper I**, male 3-month-old mice were decapitated under isoflurane anesthesia. Gastrocnemius muscle was dissected and snap-frozen in isopentane containing dry ice to prevent RNA degradation. In **paper II**, hippocampi, pre-frontal cortex, and gastrocnemius muscle, were harvested from 8-month-old males in the same manner. In **paper IV**, cell lysates of transfected myocytes and serum-treated NSPCs were harvested in RNAlater.

RNA or DNA were extracted from muscle, brain tissue, or cell lysates. Purified RNA was analyzed for integrity and purity to ensure high quality RNA samples. Quantitative PCR was performed according to MIQE guidelines for cDNA and DNA.

Comments:

Primer design was performed to ensure that cDNA primers targeted exon-exon junctions, were transcript-specific, and optimized *in silica*. All qPCR experiments included minus reverse-transcriptase controls for all samples to confirm that no genomic contamination had occurred, as well as primer pairs and minus template controls for all primer pairs to confirm that no contamination by qPCR reagents had occurred. Melt curve analysis was conducted after finishing all PCR cycles to confirm that no non-specific amplification had occurred. All primer pairs in qPCR experiments were run on standard curves of pooled cDNA samples of 5 dilution points in 10-fold steps. From the standard curve reactions, linear detection range, error, and amplification efficiency for all primer pairs could be calculated. All qPCR experiments were normalized against one or more reference genes.

Tissue and serum processing

Four weeks following irradiation, stroke, or start of BrdU-injections, mice were deeply anesthetized with a peritoneal injection of thiopental during the inactive phase of the animals.

Animals were transcardially perfused with cold saline solution followed by 4% paraformaldehyde (PFA) in phosphate-buffered solution (PBS). The brains were immersion-fixed in PFA and subsequently cryoprotected in sucrose solution after 24h. Left hemispheres were sectioned sagittally, or coronally for stroked animals, at 25 μm thickness for immunohistochemistry, using a sliding microtome. Sections were stored at 4°C in cryoprotectant solution until use.

Except for irradiated and stroked animals, blood was extracted for all animals through cardiac puncture immediately preceding transcardial perfusion. Blood was then allowed to coagulate in a low protein-binding microcentrifuge tube. After centrifugation, serum was stored at -80°C until further use.

Protein analysis techniques

Western blot

Western blot was performed to validate the upregulated protein expression of PGC-1α in gastrocnemius muscle of transgenic MCK-PGC-1α mice.

In **paper I**, male 3-month-old mice were decapitated under isoflurane anesthesia. Gastrocnemius muscle was dissected and snap-frozen. Muscle specimens were homogenized by sonication in ice-cold RIPA buffer. Both electrophoresis and electrotransfer of proteins were performed under wet and reducing conditions. Membranes were rinsed, and blocked with bovine serum albumin. Membranes were incubated with rabbit-anti-PGC-1α antibody overnight at 4°C, followed by rinsing and incubation with peroxidase-conjugated donkey-anti-rabbit antibody for 1 hour at room temperature. After washing with TBS-T, bands were visualized by chemiluminescence.

Multiplex protein analysis

In **paper II-IV**, multiplex assays were used to evaluate differences in serum protein levels of cytokines, chemokines, and myokines in transgenic MCK-PGC-1α and endurance trained animals.

Comments:

The preferable choice in analyzing the proteome in transgenic MCK-PGC-1α animals would have been a mass spectrometry-based method. The difficulty in detecting myokines through proteomics is due to a few highly abundant serum proteins making up ~99% of the protein content, which interfere with the analyses of low abundance proteins in the nano-, pico- or femtomolar range (224). We therefore decided to use immunoaffinity-based commercially available multiplex assays.

Immunohistochemistry

Immunohistochemistry was used to visualize and quantify neurogenesis and inflammatory responses, as well as other cellular markers.

An antigen retrieval step in sodium citrate was performed before immunohistochemistry with antibodies against DCX and NeuN. When staining for BrdU, sections were incubated in hydrochloric acid and neutralized in borate buffer. For all immunostainings, sections were incubated in blocking solution containing a detergent and donkey serum. Primary antibodies were diluted in blocking solution and free-floating sections were incubated at 4°C for 3 days in

goat anti-DCX, 2-4 days in rat anti-BrdU and mouse anti-NeuN, or overnight with rabbit-anti-Iba1 and rat-anti-mouse-CD68. After rinsing, sections were incubated with corresponding secondary antibodies conjugated to fluorochromes. After rinsing, sections were mounted onto glass slides and coverslipped. For BrdU/NeuN double stainings, sections were mounted onto glass slides, and after being allowed to dry, glass slides were submerged in Sudan Black in EtOH, and coverslipped.

For the immunoperoxidase method, endogenous peroxidase was first quenched with hydrogen peroxide. When staining for DCX, an antigen retrieval step was performed as described above. After blocking of non-specific binding as described above, sections were incubated at 4°C overnight with rat-anti-BrdU, overnight with rabbit-anti-GFAP, and 3 days with goat-anti-DCX. After rinsing, sections were incubated at RT for 1 hour with corresponding secondary antibodies conjugated to biotin. For amplification of the immunoperoxidase signal, sections were incubated in avidin-biotin-peroxidase complex. The staining was developed in solution containing DAB, hydrogen peroxide, and nickel chloride. Finally, sections were rinsed, mounted onto glass slides and coverslipped.

For Nissl histochemistry, rinsed and mounted sections on glass slides were stained with cresyl violet, decolorized in acetate buffer, and alcohol-treated, before coverslipping.

Comments:

In normal aging brain, and even more so with neurodegeneration, macromolecules become oxidized with lysosomes unable to degrade them. This leads to increased production of lysosomal proteins and enzymes, and abnormalities in endosomes, lysosomes, and autophagosomes (225). One such macromolecular build up from lysosomal inefficiency is lipofuscin. For aged tissue, the endogenous lipofuscin autofluorescence often needs to be quenched by treatment with a blocking agent such as Sudan Black before mounting.

Imaging and quantification

Investigator-blinded stereological quantification was performed using a wide-field microscope and stereological software. For stereological analysis, data from lost or damaged sections were estimated as the average value of the two anatomically adjacent sections. Nissl-stained sections were used for volumetric analysis of brain and lesion sizes. The lesion size was determined by the infarction area, consisting of lost and necrotic tissue, and the surrounding peri-infarct, defined as an area with a distinct reduction in neuronal density. In order to analyze astrocytic response, images for each section containing a lesion site were corrected for uneven illumination and normalized to the contralateral caudal site of the infarction using image processing software. Multiple single-lined 8-bit intensity

measurements radially projecting with fixed angle intervals from the center of the infarction were collected and averaged using ImageJ. Quantification of $Iba1^+$- and $CD68^+$- cells was performed using systematic random sampling with a counting area defined by the fixed radius from the infarct border along with systematically distributed counting frames. All Iba1-expressing/CD68-expressing cells were localized at the core of the infarction, were non-ramified and amoeboid in shape. However, not all amoeboid shaped Iba1-expressing cells were co-expressing CD68. The brain volume corresponding to the location of the photothrombotic infarct was measured on whole brain sections (including infarct) between the most anterior section, interconnecting the corpus callosum, and the most anterior section, containing the dentate gyrus. Quantification of DCX^+-cells in dentate gyrus for irradiated animals, and in cortex and corpus callosum for stroked animals, was performed. For area measurement of CD31-stained blood vessels, Fiji was used for automatic thresholding with the Huang-algorithm. For analysis of $NeuN^+/BrdU^+$ co-expression a confocal microscope was used. The total number of $NeuN^+/BrdU^+$ cells was calculated as the ratio of $BrdU^+$-cells co-expressing $NeuN^+$ multiplied by number of total $BrdU^+$-cells in the SGZ and GCL. All images were cropped and adjusted for brightness, and confocal images were level adjusted for enhanced signal-to-noise ratio per channel.

Cell culture experiments

Myoblast cell culture

Myoblasts derived from the C57BL/6J mouse fetal skeletal muscle were cultured as adherent monolayers in collagen coated flasks according to established protocols for growth of primary myoblast cultures. For proliferating conditions growth media consisted of Ham's F10 Nutrient Mix, DMEM and fetal bovine serum, supplemented with FGF-2 and penicillin/streptomycin. Cells were passaged at 80% confluency every 3-5 days for re-plating in collagen coated flasks.

ELISA

ELISA was used to determine VEGF protein concentration in myocyte lysate and conditioned media.

Twenty-four hours following plasmid transfection of myocytes, cells were washed, pelleted, and snap-frozen. Cell membranes were broken by repeated freeze-thaw cycles and centrifuged for the collection of supernatant to be used in ELISA. Additionally, conditioned media incubated with transfected myocytes as described below was used for ELISA. Samples were normalized based on protein concentrations and used in a commercially available ELISA VEGF-kit.

Plasmid preparation and transfection

Stab cultures of *E.coli* containing plasmid DNA of PGC-1α or green fluorescent protein (GFP) were streaked on agar plates and incubated. Single colonies of PGC-1α and GFP were selected with a sterile pipette and inoculated in liquid growth medium. Plasmid DNA of PGC-1α and GFP were extracted and purified from bacterial pellet. Correct plasmid DNA sizes were verified by restriction enzyme treatment and electrophoresis according to recommendations by the supplier.

Myoblasts were plated and incubated for 24 hours. At approximately 80% confluency, cationic lipid-mediated transfection was performed for introduction of PGC-1α and GFP plasmid DNA into the myoblasts. Cultures were incubated at for 24 hours and transfection medium was replaced with growth medium for NSPCs. After 48 hours of incubation, supernatant was harvested and centrifuged. Myocytes were rinsed and harvested. Conditioned media and cells were immediately frozen in -80°C until further use.

Neural stem cell culture

Using pregnant C57BL/6J animals, NSPCs were derived from subcortical cerebral brain tissue of separate mouse litters at postnatal day 3. Brain tissue was collected by sterile dissection on ice. Tissue was minced and triturated gently by pipetting for mechanical dissociation and enzymatic digestion in a solution containing papain, dispase, and DNase. Cells were passed through a cell strainer, washed, and pelleted. Cell pellet was re-suspended in warm growth media, containing Neurobasal A medium supplemented with B27, GlutaMAX and penicillin/streptomycin. Growth factors (GFs) consisting of EGF, FGF-2, and heparin, were added and cells were seeded in a suspension culture flask and grown as neurospheres. Every second day, GFs were added to the neurosphere culture. Neurospheres were passaged by pelleting, dissociation, washing, and re-resuspension in growth medium.

Comments:

The defining criteria for a neural stem cell are the ability for self-renewal and multipotency, which can be established through clonal expansion and differentiation to neurons, astrocytes, and oligodendrocytes. Neurospheres are free-floating aggregate cultures containing a heterogenous population of neuronal precursor cells with varying degrees of differentiation to neuronal and glial lineages, and are likely to contain only a few percent of neural stem cells (227). 3D cultures, such as neurospheres, are advantaged due to a higher degree of cell-cell contacts resulting in a more natural pattern of differentiation. The cell-cell interactions within neurospheres counteract the maintenance of precursor cells, which is the reason why the proportion of stem cells are so low and why cells in

the core of the neurospheres tend to differentiate (228). The interaction between differentiating cells and precursor cells may expose the stem cells to paracrine factors that promote differentiation.

The yield of NSPCs that result from preparation of SVZ is higher than for SGZ, due to a higher abundance of stem-like cells in the SVZ (229). NSPCs from SVZ also proliferate faster, compared to NSPCs derived from the SGZ (230). Taking cells into an *in vitro* culture system means a radical and complete change in their environment as they are being exposed to artificial nutrients and high concentrations of growth factors, which result in regional identities being gradually lost when are maintained in culture. For example, hippocampal NSPCs in culture lose their ability to generate Prox1-expressing neurons, a marker of granular neurons in the dentate gyrus, after a few passages (231).

Here, we have cultured NSPCs as neurospheres. Another strategy would have been to culture NSPCs as adherent monolayers (229, 231), which creates a more homogenous population of NSPCs. Further, adherent monolayers have a reduced number of cell-to-cell contacts and creates a more even exposure to the culture medium. However, adherent cultures are more difficult to generate due to a lower yield of NSPCs than for neurosphere cultures (230).

Stemness assay

Immunohistochemistry was performed to confirm that neurospheres expressed stemness markers.

Two days after passage, neurospheres were plated on coverslips coated with poly-L-ornithine. Neurospheres were fixed with PFA, washed, and blocked for unspecific binding. The cells were incubated with primary antibodies, rabbit anti-GFAP and goat anti-Sox2 at 4°C overnight. After washing, cells were incubated with corresponding secondary antibodies conjugated to fluorophores. After washing, coverslips were mounted on glass slides. Stained neurospheres for the stemness assay were visualized using an inverted confocal microscope.

Cell proliferation assay

Two days after passage, NSPCs were seeded in optical bottom microplates. The cells were incubated under growth conditions with serum or conditioned medium in a series of concentrations as specified. After 48h of incubation, the cell proliferation was assessed using a DNA dye combined with a fluorescent suppressor of non-viable nuclei. The fluorescence signal was measured in a fluorescence microplate reader. A standard curve was produced for direct conversion of fluorescent intensity values to numbers of cells per well.

Serum for cell culture use

At the age of 7 to 10 months, male and female mice, were acclimatized with locked low-profile running wheels in groups of 2-4 animals per cage (single-housed for males) for animals used in proliferation and differentiation assays, and single-housed for females used in RT-qPCR experiment. For proliferation and differentiation assays, serum was thawed and pooled in corresponding groups matched for age, sex, and running distances. For RT-qPCR, individual sera from sedentary animals and animals with the highest total running distance were used.

Comments:

Serum is typically free from platelets, because they are trapped in the fibrin meshwork of the blood clot, while plasma is prepared from blood that has been prevented from clotting and therefore is considered to be more comparable to circulating blood. Serum includes factors such as BDNF and extracellular vesicles (exosomes and microvesicles) (232) secreted by platelets during clotting, while plasma typically contain platelets, white and red blood cells, and cellular elements that affect analytical results. Serum is considered to preserve stability of analytes better than plasma, while plasma is considered to be less stable because of cellular debris.

Cell differentiation assay

Two days after passage, NSPCs were plated in an optical bottom microplate coated with poly-L-ornithine and laminin. The cells were incubated under growth conditions with sera or conditioned media in a series of concentrations as specified. Growth medium with growth factors was not included as a control in the differentiation assay since that would inhibit differentiation. The corresponding medium was replaced every two days. After 7 days, cells were fixed with PFA, washed, and blocked for unspecific binding. Cells were incubated with primary antibodies rabbit anti-GFAP, goat anti-Sox2, mouse anti-MAP2, and mouse anti-MBP, at 4°C overnight. After washing, the cells were incubated with corresponding secondary antibodies conjugated to fluorophores. Hoechst was used as a nuclear counterstain.

Systematic analysis was done for comparison across different experimental plates with each plate containing all treatment conditions for one biological replicate. Fluorescence images of all stained wells were acquired using a high-content screening microscope. The morphological analysis and the quantification of the different cell types was done after image acquisition using a high-content screening analysis software. Circularity, area and signal intensity of nuclear staining were used as parameters to identify cells through gating of detected objects per plate. Each plate containing all the compared conditions and circularity of cells was fixed for all measurements. Area threshold for cells was set high

enough in order to exclude pyknotic nuclei and low enough to exclude cell aggregates in the cell count. Signal intensity threshold for cells was set low enough to exclude pyknotic nuclei in the cell count. Pyknotic nuclei were classified as nuclear staining signals with an area less than the minimum for cells, or a signal intensity higher than the maximum threshold for cells. For proper identification of $GFAP^+$- and $Sox2^+$-cells the negative and bleed-through controls per fluorescent channel were used to determine optimal signal threshold. If a GFAP signal above a specified signal intensity threshold occurred immediately outside the perimeter of the nucleus, the cell would be counted as a $GFAP^+$-cell. If a Sox2 signal above a specified signal intensity threshold occurred within the perimeter of the nucleus, the cell would be counted as a $Sox2^+$-cell. Neurons and oligodendrocytes were quantified manually based on morphological phenotype and lack of co-labeling with GFAP.

NSPC gene expression analysis

Gene expression analysis was performed in order to validate results from proliferation and differentiation assays, taking into account the effects of serum withdrawal (see Results and Discussion).

NSPCs were plated in ultra-low adhesion plates. Growth factors were added to the media and after 3 days of incubation, growth medium was replaced by mild pelleting of cells. Cells were re-seeded on the plates and incubated for 2 more days on a rotary shaker to prevent adhesion. After 2 days of growth factor withdrawal, cells were pelleted and re-plated in low-adhesion culture plates with addition of different types of mouse sera. After 24h, cells were harvested for RNA extraction.

Statistical analysis

Data were processed and analyzed in Microsoft Excel and Graphpad Prism. Appropriate tests were chosen as specified in the text based on homogeneity of variance. Normality and homogeneity of variance was assessed by visual inspection of density plots and statistical tests, such as D'Agostino-Pearson's and Levene's test.

In **paper I** and **paper II**, PCR data were analyzed using the Pfaffl method, taking into account ratios and multiple sources of error from normalization and efficiency calculations by applying bootstrapping techniques in Qiagen REST 2009. In **paper IV**, PCR data were analyzed using the ΔΔCT method. For matched qPCR data, repeated measure ANOVA (one-way RM-ANOVA) was used if matching was effective between samples, otherwise ordinary one-way ANOVA with Tukey's post-hoc test for multiple comparisons was used. For data without normal distribution, non-parametric RM-ANOVA was used in the form of Friedman's test with Dunn post-hoc correction.

For matched cell culture data in **paper IV**, comparisons of means between two groups were analyzed using paired t-test. For repeated concentration-dependent measurements, repeated measure two-way analysis of variance (two-way RM-ANOVA) was used in conjunction with Tukey's multiple comparisons. One-way RM-ANOVA was used in conjunction with a post-test for linear trend to determine concentration-dependent effects of mouse serum or conditioned medium.

For comparison of parametric histological data between two groups Student's t-test was applied. For histological data adhering to normality and equality of variances, two-way ANOVA was used in conjunction with Tukey's post-hoc test. For repeated measurements of histological and running data, two-way RM-ANOVA was used. In **paper III**, three-way ANOVA was performed in IBM SPSS Statistics.

For multiplex assay analyte data, normality was determined by visual inspection of density plotted logged and unlogged data. In **paper II** and **paper III**, protein concentration data were evaluated by using a two-tailed t-test for normally distributed analyte data and Mann Whitney test for non-normally distributed analyte data. In **paper III**, analyte data adhering to normality and equality of variances was analyzed using two-way ANOVA in conjunction with Tukey's post-hoc test. For non-parametric analyte data, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test was used as a non-parametric equivalent of the two-way ANOVA. Running distances were analyzed against protein concentrations in wildtype and transgenic mice using either linear regression for data adhering to normality, or Kendall rank correlation for parametric and non-parametric data. Linear correlation coefficient and p-values were calculated with Pearson statistics using the limma package in R. Kendall rank correlation was used to calculate ranking correlation between running distance and cytokine concentrations in wildtype and transgenic mice. Kendall's tau coefficient and corresponding p values were calculated based on non-parametric tau statistics. In **paper II** and **paper III**, all multiplex analyte data was treated systematically without discrimination, and false discovery rate (FDR) was calculated with Benjamini-Hochberg using an online calculator to adjust for multiple statistical analyses. In **paper I**, Cohen's d and power were calculated post-hoc for t-test regarding differences in infarct sizes (independent samples) using G*Power. In **paper I**, statistical outliers were detected as specified in the Results section for the infarct volume data set, using ROUT and Grubbs method. We used a significance level of 0.05 for all test, except for the rigorous FDR adjustments in **paper II** where we selected a significance level of 0.1. Values were expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

In this chapter the main results of the included papers are presented and discussed in the order of the specified Aims:

In **paper I**, we investigated whether MCK-PGC-1α animals with improved muscle metabolism would display an altered response to acute brain insults. In particular, we studied morphological outcome, inflammatory responses, and neuroplasticity mechanisms, after irradiation and cortical stroke.

In **paper II**, we investigated whether MCK-PGC-1α animals with improved muscle metabolism would show enhanced hippocampal neurogenesis in aging, and if this effect would be sex-dependent.

In **paper III**, we investigated whether MCK-PGC-1α animals with an endurance muscle phenotype would display different exercise-induced effects on hippocampal neurogenesis, and if this effect would be influence by a running wheel paradigm. We also investigated whether exercise or muscle-specific PGC-1α overexpression releases exercise-inducible factors into the circulation that could contribute to exercise-induced effects on the CNS.

In **paper IV**, we investigated whether exercise or muscle-specific PGC-1α overexpression could influence serum-induced neural stem cell responses *in vitro*. We also evaluated the effect of conditioned media from PGC-1α-transfected myocytes on neural stem cell responses *in vitro*.

Validation of PGC-1α overexpression in skeletal muscle

First, we validated that the MCK-PGC-1α transgenic mouse model displayed an endurance exercise muscle phenotype. In **paper I**, we observed that MCK-PGC-1α had upregulated *PGC-1α* mRNA levels, protein levels, and downstream effector genes, such as *Fndc5*, *Vegfb*, *Il15,* and *Mb,* in skeletal muscle, which we detected at levels comparable to previous studies (2, 120, 138), see Figure 9. PGC-1α-overexpression induces mitochondrial biogenesis and we detected a robustly increased ratio of mitochondrial to nuclear DNA copy number in skeletal muscle of transgenic animals, which is a good measure of mitochondrial density (233). In **paper II**, we again validated the phenotype of older 8-month-old MCK-PGC-1α animals that showed comparable mRNA levels of *PGC-1α* and downstream effector genes, including *Timp4* and *Ctsb* (cathepsin B). We did not evaluate mRNA levels of *Bdnf*, which later has been found to upregulated in muscle of MCK-PGC-1α animals (122).

As discussed in **paper III**, the protein products of each of these downstream effector genes have been identified to be secreted from muscle and to have neurotrophic properties.

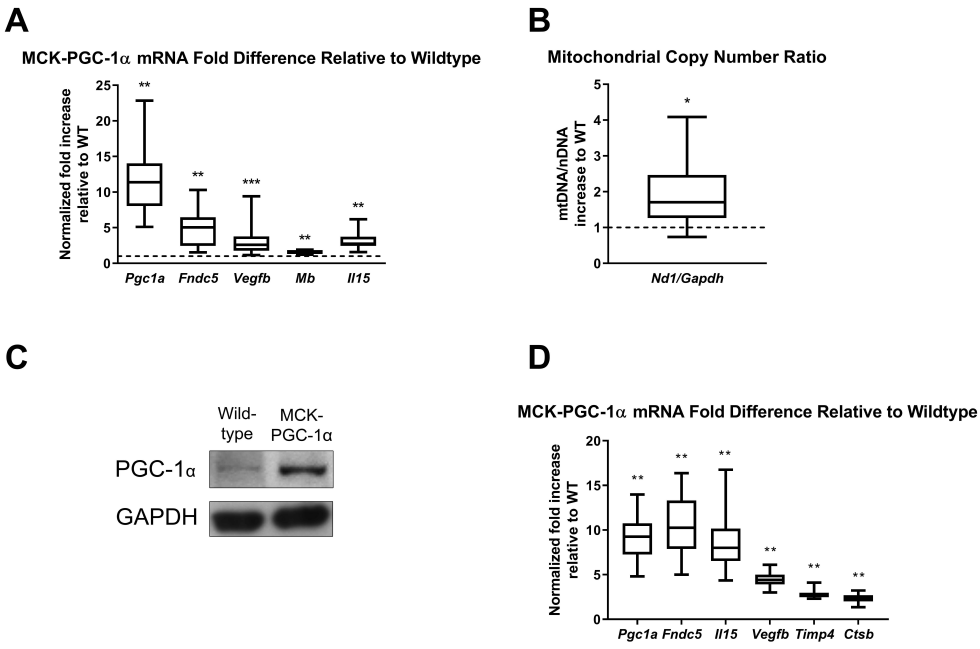

Figure 9. Validation of PGC-1α overexpression in skeletal muscle. Graph **(A)** shows relative mRNA levels of *Pgc1a* and downstream effector genes in gastrocnemius of 3-month-old animals. Graph **(B)** shows ratio of mitochondrial to DNA copy number relative to wildtype. Image **(C)** shows western blot on pooled samples with antibody against PGC-1α (GAPDH as loading control). Graph **(D)** shows relative mRNA levels of *Pgc1a* and downstream effector genes in gastrocnemius of 8-month-old animals. Data expressed as median, with interquartile range as box, with minimum and maximum values as whiskers for wildtype and MCK-PGC-1α animals (Pfaffl method, resampling test; n=5-6; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Cranial irradiation and Cortical stroke

Skeletal muscle PGC-1α overexpression does not protect against irradiation-induced reduction of hippocampal neurogenesis

In **paper I**, using a model of cranial irradiation, we studied the potential protective or regenerative effects of chronic muscular overexpression of PGC-1α on hippocampal neurogenesis after ionizing radiation (IR). Animals were injected with BrdU from day 3 to 8 post-irradiation and analyzed 4 weeks after IR.

We found that the number of newly generated, BrdU-positive, cells in the neurogenic region of the DG, consisting of the SGZ and GCL, was reduced by IR treatment without any difference between wildtype and transgenic animals. Similarly, DCX-positive immature neurons were reduced by IR, but without significant difference with respect to genotypes. In addition, we quantified $Ki67^{+}$-cells in the DG that showed a tendency toward irradiation-induced reduction of proliferation 4 weeks after IR. The number of newly generated neurons determined by NeuN/BrdU co-labeling, was reduced by irradiation treatment without any differences between the genotypes, see Figure 10. Furthermore, the number of newborn cells in the DG, ML, and hilus, as well as the regional volumes of GCL, ML and hilus, showed no differences between genotypes.

The sensitivity of immature neurons to irradiation increases as a function of age (234). By using a cranial irradiation dose of 10 Gy, Andres-Mach et al. found that there is an 90% decrease in proliferation within the SGZ and an 80% decrease in the number of DCX positive cells within the DG for 4-month-old irradiated animals compared to age-matched controls (234). Based on age-dependent decline of neurogenesis and age-dependent increase in radiation sensitivity the irradiation dose in this study was limited to 4 Gy for 4-month-old mice to have a sufficient portion of neural progenitors and immature neurons left to study. However, using this dose we achieved only a mild decrease in neurogenesis. Therefore, we cannot exclude that a higher irradiation dose may have uncovered a difference in post-irradiation neurogenesis levels between genotypes.

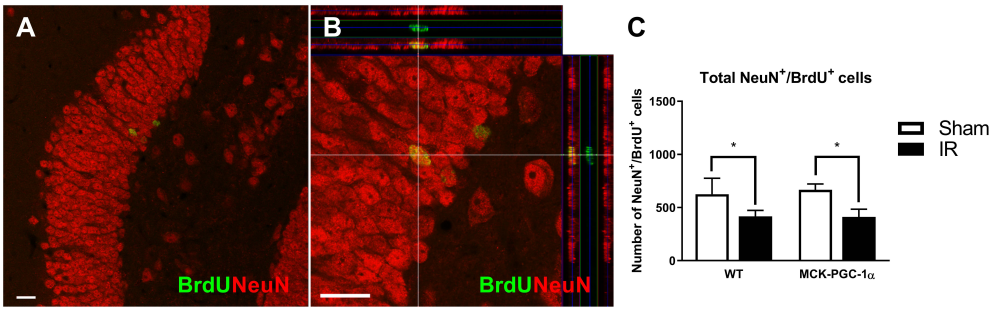

Figure 10. Overexpression of PGC-1α in skeletal muscle does not protect against irradiation-induced reduction of hippocampal neurogenesis. Images show **(A)** confocal image overview of $BrdU^+/NeuN^+$ immunostaining of the DG 4 weeks after irradiation and **(B)** confocal image from quantification, with corresponding graph **(C)** showing total number of $NeuN^+/BrdU^+$ cells in the combined area of SGZ and GCL (see Methodology section for calculation). Data expressed as mean ± SEM for sham and irradiated animals (two-way ANOVA; n=7-8; *, $p<0.05$; **, $p<0.01$). Scale bars = 20 µm. *IR, ionizing radiation; SGZ, subgranular zone; GCL, granular cell layer.*

Skeletal muscle PGC-1α overexpression does not affect neuroinflammatory response and is not favorable for morphological outcome after cortical stroke

In **paper I,** we investigated the potential protective and regenerative effects of chronic muscular overexpression of PGC-1α on the morphological outcome after cortical stroke. Animals were injected with BrdU day 7 to 10 after photothrombotic stroke and analyzed 4 weeks after stroke.

With regard to inflammatory responses, stroke-lesioned animals displayed a higher density of microglial cells ($Iba1^+$) in the peri-infarct region compared to sham-lesioned controls, but no differences existed between wildtype and transgenic mice, see Figure 11. There were no significant differences in activated microglia between the genotypes as judged by density of $CD68^+$-cells in the peri-infarct area. Microglial activation index was calculated as the fraction of $Iba1^+$-cells co-expressing $CD68^+$, which showed no difference between wildtype stroke and MCK-PGC-1α stroke group. Reactive astrocytosis was measured as the intensity of GFAP expression within the peri-infarct region, which revealed a significant treatment effect from photothrombosis on GFAP intensity, representative of the astrogliotic response to the injury, but no difference could be detected between genotypes.

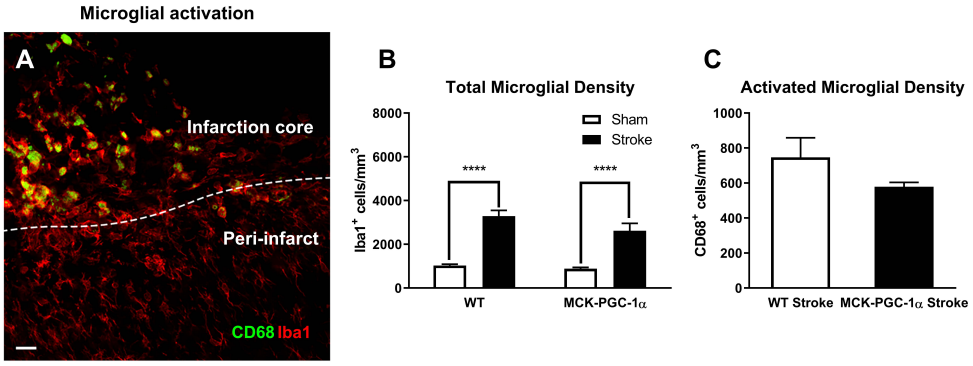

Figure 11. Overexpression of PGC-1α in skeletal muscle does not affect microglial response after cortical stroke. Image **(A)** represents immunohistochemical staining of Iba1/CD68 at 4 weeks after photothrombotic stroke at the infarct border. Graphs show **(B)** density of total microglia ($Iba1^+$; two-way ANOVA) and **(C)** activated microglia ($CD68^+$; t-test) in both infarct and peri-infarct zones for stroked wildtype and MCK-PGC-1α mice (WT, n=7; MCK-PGC-1α, n=5) or for sham treatment (WT, n=3; MCK-PGC-1α, n=3). Scale bar = 20 µm. Data presented as mean ± SEM (****, $p<0.0001$).

PGC-1α regulates the antioxidant defense that may play a role in inflammation, as oxidative stress can induce an inflammatory response through activation of the redox-sensitive NFkB (158). As discussed in **paper I,** inflammatory response after cortical ischemia peaks within a week to gradually diminish afterwards. Even though we do not see any impact on inflammatory responses the time point for analysis of inflammatory response chosen in this study may have missed potential differences in a more acute phase of the inflammatory response.

We detected increased levels of VEGF mRNA in PGC-1α overexpressing muscle tissue. Since VEGF is released into the circulation following exercise and has been identified as the most important pro-angiogenic factor in most tissue (116), the increased expression in skeletal muscle overexpressing PGC-1α may affect the microvascular system in the CNS. However, we found that vascularization in the neocortex, determined by the mean vessel density (based on CD31-staining of endothelial cells), was unchanged between WT and MCK-PGC-1α.

In terms of morphological outcome, transgenic mice had on average 57% greater infarct volume in comparison to the wildtype group, see Figure 12. There was no difference in brain volume between the two groups, and the relative amount of necrotic tissue loss to brain volume was also significantly higher in the MCK-PGC-1α stroke group. The peri-infarct region, defined as an area with a distinct reduction in neuronal density, showed no difference between wildtype stroke and MCK-PGC-1α stroke group.

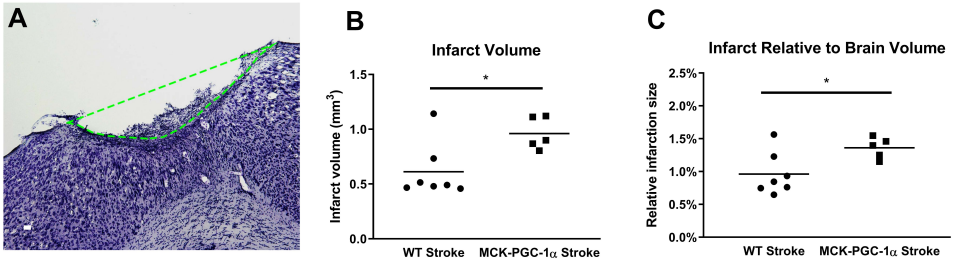

Figure 12. Overexpression of PGC-1α in skeletal is not favorable for the morphological outcome after cortical stroke. Lesion size in wildtype (n=7) and MCK-PGC-1α (n=5) mice 4 weeks following photothrombotic stroke. Image **(A)** shows area measurement of infarct size marked with dashed green line performed for each section, along with graphs showing a significantly higher **(B)** volume of tissue loss ($p=0.02$; t-test) and **(C)** relative tissue loss to brain volume ($p=0.03$; t-test) after stroke in MCK-PGC-1α compared to wildtype animals. Scale bar = 50 µm. Data presented as mean ± SEM (*, $p<0.05$).

In **paper I** we discuss possible mechanisms out of the scope of the study that could have led to larger infarcts in PGC-1α animals. For example, the PGC-1α pathway is known to increase mitochondrial biogenesis and ROS production after cerebral ischemia, which may exacerbate mitochondria-dependent apoptotic processes (206).

In the study we used photothrombosis as a model for cortical stroke (discussed in Methodology). We cannot rule out that MCK-PGC-1α animals would have shown a different outcome if we instead would have chosen a model producing a larger penumbra, such as transient or permanent middle cerebral artery occlusion, or a re-perfusion injury model of vasoconstriction, such as intracerebral injection of endothelin-1.

We did not investigate behavioral outcomes after irradiation or photothrombotic stroke in the MCK-PGC-1α animals. Even though it neuroanatomically is unlikely, we cannot exclude the possibility that transgenic animals would have more or less motor deficits after stroke to the motorsensory cortex.

Aging and Voluntary running

Skeletal muscle PGC-1α overexpression does not protect from age-dependent decline in neurogenesis

In **paper II**, we investigated the potential longitudinal protective effects from chronic muscular overexpression of PGC-1α on hippocampal neurogenesis in aging along with possible differences between the sexes. In this study, group-housed female and male animals were injected with BrdU during 5 days and analyzed 4 weeks later.

We found that the number of $BrdU^+$-cells in the GCL was substantially decreased with aging in both WT and transgenic animals of both sexes, but no difference existed between genotypes or sexes. Similarly, the number of newly generated mature neurons determined by $NeuN^+/BrdU^+$ co-labeling decreased with aging without any differences between either genotype or sex for 3- and 11-month-old animals. The number of immature neurons were reduced with aging, but there were no significant differences for 3- and 11-month-old animals with respect to either genotype or sex. The orientation of DCX^+ neural progenitor cells in the DG indicates a state of maturity. We analyzed DCX^+-cells in 11-month-old animals and found no difference in numbers of DCX^+-cells with parallel or perpendicular orientation between genotypes and sexes, indicating no difference in the maturation rate of immature neurons.

In this study, we have not controlled for estrogen levels in female animals, but found no differences in neurogenesis between sexes neither in young nor in older mice, which is supported by previous reports (235).

Even though MCK-PGC-1α mice have been reported to have unchanged levels of gene expression for a number of neurotrophic factors in the hippocampus (138), MCK-PGC-1α mice are protected from a neuroinflammatory reduction in hippocampal BDNF, GDNF, and VEGF, indicating that muscular PGC-1α overexpression could mediate exercise-induced protection from stress-induced reduction in neurotrophic growth factors in the brain. MCK-PGC-1α animals also have upregulated circulating levels of irisin, BDNF, and IL-15, which are linked to neuroprotection (122). However, we found no difference in *Bdnf* gene expression between genotypes in the hippocampus or pre-frontal cortex, confirming the findings of a previous report (138).

Skeletal muscle PGC-1α overexpression is not sufficient to mimic running-induced neurogenesis

In **paper III**, we investigated potential protective and regenerative effects of chronic muscular overexpression of PGC-1α in exercise and aging. Wildtype and transgenic female animals were single-housed and subjected to voluntary running at 2 and 10 months of age. At start of running, animals were injected with BrdU to label newly generated cells to be analyzed 4 weeks later.

We found that average daily running distance was similar for both young female wildtype and transgenic animals, and was reduced in older female wildtype and transgenic animals. Compared to females, average daily running distance in males was considerably lower in young male animals and was further decreased in older male animals. Female mice have been observed to be more active than males and tend to run longer distances with higher velocities, but for the same duration, in a running wheel (87, 236). One possible explanation of this may be that female mice seem have better respiratory abilities with differences in energy metabolism (84).

We did not observe any difference in voluntary running between genotypes, which stands in contrast to the enhanced endurance capacity that these transgenic animals have been reported to display. Calvo and colleagues reported that overexpression of PGC-1α in muscle greatly improved short-term exercise performance in voluntary running, measured by single-housing animals with running wheels for 72h, and a forced exercise paradigm (159). In our data, we see that voluntary running activity increases substantially over 4 weeks, a period over which no difference was detected between genotypes. However, if pushed by forced running MCK-PGC-1α mice should outperform wildtype animals due to a higher peak oxygen uptake in muscle (159).

Aging negatively impacts running velocity, representing a decrease in muscle mass, muscle strength, as well as more slow-twitch fibers and fibrosis with higher age (237). A part of this effect is due to loss of myoregenerative capabilities in satellite cells dependent on lost Notch signaling and activated Wnt signaling by circulating factors (e.g. cytokines) (238). Bartling and colleagues reported that differences in running activity for females and males gradually diminish with age (236) and attributed this to age-related changes in metabolism (84) and hormonal regulation, making exercise capacity in females and males more alike. See **paper III** for a discussion on age-related and sex-dependent differences in running capacity, as well as sex differences in neurogenesis and exercise-induced neurogenesis.

We found that the number of $BrdU^+$-cells in the SGZ and GCL of the DG was decreased with aging and increased by exercise in both wildtype and transgenic animals, but no differences existed between genotypes, see Figure 13. The number of newly generated mature neurons determined by NeuN/BrdU co-labeling was

reduced with aging, but exercise only increased numbers of new mature neurons in 3-month-old animals. Numbers of immature DCX$^+$-neurons were reduced by aging and increased by exercise for both genotypes. Number of DCX$^+$-cells and running distance was not correlated in running 11-month-old animals, male and females combined, for either wildtype or transgenic animals. There were statistically significant differences in numbers of DCX$^+$ progenitor cells with parallel (early) and perpendicular (late) orientation of the lead process in relation to the GCL border for female 11-month-old animals between sedentary and running animals, but no differences between genotypes were observed.

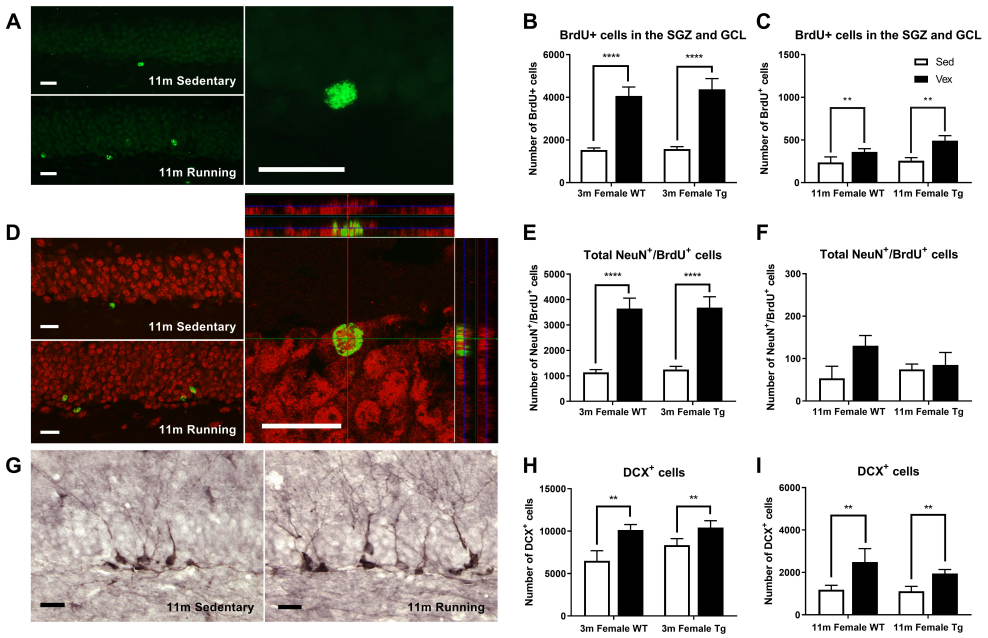

Figure 13. Overexpression of PGC-1α in skeletal muscle does not alter exercise-induced amelioration of age-dependent decline of hippocampal neurogenesis. (A) Images showing BrdU immunostainings of DG for sedentary and running 11-month-old females 4 weeks after first BrdU-injection and start of running, with corresponding graphs **(B, C)** representing number of BrdU$^+$-cells in the SGZ and GCL for 3- (two-way ANOVA; n=6-10; running effect, ****, $p<0.0001$) and 11-month-old animals (two-way ANOVA; n=6-9; running effect, **, $p<0.01$). **(D)** Images of NeuN/BrdU immunostainings of DG for sedentary and running 11-month-old females with confocal images of co-localized NeuN$^+$/BrdU$^+$-cells with corresponding graphs **(E, F)** representing number of NeuN$^+$/BrdU$^+$-cells for 3- (two-way ANOVA; n=5-9; running effect, ****, $p<0.0001$) and 11-month-old animals (two-way ANOVA; n=6-9; n.s.). **(G)** DCX immunostainings of DG for sedentary and running 11-month-old females with

corresponding graphs **(H, I)** showing number of DCX^+-cells for 3- (two-way ANOVA; n=6-10; running effect, **, $p<0.01$) and 11-month-old animals (two-way ANOVA; n=5-10; running effect, **, $p<0.01$). Data expressed as mean ± SEM. Scale bars = 20 μm.

Endurance exercise increases proliferation, differentiation (56), and accelerated maturation of adult-born DG neurons (73). As expected, we observed a robust exercise-induced increase in neurogenesis measured as newborn $BrdU^+$-cells, new immature DCX^+-neurons, and newly generated mature $BrdU^+/NeuN^+$-neurons. As for the lack of effect from exercise on new mature neurons in older animals, this has previously been described in a study where chronic voluntary wheel running prevented age-related decline in proliferation and immature neurons, which however did not lead to more newborn mature neurons (62).

Here, we have investigated changes in neurogenesis after 4 weeks of voluntary running. It is however possible that acute or longer-term exercise would have yielded different results. Due to the fact that MCK-PGC-1α animals have higher peak aerobic capacity it is possible that the result would have been different if we had subjected animals to a forced exercise paradigm, pushing the transgenic mice to outperform the wildtype animals. However, one study showed a greater increase in hippocampal neurogenesis by mild compared to intense forced running on a treadmill in rats (88), which can be explained by higher levels of stress from intense exercise compared with mild exercise.

Skeletal muscle PGC-1α overexpression and running-induced serum cytokine and myokine profile

In **paper III**, we also sought to determine if skeletal muscle activation via PGC-1α overexpression release exercise-inducible molecules into the circulation that contribute to exercise-induced effects on the CNS. To investigate possible differences in serum proteins associated with overexpression of PGC-1α in skeletal muscle, running, or a combination of both, we housed 11-month-old male wildtype and transgenic animals individually and subjected them to voluntary running during 4 weeks. After 4 weeks of running, blood was extracted to determine levels of cytokines, chemokines, and myokines in serum. Analysis after FDR adjustment for analyte data showed that musclin was significantly upregulated at 4-fold higher concentration in transgenic serum, in line with a previous study on MCK-PGC-1α animals (170), see Figure 14. Several analytes that showed significant differences before FDR-adjustments, but only a trend after FDR, require confirmation in future experiments. We observed trends toward increased levels of myokines and reduced levels of pro-inflammatory cytokines in transgenic animals, including upregulation of eotaxin with running, upregulation of oncostatin in transgenic animals, and downregulation of MCP-1, MCP-3, IL-5, MIP-1beta, and myostatin, in transgenic animals.

Musclin was first described over 10 years ago as a myokine closely linked to nutritional changes and produced almost exclusively in fast-glycolytic type IIb myofibers (239). Subbotina and colleagues reported musclin as an exercise-responsive myokine enhancing endurance capacity through mitochondrial biogenesis (240). On the other hand, musclin has also been described to mediate high-fat diet-induced effects such as insulin resistance and hypertension (239).

We observed trends towards decreased pro-inflammatory cytokines. Cytokines and chemokines have complex functions in the brain due to simultaneously being mediators of neuroinflammation and neurodegenerative diseases, but also having beneficial functions as neuromodulators. Overexpression of PGC-1α in skeletal muscle cells *in vitro* has been reported to downregulate the pro-inflammatory genes IL-6 and TNFα. Muscle-specific overexpression of PGC-1α in mice does not alter systemic levels of IL-6 and TNFα, but strongly inhibits gene expression of pro-inflammatory cytokine IL-12, as well as increases anti-inflammatory cytokines CCL1 and TGFβ in skeletal muscle, overall indicating an anti-inflammatory profile of the PGC1α pathway (158).

A recent study by Peng and colleagues found that MCK-PGC-1α mice had upregulated serum levels of the myokines irisin, BDNF, and IL-15 (122). The commercial multiplex assays employed in our study included quantification of irisin and BDNF in serum. However, the assay was not sensitive enough to enable statistical comparisons between the groups.

We did not observe any significant effect of exercise on the studied serum proteins. This is in accordance with a previous study by Jeon and colleagues, who were unable to detect differences in a panel of 50 cytokines in serum of 20-month-old mice subjected to treadmill running (241).

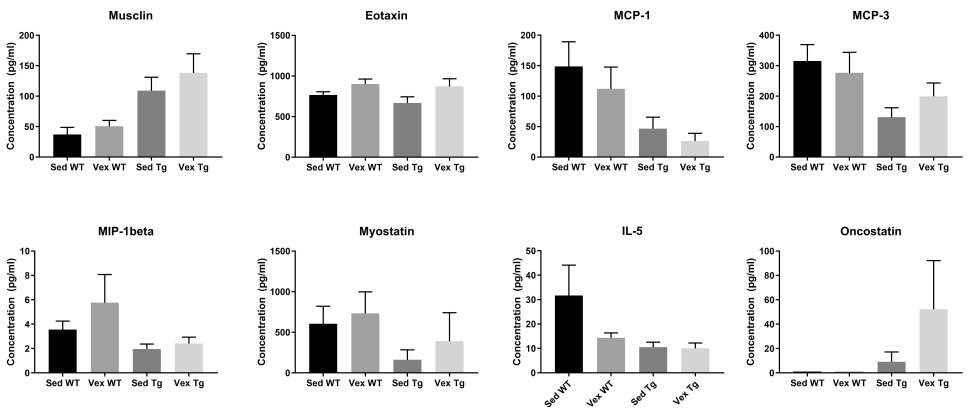

Figure 14. Levels of cytokines and myokines in mouse serum. Graphs showing protein concentrations of analytes with differences, or tendencies toward differences, between genotype and running (n=7-11). *WT, wildtype; Tg, transgenic; Sed, sedentary; Vex, voluntary exercise.*

Neural stem cell responses in vitro

Effect of exercise and muscle-specific PGC-1α overexpression on neural stem cell responses

In **paper IV**, we sought to determine if skeletal muscle activation via PGC-1α overexpression releases exercise-inducible molecules into the circulation that contribute to exercise-induced effects on the CNS. Serum from wildtype and transgenic mice was harvested following 4 weeks of running to evaluate the response of NSPCs in terms of proliferation and differentiation. Harvested exercise-conditioned and transgenic sera were added to culture medium without growth factors for a 2-day incubation period to investigate the response of NSPCs.

Initially, we evaluated the effect of sedentary wildtype serum on proliferation of NSPCs and found a concentration-dependent effect on proliferation, see Figure 15. In subsequent experiments, we compared exercise-conditioned and transgenic sera over a series of concentrations. Similarly, we observed a higher proliferative response with increasing serum concentrations, but without differences between exercise-conditioned and transgenic sera.

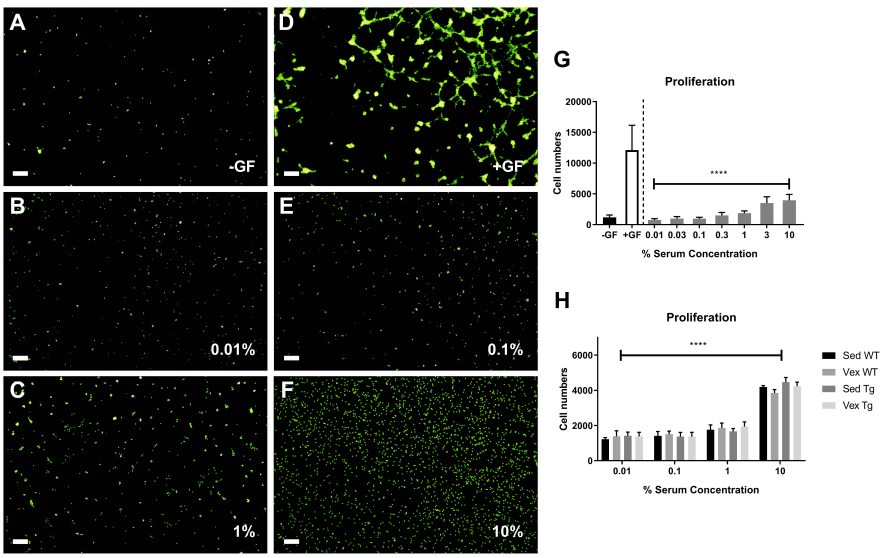

Figure 15. Proliferative response of NSPCs upon treatment with sera from mice subjected to voluntary running or with chronic muscle-specific PGC-1α activation. (A-F) Fluorescence images from proliferation assay showing viable cells in green after incubation for 2 days in 0.01%, 0.1%, 1% and 10% sedentary transgenic serum. Graphs show proliferative response of NSPCs treated with a range of serum concentrations from **(G)** sedentary wildtype animals only (one-way RM-ANOVA; post-hoc test for linear trend, ****, $p<0.0001$); n=3 biological

replicates) and **(H)** sedentary or running, wildtype or transgenic, animals (two-way RM-ANOVA; concentration effect, ****, $p<0.0001$; treatment effect, n.s.; n=4 biological replicates). Dashed line indicates that '–GF' and '+GF' have not been included in the statistical analysis. Scale bars = 20um. Values are shown as mean ± SEM. *, $p<0.05$. *GF, growth factors; '-GF', culture medium without GF; '+GF', culture medium with GF; Sed, sedentary; Vex, voluntary exercise; WT, wildtype; Tg, transgenic.*

We further evaluated NSPC differentiation as a response to exercise-conditioned and transgenic sera. Cell numbers increased with a concentration-dependent effect without any difference overall between mouse sera. Concentration-dependent effects were observed for astrocytes and oligodendrocytes, but without any difference between treatment groups. No concentration or treatment effects existed for $MAP2^+$-neurons, $Sox2^+$-progenitors or $GFAP^+/Sox2^+$-stem cells.

To validate our findings, we also investigated the transcriptional response of genes involved in cell-cycle regulation and differentiation, including withdrawal of growth factors for 2 days. After withdrawal of essential growth components from neural stem cells, cell-cycle related genes remain upregulated for 24 hours (242). Neuronal fate markers are upregulated within 6-8 hours with DCX being robustly upregulated over the first 1-3 days from the start of differentiation (243). We observed a robust upregulation of gene expression in proliferative genes, i.e. PCNA, E2F1, and MCM2, from the addition of growth factors to NSPCs. Accordingly, upon growth factor treatment we detected a large decrease in expression of genes involved in differentiation, i.e. DCX and GFAP. However, we did not observe any difference in gene expression from treatment with exercise-conditioned or transgenic sera compared to sedentary and wildtype.

We were unable to detect any difference in serum-induced response on proliferation from exercise. The increase in proliferation with higher serum concentrations is a well-known phenomenon for cell cultures and indicates that the assay is capable of detecting serum differences.

As discussed in Methodology, there are important differences between plasma and serum. We cannot exclude the possibility that the use of plasma would have yielded different results on neural stem cell behavior.

We were unable to detect differences in stem cell differentiation between serum from exercising versus sedentary animals. Serum treatment shifts differentiation to a glial lineage, an effect that is stronger with higher concentrations (244) and may largely be mediated by glucocorticoids (245). However, the low molecular weight fraction of serum, as opposed to whole-serum, has been proposed to be able to promote neural differentiation, while the serum fraction above 100 kDa could be toxic for cultured neurons (246). Many neurotrophic factors, such as (e.g. EGF, FGF-2, IGF-1, PDGF, cytokines, polyamines, and vitamins), are a part of

the molecular weight fraction of serum below 100kDa, this sub-100kDa serum fraction in conjunction with FGF-2 promote neuronal differentiation in rodent NSPCs (247). However, the interpretation of this finding is difficult since FGF-2 has been demonstrated to promote neuronal differentiation on its own (248). Peng and colleagues evaluated different molecular weight fractions of mouse serum from MCK-PGC-1α mice on kidney tubule cells (122). They found that only the 10-50 kDa fraction could improve mitochondrial function in kidney cells by significantly increasing ATP-coupled respiration and maximal respiratory capacity. Using mass spectrometry, the serum fraction was found to contain irisin with the effects of the serum fraction being blocked by an antibody against irisin, indicating that irisin mediated these effects. Therefore, the possibility remains that the response to a low molecular weight fraction of serum could have yielded different result in our assays.

Inflammatory mediators are known to inhibit neurogenesis, and overexpression of PGC-1α downregulates inflammatory activity through NFkB inhibition (249), a transcription factor that is also involved in neuronal differentiation. Despite potent systemic changes in MCK-PGC-1α animals that we and others have observed (122, 138, 153, 159, 164, 170, 171, 176) we did not detect any difference in neural stem cell response when treating with serum from MCK-PGC-1α and wildtype animals, independent of exercise activity or serum concentration.

We collected sera from 7- to 10-month-old wildtype and transgenic mice. Factors inhibiting neurogenesis could exist in the blood of older mice that could inhibit the exercise-induced effects on neural stem cell behavior. It is known that aging factors increase in the blood of older mice, such as CCL11 and β2-microglobulin, which have been found to impair neurogenesis and cognitive function (202), whereas anti-aging factors, which have protective effects, have been found to decrease (201). See **paper IV** for a discussion on the use of older serum that contain glucocorticoids and eotaxin/CCL11, among other factors being able to negatively impact neurogenesis. It is possible that using blood from younger animals could have yielded a different effect of exercised serum on neural stem cell behavior.

We collected serum after 4 weeks of voluntary wheel running in the inactive phase of the animals. Due to the difference in regulation of exercise factors after an acute exercise bout and regular exercise it is also possible that acute exercise could have yielded different effects on neural stem cell behavior.

Precursor cells from different brain regions do not behave identically under identical culture conditions (250). We use NSPCs derived from subcortical tissue of early postnatal mice. The two main neurogenic regions in the subcortical regions are the SGZ and SVZ. Due to the inherent higher capacity of NSPCs derived from SVZ to proliferate in cell culture, in comparison SGZ, it is likely that most NSPCs that we have cultured have originated from the SVZ. Since

exercise may be limited to induce neurogenesis primarily in the SGZ, and not in the SVZ (67), this could mean that the NSPCs from the two neurogenic regions intrinsically have different propensities to respond to exercise-induced changes. We use subcortical brain tissue which may give away spatial information and potential insight into regional differences of NSPCs. Therefore, it is possible that using NSPCs derived from subcortical tissue, rather than hippocampal NSPCs, could have influenced neural stem cells response for the different mouse sera (see Methodology section for commentary).

Effect of conditioned medium from PGC-1α-transfected myocytes on neural stem cells responses

In **paper IV**, we sought to determine the effect of conditioned medium from PGC-1α-transfected myocytes on the response of neural stem cells. Medium was harvested from myocytes transfected with plasmid vectors containing either a PGC1 or GFP construct under the CMV promoter. Media were conditioned by incubation with transfected myocytes for 48 hours before being harvested. At this point the myocytes had turned into immature myotubes, while ten days after transfection myotubes developed into mature myotubes. In PGC-1α-transfected myocytes, *Pgc1a* mRNA levels were elevated 250-fold with a 3-fold increase in VEGF protein levels compared to GFP-transfected myocytes 24 hours after transfection, see Figure 16. However, no elevation of VEGF protein levels could be detected in the PGC-1α-conditioned media, possibly due to the short half-life of VEGF (135).

PGC-1α upregulation induces expression of VEGF, which regulates angiogenesis and vasodilatation in skeletal muscle (2). VEGF is considered to be released into the circulation from muscle during exercise, but also to be induced in the hippocampus following exercise, where it acts to promote angiogenesis, BDNF expression, synaptic plasticity, and neurogenesis (105, 107). Interestingly, Rich and colleagues show that skeletal muscle VEGF is essential for exercise-induced neurogenesis, even though the exact mechanism has not been clearly established (137).

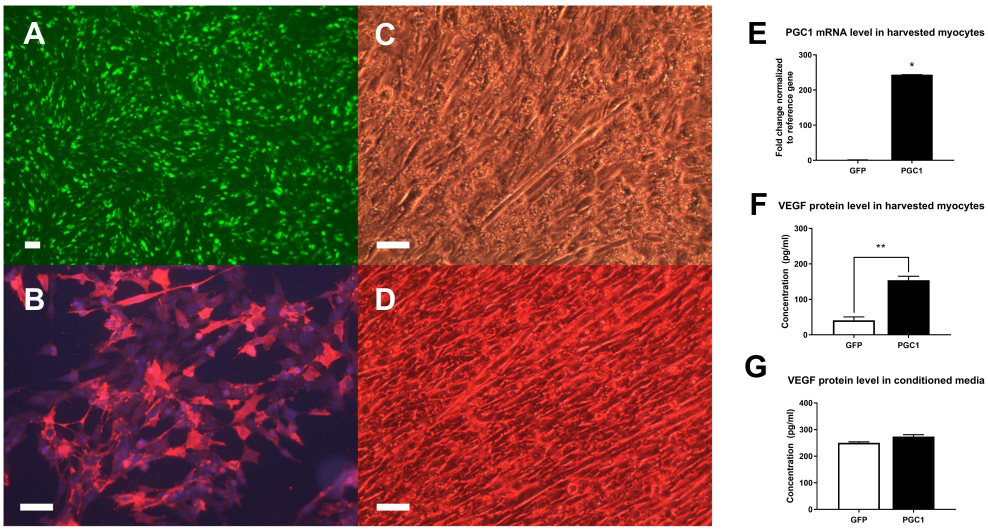

Figure 16. Transient transfection in myoblast cultures and validation of upregulated target and downstream protein. Images showing **(A)** myoblast culture transfected with GFP plasmid DNA and **(B)** immunocytochemistry against GFP (red) with DAPI (blue), **(C)** myocytes developed into immature myotubes 3 days after transfection (at the time of media collection), which **(D)** continue to develop into mature myotubes 10 days after transfection. Graphs show **(E)** relative mRNA levels of *PGC-1α* in myocytes (normalized to reference gene *18s*) (paired t-test; *, $p<0.05$; n=5-6), **(F)** protein levels of VEGF in myocytes (paired t-test; **, $p<0.01$; n=3), and protein levels of VEGF in conditioned medium (paired t-test; n.s.; n=3). Values are shown as mean ± SEM.

In a proliferation assay, we found a concentration-dependent effect from treatment with conditioned media and a higher proliferative response in NSPCs from incubation with conditioned media coming from PGC1α- compared to GFP-transfected myocytes, see Figure 17.

Myocytes used in this study were allowed to differentiate for 3 days at the time of medium collection, which is comparable to a previous study in which myocytes had differentiated for 2 days (144). It is known that the gene expression and proteomic profile of myoblast differ from differentiated myotubes (251). It is possible that upregulation of PGC-1α in fully differentiated myotubes could have yielded other results. Primary myoblasts were used to allow for adequate transfection efficiency with transient lipotransfection of plasmid DNA. However, it may have been preferable to transduce primary myoblast with a retroviral vector containing a mammalian antibiotic resistance gene (e.g., puromycin, basticidin), which would have enabled selection of a stable cell culture after transduction. A homogenous cell culture prepared by selection of transduced cells would have increased reproducibility by eliminating the variation associated with repeated transient transfections. This would have allowed the continued culturing and

selection of myoblasts retrovirally transduced with an inducible gene expression, such as Tet-on (252), and enabled us to turn on the PGC-1α expression in fully differentiated myotubes.

Since the conditioned medium contains metabolites from the myocyte culture it is also possible that we would have observed different results by purifying proteins in the conditioned media through protein precipitation (2) or through dialysis.

Several potential myokines regulated by the PGC-1α pathway could mediate the observed proliferative effect. For example, FNDC5, or irisin, is upregulated by PGC-1α overexpression and is increased in the circulation following exercise training (130). Irisin increases proliferation of NSPCs at pharmacological doses *in vitro* (126), and FNDC5 is reported to upregulate hippocampal BDNF levels (3), which could increase proliferation of NSPCs by different signaling pathways (253).

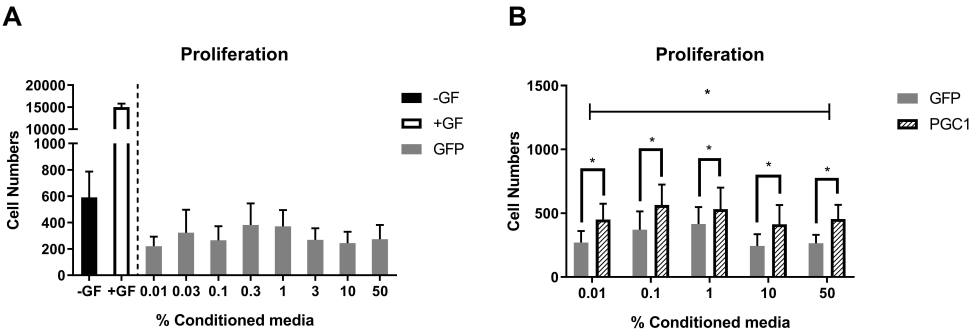

Figure 17. Proliferative response of NSPCs upon treatment with conditioned media from PGC-1α-overexpressing myocytes. Graphs show proliferation analysis of NSPCs treated with a range of concentrations of conditioned media from **(A)** GFP-transfected myocytes (RM-ANOVA, Tukey multiple comparisons; n.s.; n=3) and **(B)** comparison of conditioned media from PGC-1α- and GFP-overexpressing myocytes (RM-ANOVA; treatment group effect, $F(1,4)=8.0$, $p=0.05$; concentration effect, $F(4,16)=3.6$, $p=0.03$; n=5 biological replicates), showing a higher proliferative response with the PGC-1α-transfected conditioned media. Dashed line indicates that '–GF' and '+GF' have not been included in the statistical analysis. Values are shown as mean ± SEM. *, $p<0.05$. *GF, growth factors; '-GF', culture medium without GF; '+GF', culture medium with GF.*

CONCLUDING REMARKS

In this chapter we summarize and discuss the conclusions made in each paper in relation to the aims of this thesis.

In **paper I**, we found that muscular overexpression of PGC-1α does not exert beneficial effects on adult neurogenesis after cranial irradiation, or on the morphological outcome after cortical stroke, indicating that PGC-1α overexpression in muscle is not sufficient to phenocopy exercise-induced neuroprotection in these injury models.

In **paper II**, we found that muscular overexpression of PGC-1α does not exert beneficial effects on adult neurogenesis, independent of sex or age, indicating that PGC-1α overexpression in muscle is not sufficient to phenocopy exercise-induced neurogenesis in aging.

In **paper III**, we found that muscular overexpression of PGC-1α does not increase basal neurogenesis, or augment exercise-induced neurogenesis, independent of age, indicating that PGC-1α overexpression in muscle is not sufficient to phenocopy exercise-induced effects on neurogenesis.

In **paper IV**, we found that serum from animals subjected to voluntary running and animals with muscular overexpression of PGC-1α did not affect proliferation or differentiation of neural stem cells. This suggests that circulating factors induced by exercise, or muscle-specific PGC-1α overexpression, are not sufficient to directly influence neural stem cell behavior.

Based on previously reported upregulation of systemic factors with neurotrophic effects in MCK-PGC-1α mice (2), we hypothesized that constitutive muscular PGC-1α overexpression could result in increased basal neurogenesis, and that exercise could act in synergy with this effect. Further, MCK-PGC-1α animals were reportedly protected from stress-induced neuroinflammation (138), and we hypothesized that a similar protective effect could emerge in other neuroinflammatory conditions that have been shown to be benefited by exercise, such as cranial irradiation, cortical stroke, and age-dependent decline of neurogenesis. However, we found no protection by muscle-specific overexpression of PGC-1α in models of brain injury and no difference between genotypes regarding basal neurogenesis, independent of age or sex. Furthermore, we did not see any further improvement in exercise-induced neurogenesis in MCK-PGC-1α animals. The lack of synergistic effect is in line with a finding that endurance training increased PGC-1α mRNA levels in gastrocnemius by two-fold in WT, while no further increase could be seen in MCK-PGC-1α animals (171). Moreover, muscle-specific PGC-1α overexpression did not augment metabolic effects from exercise (254).

We also hypothesized that effects from exercise or muscle-specific PGC-1α overexpression may be present upon treatment of neural stem cells *in vitro*. However, we found no effect of exercise or muscle-specific PGC-1α overexpression from whole-serum treatment on neural stem cell proliferation or differentiation. Metabolic effects of acute exercise bouts are very different from metabolic adaptations to regular exercise training, with only a few myokines being upregulated in both acute and regular exercise (255). Chronic expression of PGC-1α could be regarded as an adaptive response to long-term regular physical exercise and may not have the same effects on hippocampal neurogenesis as an acutely elevated expression of the transcription factor. For this reason, we also sought to investigate the acute effects of upregulating the PGC-1α pathway by acute overexpression in myocytes. We hypothesized that an acute overexpression of PGC-1α in myocytes may secrete factors into the condition media, which may influence neural stem cell behavior. We observed a slight increase in neural stem cell proliferation from conditioned media coming from PGC-1α-transfected myocytes.

In order to finalize our studies and further validate our findings, we are in the process of producing immunoblots for PGC-1α protein expression in gastrocnemius muscle homogenate of wildtype and MCK-PGC-1α animals, as well as for PGC-1α-transfected myocytes.

In several experiments we have demonstrated the lack of beneficial effects from chronic muscle-specific overexpression of PGC-1α on neurogenesis and protection from brain insults. We find that chronic muscle activation through the PGC-1α pathway is not sufficient to mimic exercise-induced effects on neurogenesis or protection from brain insults, and that serum factors secreted by exercise or chronic muscle activation through the PGC-1α pathway is in physiological levels not able to directly influence neural stem cell behavior *in vitro*. We have studied neurogenesis for the importance of this phenomenon in exercise-induced effects on the CNS and in cognition. However, exercise-induced effects on cognition are also correlated to enhanced neuronal and synaptic plasticity (256). While neurogenesis is important for exercise-induced improvements in spatial memory, it is not required for improvements in motor performance or contextual fear conditioning (102, 257). It should therefore be noted that we have not studied electrophysiological or behavioral aspect of exercise-induced effects on the brain.

Models of muscular PGC-1α overexpression

In cell culture studies, adenovirus-mediated PGC-1α overexpression in C2C12, L6 (258), and primary rat myotubes (259), resulted in increased GLUT4 expression and glycogen build-up, suggesting a PGC-1α-mediated enhancement of glucose homeostasis in skeletal muscle. This was supported by the finding that enhanced insulin-stimulated glycogen synthesis by antidiabetic agents in primary human muscle cells was mediated by GLUT4 upregulation via PGC-1α induction (260). Similarly, electroporation of PGC-1α that resulted in ~25% increase in PGC-1α protein expression in tibialis anterior skeletal muscle yielded an increased GLUT4 expression and glucose uptake *ex vivo* (261).

However, in a transgenic mouse model of muscle-specific overexpression of PGC-1α (10-fold increase in gene expression) that displayed red colored muscle phenotype characteristic of oxidative muscle, Miura and colleagues unexpectedly observed a downregulation of GLUT4 mRNA (262). Choi and colleagues used MCK-PGC-1α animals with a 6-fold increase in PGC-1α gene expression in muscle composed of type II fibers (175), which is within the range of the increase observed after exercise training (10-13-fold increase according to Miura and colleagues), and similar to that found in type I muscle fibers (262). MCK-PCG-1α mice had increased mitochondrial function, but displayed aggravated fat-induced muscle insulin resistance (175). In MCK-PGC-1α muscle there was a 140% increase in mitochondrial density, but only a 60% increase in ATP synthesis, suggesting a partial downregulation of activity per unit of mitochondrial mass. The results from *in vitro* and *ex vivo* studies may also indicate that an acute overexpression could be responsible for the beneficial effect on glucose homeostasis.

Wende and colleagues used a transgenic mouse model of inducible PGC-1α overexpression in skeletal muscle and found that overexpression of PGC-1α reduced high-intensity exercise capacity after 3-4 weeks following induction, explained by a decreased ability to utilize glycogen during exercise. Muscle-specific activation of PGC-1α increased muscle glucose uptake, suppressed glycolysis and glycogen breakdown, normally stimulated by insulin, leading to an increase in glycogen fuel depot similar to endurance training. In addition to the temporal differences in PGC-1α expression, the model used by Wende and the MCK-PCG-1α model likely exhibit differences in expression levels (153, 160).

The many faces of PGC-1α add to the complexity of the regulation and functions of this gene. Baar and colleagues found that PGC-1α has at least two protein isoforms, one full-length form and one smaller 34-kDa form (263). Later, it was demonstrated that transcription of the canonical exon (exon 1a) was increased by high intensity exercise and AICAR, which gave rise to the PGC-1α-a (PGC-1α1) isoform, while the alternative exon 1b drove expression from low-, medium- and

high-intensity exercise, AICAR, and clenbuterol (β_2-receptor agonist), gave with alternative splicing rise to PGC-1α-b (PGC-1α4) and PGC-1α-c (264). Various isoforms appears to have different functions (265). Truncated forms of PGC-1α have been reported to be more stable and primarily regulate angiogenesis, while full-length forms primarily regulate mitochondrial biogenesis. It should be noted that there are differences in how muscle-specific PGC-1α overexpressing transgenic mouse models have been produced, which makes it difficult to make direct comparisons between models (153, 261, 262). Two independent PGC-1α1 transgenic mouse models have been observed to exhibit different phenotypes. For example, MCK-PGC-1α animals are reported to prevent denervation and fasting-induced skeletal muscle atrophy (164), while another model displayed increased muscular atrophy (266) and dilated cardiomyopathy (267).

Metabolic effects in MCK-PGC-1α mice are activity-dependent

MCK-PGC-1α mice have increased vO2max, but no change in whole-body glucose homeostasis or insulin sensitivity under either fed and fasting sedentary conditions (159). Thus, the PGC-1α transgenic mice do not exhibit differences in multiple metabolic parameters under basal conditions, but display lower respiratory exchange ratio during exercise, indicating an enhanced fat metabolism and fuel utilization. PGC-1α promotes anabolic processes, including synthesis and storage of intramyocellular glycogen (160) and lipids (268). Increased intramyocellular lipid droplets is a signature of increased fuel storage related to exercise training, with the term "athlete's paradox" implying that high oxidative capacity and increased insulin sensitivity from endurance exercise occurs in parallel with accumulation of glycogen and intramyocellular lipid. Lipid accumulation is not an issue under physiological conditions due to constant substrate turnover in exercise (269). Under normal physiological conditions of exercise, PGC-1α induces a coordinated program of increased energy delivery, mitochondrial biogenesis, and fatty acid oxidation to meet the increased energy demands of working skeletal muscle (175). Despite the strong promotion of an exercised muscle phenotype, elevated expression of PGC-1α in sedentary mice exacerbates diet-induced insulin resistance (175), due to intramyocellular lipid accumulation from increased fatty acid uptake and re-esterification exceeding mitochondrial fat oxidation. However, elevated expression of PGC-1α in exercising mice enhances exercise-induced improvements in glucose homeostasis, oxidative capacity and lipid partitioning (176, 268).

MCK-PGC-1α mice may have too high PGC-1α levels

According to a review, the physiological induction of PGC-1α from acute and exercise training should be between ~50-300% increase in mRNA and protein levels (270). Supraphysiological PGC-1α production >600% induces many undesirable consequences, including abnormal mitochondrial proliferation,

disruption of myofibrillar architecture, intramuscular lipid accumulation, and insulin resistance (175, 262). The fatty acid transporter FAT/CD36 is a PGC-1α-inducible gene that has been linked to insulin resistance due to its function to increase uptake of fatty acids outside the cells capability for oxidization, resulting in lipid accumulation (270). A modest increase in PGC-1α expression by ~25%, limited FAT upregulation and lipid accumulation, as well as improved fatty acid oxidation, insulin signaling and glucose transport (161, 261). The benefit of physiological overexpression is supported by a study using whole-body overexpressing PGC-1α transgenic mice, at a ~2-fold increased gene expression, which displayed improved muscle insulin resistance, an effect which was neutralized by high-fat diet (271). Therefore, it is possible that the PGC-1α dose has to be carefully titrated to avoid unwanted effects. A transgenic mouse model with modest overexpression of PGC-1α may have displayed different effects on the CNS than what we have observed in the MCK-PGC-1α. However, it is not known if a modest upregulation of PGC-1α would have yielded the same response in secretable myokines (2, 122).

Mimicking exercise-induced effects on the CNS

Evidence for effect of exercise factors on the brain

Exercise improves general brain health, brain plasticity and cognition, and evidence indicates that factors secreted by peripheral organs are involved in this regulation. For example, the PGC-1α-inducible myokine FNDC5 can induce acute effects in the brain when adenovirus with a FNDC5 construct is injected in the liver (3) or intravenously (123). Further, the pro-angiogenic PGC-1α-inducible factor VEGF in muscle has been found to be necessary for exercise-induced neurogenesis (2, 137). Likewise, skeletal muscle activation by AICAR and GW can induce short-term effects on neurogenesis, neuroprotection, and cognition (4, 182, 272, 273). The AMPK- and PGC-1α-inducible myokine cathepsin B can pass through the BBB to enhance hippocampal BDNF levels, neurogenesis, learning and memory (109). Apart from these factors, also other factors secreted by muscle, adipose tissue (e.g. adiponectin), and liver (e.g. FGF-21, IGF-1), mediate exercise-induced effects on neurogenesis, cognitive function, appetite and metabolism. Exercise-induced conversion of the neuroinflammatory tryptophane metabolite kynurenin to kynurenic acid is enhanced in MCK-PGC-1α animals (138), indicating the importance of peripheral metabolism in exercise-induced effects on the brain. Finally, MCK-PGC-1α have upregulated levels of circulating myokines with neurotrophic properties such as irisin, BNDF, and IL-15, capable of having potent effects on the CNS (122).

The difficulty in mimicking exercise-induced effects on the CNS

Evidence from the above-mentioned studies suggest that muscle-derived exercise-induced signaling influences neuroplasticity under physiological and pathophysiological conditions. From our studies, we found that muscle-specific PGC-1α overexpression did not improve recovery or protection from neurological conditions such as irradiation-injury to the brain, ischemic stroke, or age-related cognitive decline. Further, we found no differences between genotypes in basal or exercise-induced levels of neurogenesis, independent of age or sex. Our results based on whole-serum and protein analysis of serum, suggest that factors induced in the circulation by exercise, or by overexpression of PGC-1α in skeletal muscle cells, are not able to directly affect neural stem cell behavior. In summary, we conclude that forced expression of the PGC-1α pathway in skeletal muscle is not sufficient to mimic exercise-induced neurogenesis.

An acute peripheral overexpression of FNDC5 has been reported to increase BDNF expression in the hippocampus (3). Intravenous injection of an adenoviral vector, which upregulated the circulating level of FNDC5 improved synaptic plasticity and memory impairment in a mouse model of Alzheimer's disease (123). We find that upregulation of the PGC-1α/FNDC5 pathway in muscle is not sufficient to achieve exercise-induced effects on the CNS and that upregulation in other tissues may be necessary (3, 123). The question also remains if the long-term effects of exercise on the brain could be achieved sustainably by pharmacological or genetic therapy alone. Pharmacological activation of the AMPK pathway in skeletal muscle, which also reliable upregulates the PGC-1α pathway, indeed leads to a short-term increase in hippocampal BDNF expression and proliferation in the DG. However, after a few weeks of treatment these effects were lost and replaced with overexpression of pro-apoptotic and inflammatory genes in muscle (182). The study mentioned above, by Lourenco, and a study by Choi that reported improved cognition in an Alzheimer's mouse model by combined treatment with intravenous injections with AICAR and intracerebral injection with BDNF (272), both reported effects of treatment up to a 2-week time point. Our results from chronic upregulation of PGC-1α in muscle is relevant in predicting sustainable effects from activation of exercise-induced pathways in muscle, particularly considering the sustained upregulation of potent systemic factors in the circulation reported for the MCK-PGC-1α model (122). In contrast to these acute models of FNDC5 pathway stimulation, our chronic muscle activation model factors in complex compensatory counter-regulation that may occur over time.

Exercise induces adaptations in every organ of the body (274) with profound benefits for neural, immunological, vascular, and metabolic systems. Exercise is a complex stimulus affecting a range of tissues and cellular processes, that up- and downregulate thousands of genes with both temporally and spatially distinct

patterns. The mechanisms by which different modes of exercise may affect brain function remain to be elucidated. Further, caution should also be used when extrapolating factors to potential therapeutic treatment with seemingly healthy factors secreted by muscle cells may also have detrimental effects in other conditions (118). For instance, the proliferative mediator IGF-1 can also promote cancer (275) and decrease life span (276).

Controversies in the field of exercise-mimetics

Since the start of this PhD project in early 2012, several publications have emerged to fill the knowledge gaps in the field, which in some ways have changed our original assumptions and hypotheses. There is a need to continuously assess the merit of reported findings in the scientific community. Two papers reporting on positive metabolic, protection from muscle wasting and improvement of mitochondrial myopathy disorder in MCK-PGC-1α mice, were later retracted in 2016 (277, 278). In one of the retracted papers, MCK-PGC-1α mice was reported to have improved metabolic responses as evident by increased insulin sensitivity and insulin signaling, as well as being protected against systemic chronic inflammation observed during normal aging (278).

Irisin, meteorin-like, and BAIBA, are all exercise-inducible myokines regulated by PGC-1α, which have been reported by the Spiegelman lab. The best described factor of these is irisin, which was first reported in 2012. Irisin is produced by cleavage of the transmembrane protein FNDC5 for release into the circulation, triggering the appearance of inducible brown adipose tissue within white fat, as well as being increased in the circulation from exercise in humans (2, 130). This paper immediately received a lot of attention, even in the lay press and spurred a number of follow-up studies. However, observed levels of circulating irisin were highly variable and contradictory (279). Timmons found that muscle FNDC5 mRNA expression was increased only in a minority of older endurance-trained human subjects and that exercise-induced improvements in insulin sensitivity were not associated with FNDC5 gene expression (280). In another study, PGC-1α mRNA levels were only upregulated 4-fold in young and 2-fold in older men after an acute exercise bout, but not after endurance training (281). Due to the fact that commercially available ELISA kits used in publications to measure irisin were unspecific and detected cross-reacting proteins, Albrecht and colleagues concluded that irisin was not proven to be an exercise-inducible protein (129). However, the Spiegelman lab used tandem mass spectrometry to report that irisin was increased from 3.5 ng/ml in sedentary humans to 4.3 ng/ml in individuals that underwent 8 weeks of high intensity aerobic training, which can be considered a relatively small increase and the observation was based on 10 individuals (130). Due to these marginal differences in humans, and the previously used unspecific ELISAs, the extent circulating irisin is increased in humans needs to be validated in future studies.

CONCLUSIONS

Exercise leads to many physiological changes in the body and brain, including improved cardiovascular fitness, reduced inflammatory status, reduced oxidative stress, increased cerebral blood flow with improved exchange of oxygen, nutrients, metabolites in the brain, all which likely have a substantial role to play in exercise-induced effects on the CNS. Exercise exerts these effects on the body and brain by activating a complex network of pathways in different cell types, tissues, and organs, in a periodic and dynamic manner (274). Through the use of transcriptomics, proteomics, and metabolomics, attempts have been made to map out exercise pathways in muscle and exercise-induced factors in the blood. Even though a multitude of exercise factors have been identified, it is still unclear if and how much these factors contribute to exercise-induced effects. This would require comprehensive evaluation for each of these factors, in loss-of-function, gain-of-function and pharmacological targeting studies. It is a non-trivial endeavor due to the fact that patterns of factor expression differ between individuals, studies, species, modes of exercise, etc. The study of exercise-activated pathways by genetical and pharmacological approaches allows evaluation of the combined effect of downstream factors of certain signaling pathways that enable easier identification of factors. Through these studies, a few candidates released into the circulation from exercise have been identified as capable of inducing exercise-induced effects on the brain.

Even though genetic and pharmacological approaches of muscle activation have reported potent effects on the CNS, none have yet to report sustainable effects, possibly due to compensatory adaptations occurring in longer-term treatment. Exercise-induced neurogenesis is one of the most reproducible findings of exercise-induced changes on the rodent brain and has a central role in exercise-induced brain plasticity and cognition. We demonstrate that a sustained upregulation of the muscular PGC-1α pathway, despite potent systemic changes, is not sufficient to phenocopy exercise-induced neurogenesis. From this, we conclude that upregulation of the PGC-1α pathway in skeletal muscle is not sufficient to directly induce sustainable exercise-induced effects in the brain.

The study of PGC-1α, together with its related molecular pathways and downstream targets, contributes to our understanding of exercise-related benefits on the brain. The essential role of PGC-1α in mitochondrial metabolism and regulation of inflammation has made it a possible therapeutic target for a wide range of diseases. The findings presented here offer new insights for continued studies of PGC-1α, and provide evidence that upregulation of this co-activator under certain pathological conditions can even have detrimental effects.

Our findings raise doubts that pharmacological targeting of exercise-induced systemic factors can achieve sustainable effects on the CNS. However, pharmacological strategies based on exercise-induced effects remain an intriguing concept, even though a limited range of exercise-induced effects need to be aimed for. As previously discussed regarding the metabolic effects of artificial skeletal muscle activation, the necessity to actually consume energy will be an important limitation in exercise-mimicking manipulations. Perhaps such pharmacological possibilities could involve exercise adjuvants for enhancing health-promoting effects (282). It is also likely that more than one pathway will have to be targeted in order to achieve an exercise-mimicking effect (283).

Future perspective

Further studies are needed to determine if a modest overexpression of PGC-1α and downstream factors in skeletal muscle could confer sustainable exercise-induced effects in the CNS. Furthermore, continued studies are required to determine what other pathways in muscle and other tissues, as well as which circulating signals that could be important for mediating exercise-inducing changes on the brain. Especially, the findings in thesis highlights the necessity of understanding temporal aspects of crosstalk between brain and other tissues during exercise. A thorough understanding of the complex molecular mechanisms underlying exercise-induced effects on the body and brain could enable the discovery of new pharmacological targets, the identification of biomarkers for measuring health status, and the optimization of physical therapy based on genetics and molecular responses. Novel treatment strategies could aid in the management of neurological and neurocognitive impairments, as well as to increase quality of life in cancer and stroke survivors.

ACKNOWLEDGEMENTS

I have so many people to thank for my PhD thesis. My deepest and sincerest gratitude goes out to all the incredible people I have had the pleasure of working with over the years.

To my main supervisor, **Georg Kuhn**. Thank you for taking me on as a PhD student, for your support and belief in this project, and for allowing me to manage the project on my own. Your door has always been open, and you have always managed to sound encouraging no matter what bad news I have brought to the table. Your curiosity, and vast knowledge is a true inspiration for me.

Klas Blomgren, my previous main supervisor and mentor. Thank you for welcoming me into your group as a master's student more than a decade ago, and then taking me on as a PhD student a couple of years later. Thank you for believing in me, and continuously offering your support and wisdom.

Mats Börjesson, my co-supervisor. Thank you for taking on the role as co-supervisor in the middle of my PhD project. I have greatly appreciated your support, scientific input, and constant availability.

Reza Motalleb, thank you for having my back for the past few years (literally) and for contributing to this thesis. Thank you for all the long hours in the lab together, and all our scientific and philosophical conversation. I will miss having you as my room-mate and, most of all, your daily comical relief.

Thank you to all my previous students that have put in work into this PhD thesis (in chronological order): **Mar Larrosa-Flor**, **Jonas Bergqvist**, **María Nazareth González-Alvarado**, **Dilip Kumar Malipatlolla**, **Anna Vidal**, **Parthenia Savvidi**, **Chinenye Onyeanwu**, and **Eva Brigos**. You have all done terrific work, and I am proud and grateful to have had you as my students. I wish you the very best in the future.

Thank you also to everyone else how have helped with different aspects of the thesis. **Ahmed Osman**, for help with immunohistochemistry and phototrombotic stroke. **Peidi Liu**, for assistance with multiplex assay statistical analysis. **Gunilla Runström**, for assistance with the multiplex assays. **Keerthanaa Balasubramaniam**, for assistance with ELISA. **Anna Tjärnlund-Wolf**, for guidance and input in early projects. **Emil Egecioglu**, for initial help with setting up running wheels. **Anna-Lena Leverin**, for technical support on western blot.

The Center for Cellular Imaging, for expertise in imaging and technical assistance.

Rita Grandér, **Anne-Marie Alborn**, and **Birgit Linder**. Thank you for your wonderful personalities and kindness. You have always been there for me when I needed help. Thank you for introducing me to the lab and for all the assistance throughout my PhD thesis.

Special thanks go out to past and present members of Center for Brain Repair and Rehabilitation (CBR) for all their help and support during my PhD studies. **Changlian Zhu**, for all help and scientific discussions over the years. **Virginia Claudio**, for sharing your insights and for contributing to a friendly office environment. **Michelle Porritt**, for a fun time on the S-floor, help with protocols, and scientific input. **Ahmed Osman**, for experimental assistance and good laughs. **Marie Kalm**, for your advice and scientific input. **Olle Lindberg**, for technical support and great discussions. **Henrik Landgren**, for initial input early on in the project and for breaking my back in the downhill racing track. **Cecilia Bull**, for your encouraging curiosity and openness. **Dilip Kumar Malipatlolla**, for your kindness and laughs. **Piyush Patel**, for your kindness and helpfulness. **Jenny Nyberg**, for all the entertaining discussions. **Simon Skau**, for all inspiring and educational discussions. **Karolina Roughton**, for being a fun and energetic roommate. **Fredrik Larsson**, for fixing my bike and being a great roommate. **Martina Boström**, for help with antibodies and for good talks in the stereology. **Marie Kalm** and **Niklas Karlsson**, for the much-needed support and an introduction to science. **Giulia Zanni**, for all the laughs. **Andrew Naylor**, for all the good conversations.

Special thanks also go out to colleagues on the 3rd floor, **Eric Hanse**, for input on initial projects and for the time together in the faculty board. **Henrik Seth** and **Henrik Michaëlsson**, for a fun and rewarding collaboration.

Thank you to all colleagues on the S-floor for contributing to an interesting time and a dynamic work environment: **Malin Johansson, Keerthanaa Balasubramaniam, Davide Lovera, Katarina Tomazin**, **Kevin Weiss**, **Prince Nana Twum**, and **Chesia Testa**, and members the Zhu group, **Tao Li**, **Yanyan Sun**, **Juan Rodriguez**, **Kaiming Huo**, **Yafeng Wang**, and **Yiran Xu**. Thank you also to all past colleagues on the former 4th floor. **Wei Han**, **Ahmed Osman**, **Kai Zhou**, **Cuicui Xie**, **Åsa Persson Sandelius**, **Charlotta Blom**, **Nina Hellström Erkenstam, Malin Blomstrand**, **Thomas Padel**, **Elena Di Martino**, **Maurice Curtis**, **Ina Nordin**, **Anke Brederlau**, **Lina Bunketorp Käll**, **Heléne Andersson**, **Jenny Zhang**, **Yoshiako Sato**, and **Tomoyo Pschiishi**. Thank you for all the enjoyable fika breaks in the lunch room, inspiring discussions, and laughs, contributing to a cheerful place to work.

I also want to thank all colleagues on the current 4th floor. **Ali Komai**, for hugs. **Peter Micallef**, for great laughs. **Saliha Musovic**, for being really nice. **Ahmed Alrifaiy**, for all the smiles and sporadic conversations. **Michael El Hachmane**, **Kim Eerola**, **Massimo Muratorem**, **Eduard Peris**, **Belén Chanclón García**, **Charlotta Olofsson**, and **Ingrid Asterholm.**

I also want to thank past and current colleagues on the S-floor. **Mia Ericsson**, for all the interesting discussions on life in general and on the PhD education. **Amir Lotfi**, for a great conference in Chicago together. **Louise Adlermark** and other members of the addiction biology unit on the S-floor for contributing to a friendly environment. Thank you also to other people in the physiology building who I have had the pleasure to get to know: **Marcus Ulleryd**, **Peter Smith**, and **Mats Sandberg**.

Also thank you to **Gunnel Nordström**, **Oscar Bergström**, **Markus Johansson**, and **Kirsten Toftered**, for all help with IT and administrative tasks.

Thank you also to all fellow PhD students in the Faculty PhD student council, for making the PhD period more enjoyable and meaningful.

Finally, even though the decade-long journey to my PhD has been filled with laughter and many dear memories, it has not been an easy road. But as they say, nothing in the world is worth having unless it means effort, pain, and difficulty. With this in mind, I am thankful for having such wonderful family and friends who have supported me throughout this time. Above all, I am lucky for having such a loving and understanding wife who have stood by my side during the whole process. **Anna**, I love you and our children with all my heart, and I would sacrifice everything for you in the blink of an eye.

Financial support for this thesis was given by Barncancerfonden, Göteborgs Läkarsällskap, Drottning Silvias Jubileumsfond, Wilhelm & Martina Lundgrens Vetenskapsfond, and Stiftelsen Fru Mary von Sydows donationsfond.

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