THE SAHLGRENSKA ACADEMY

Effects of Muscular Overexpression of PGC-1alpha on Hippocampal Neurogenesis

Degree Project in Medicine

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Programme in Medicine

Gothenburg, Sweden 2017

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LIST OF ABBREVIATIONS

- **BDNF** brain-derived neurotrophic factor
- **BrdU** 5-Bromo-2-deoxyuridine
- **CNS** central nervous system
- **DG** dentate gyrus
- **fMRI** functional magnetic resonance imaging
- **GABA** neurotransmitter gamma aminobutyric acid
- **GCL** granular cell layer
- **IGF-1** insulin like growth factor 1
- **IR** ionizing radiation
- **MCL** molecular cell layer
- **PGC-1a** peroxisome proliferator-activated receptor gamma co-activator 1-alpha
- **RT** radiotherapy
- **SGZ** subgranular zone
- **VEGF** vascular endothelial growth factor

ABSTRACT

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Radiation therapy is an important treatment modality for tumors in the central nervous system. However, ionizing radiation (IR) gives rise to long-term cognitive deficits in patients, such as intellectual and memory impairments and this is believed to be partly due to irradiation-damage on adult neurogenesis, birth of new neurons, in the dentate gyrus of the hippocampus in the brain. Studies on rodents have shown that voluntary running can restore adult hippocampal neurogenesis after IR. Further, one study have reported a potential link between exercise and adult neurogenesis through a PGC-1a/FNDC5 pathway. Here, we examine if expression of the muscle exercise gene PGC-1a contributes to beneficial effects on recovery after cranial IR in mouse using a transgenic mouse model that constitutively overexpress PGC-1a in skeletal muscle. Male animals were subjected to whole brain IR (4 Gy) at 4 months age. Animals were given daily intraperitoneal injections of BrdU (50 mg/kg) from day 3 to 8 post IR. For voluntary exercise, female animals were single housed in cages with free access to running wheels. After 5 days of acclimatization, half of the running wheels were unlocked and animals were given daily intraperitoneal injections of BrdU (50 mg/kg) for 3 consecutive days. Animals were euthanized 4 weeks after IR, or unlocking of running wheels, for immunohistochemical analysis. We did not find any changes in baseline or exercise-induced levels of neurogenesis, or difference in baseline or irradiation-reduced levels of neurogenesis. In summary, we conclude that forced muscular expression of PGC-1a does not have a beneficial effect on hippocampal neurogenesis after IR in mice. This suggests that overexpression of PGC-1a in skeletal

muscle is by itself not sufficient to mimic exercise-induced recovery after IR.

Key Words: Neurogenesis, hippocampus, PGC-1a, irradiation, voluntary exercise.

INTRODUCTION

The cellular and molecular mechanisms underlying exercise-induced changes in brain function are currently being characterized in rodent models. By understanding the cellular and molecular consequences of wheel running in the rodent brain, it may be possible to develop novel therapeutic interventions that mimic the positive effects of exercise. These potential pharmaceuticals will particularly be good for those who are unable to engage in vigorous physical activity (coma, spinal cord injury, etc) or in patient groups where the adherence rate for voluntary exercise programs are low and compliance would likely be far higher for an exercise pharmacomimetic, e.g children and elderly weak patients. Uncovering the molecular and cellular linkages between physical exercise and hippocampal neurogenesis is critical to advancements towards the creation of neuroprotective agents that can be administered to patients who risk long-term neurocognitive sequelae after radiation therapy and/or suffer from neurodegenerative diseases. This degree project in medicine have contributed to a bigger PhD project that studies the molecular mechanism behind the positive effects from exercise on adult neurogenesis in the brain hippocampus. The following paragraphs describes what neurogenesis is.

Neurogenesis and regenerative medicine

The central nervous system (CNS) was widely perceived as a structurally fixed, non-cellular organ that is incapable of regeneration, until Altman's pioneering studies that provided the first anatomical evidence for the presence of newly generated dentate

granule cells in the postnatal rat hippocampus (1). Despite his findings, the non-regenerative capability of the adult damaged brain still remained as an accepted scientific dogma for decades. However, accumulating evidence indicated that neurons and astrocytes can be generated from isolated cells of the adult mammalian CNS (2). This birth of neurons is the process by which neurons are generated from neural stem cells and progenitor cells in the postnatal brain, now termed adult neurogenesis. Later, Peter Eriksons' 1998 study confirmed that new neurons are also generated in the hippocampus of adult humans (3). Adult neurogenesis takes place in at least two cytogenic niches, the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (4). On top of this, regenerative medicine has emerged as a new scientific field advancing stem cell therapy for treating brain disorders, with emphasis on either transplanting exogenous stem cells or amplifying endogenous stem cells via neurogenesis (5, 6). The later, is subject in this project paper.

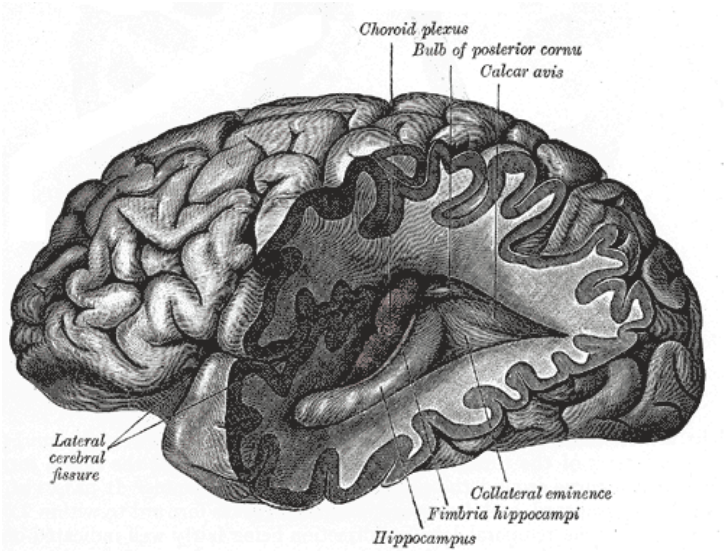

Figure 1: The hippocampus is located within the medial temporal lobe of the human brain hemispheres (7)

Hippocampus: anatomy, function and neurogenesis

The hippocampus, or the hippocampal formation, that holds the SGZ which is one of the two locations for adult neurogenesis in the brain, is a bilateral structure found within the medial temporal lobes of the human brain hemispheres (Fig 1). The hippocampal formation belongs to the limbic system and is considered to play a key role in the learning process, short-term memory and spatial navigation (8), but the precise nature of this role remains widely debated (8-11).

The hippocampus consists of different anatomical parts (Fig 2). The SGZ is outlined by the border between the granular cell layer (GCL) and hilus. The SGZ contain stem cells and progenitor cells. They play a key role in maintaining hippocampal functions such as memory and learning. They are also considered to be of importance in the functional recovery after CNS injury due to their regenerative potential (12-15). The GCL, together with the SGZ defines the structure of the dentate gyrus (DG) of the hippocampal formation.

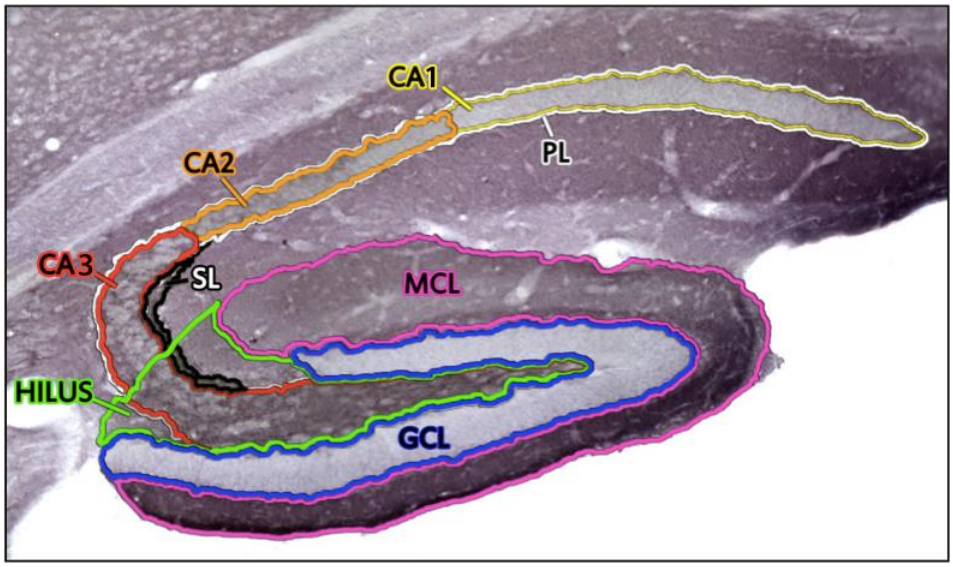

Figure 2: Anatomical description of the different hippocampal layers in mice. The pyramidal layer (PL) is divided into three subareas (CA1- 3). The stratum lucidum (SL) is a separate area alongside the PL. The SGZ is outlined by the border between the GCL and hilus. Colors outline the areas: GCL (blue), molecular cell layer (MCL, magenta), Hilus (green), PL (white), CA1 (red), CA2 (orange), CA3 (yellow) and SL (black). Used and edited by permission from Lars Karlsson.

Function of the Dentate gyrus

One of the main objectives behind research within this field is to find means to ameliorate the pathological effects from for example neurodegenerative diseases and long-term neurocognitive sequelae after radiotherapy (RT). Examples of such neurocognitive sequelae are varied kinds of memory impairments. As stated above, the hippocampus play an important role in memory, but it's still extensively researched about which and how the different anatomical parts of the hippocampus contribute to the memory function. In the scope of this degree project, the function of the DG is central as it is the site for adult neurogenesis, thereby most relevant to discuss. The most common theory of the function of the DG of the hippocampal formation is *pattern separation* of sensory information to the hippocampus. As events are experienced, cortical inputs from the enthorinal cortex reach the hippocampus via the perforant path, which projects to both DG and CA3 (16). It is believed that DG compresses and conjunctively encodes multimodal sensory and spatial representations about the environment and projects it further downstream in the hippocampus for processing, which results in minimal interference between overlapping spatial or contextual information. Both experimental lesion studies in rodents and neurocognitive testing and fMRI studies in humans supports this role of the DG (17, 18). However, because "pattern separation" is a feature of most brain circuits, some scientist argue it is better to refer to DG function as controlling "memory resolution" (19), or describe DG role

as ”binding” (20), but this difference in terminology is mostly semantic.

Function of Adult Neurogenesis.

Extensive research, using several different approaches, increasingly suggest that the role of the adult neurogenesis is highly complex and affects multiple aspects of learning, as opposed to having a clearly definable function (17). However, as the adult neurogenesis takes place in the dentate gyrus, it is believed to play a critical role in pattern separation. During the maturation process (see below) of the adult-born granular cells (GC), studies have shown that these immature neurons may be hyperexcitable, in comparison to more mature granular cells, suggesting that their functional contribution to DG function is different, from more mature GC in the DG network (21, 22). In short, the common theoretical framework for adult neurogenesis role in DG pattern separation, is that a continuous neurogenesis results in a combination of signals from the DG to the CA3 that consists of two separate populations;

a) one population of broadly tuned GC that weakly encode most of the features of the environment, and
b) one sparse population of sharply tuned GC that strongly encode only features that have been previously experienced.

Together, they promote high memory resolution/pattern separation while at the same time keeping already formed memories distinct, and minimizing interference in downstream networks (19). This is consistent with some experimental studies, for example, when adult neurogenesis is blocked either by IR or by genetic ablation, discrimination is impaired leading to generalization and inability to distinguish context/object A from B. In contrast, when neurogenesis is stimulated by genetic manipulations or by exercise, discrimination is enhanced (18).

Maturation process of new born granular cells in the adult hippocampus

Because neurogenesis in the adult hippocampus is the process in which new cells are born to become neurons, naturally, this process consists of a couple of general, developmental phases (23) (Fig 3) which can be tracked and analyzed using different cell markers. The process begins with an expansion phase where radial glia-like stem cell with the capacity for self-renewal, start to multiply through cell division within the SGZ occurs (24, 25). Thereafter, there are at least two consecutive amplifying lineages of progenitor cells where immature cells with the ability to differentiate into neurons start to accumulate and migrate into the GCL (25). After the expansion phase comes the survival and elimination phase, where the early survival phase of the amplified stem cells begin with the exit from the cell cycle. The cells begin to extend their axons and dendrites towards the molecular layer perforant path-associated cells in MCL in order to receive their first synaptic input and progress into the postmitotic maturation phase. The main synaptic input to the new cells is GABAergic and drives the ongoing maturation until the glutamatergic input from the entorhinal cortex (adjacent to the CA1 area of the PL) sets in (26). The majority of the cells fail to establish functional connections during this stage and are therefore eliminated through an apoptotic process. The glutamergic input drives the late survival phase that is a continuation of the maturation process for the neuronal cell and its dendritic spines. Once glutamergic synaptic connections have been made, the new neurons go through a phase of increased synaptic plasticity where the threshold for long-term potentiation is lowered. The total time for the neurogenetic process, when the differentiated cell becomes indistinguishable from old neuronal cells, is approximately 7 weeks (26, 27). Mature neurons express the postmitotic neuronal marker NeuN, the most widely used indicator of mature neurons (28). 5-Bromo-2-deoxyuridine (BrdU) has been a principal marker

for mitotic cells in studies of adult neurogenesis (29). BrdU is an analog to thymidine that can be administered systematically. BrdU is a false base that competes with endogenous thymidine during the synthesis phase of the cell cycle. Ki-67 is also a mitotic marker that can be used as an alternative, endogenous marker for proliferation (30). The antibody Ki-67 identifies a cell cycle associated protein that appears to be essential for cell cycle progression, but the exact function of the protein is unknown. The Ki67 marker appears in cells during late G1, S, G2 and M phases, but not in cells in late G0- and early G1 phase. One study has shown that Ki67 immunohistochemistry yield higher number of proliferating cells compared to BrdU (30). In contrast to Ki67, or other endogenous markers of cell division, BrdU identifies cells dividing at the time of marker injection, not when the tissue specimen was fixated, in which the tissue is prepared with solutions to immobilize antigens while retaining cellular and subcellular structure. Thus, BrdU allows the labeling of a cohort of cells undergoing division at the known time when BrdU was applied. This allows researchers to use various study designs, where they can control the interval between the time-point of birth-marking by BrdU-injections/BrdU-incorporation and the collection of tissue and its analysis. This interval can be set to days, weeks or years, and gives the labeled cells sufficient time to differentiate and mature into neurons. It is this fundamental difference that is the basis for the identification of new neurons with the BrdU method. However, because the synthesis phase only comprises half or less of the entire cell cycle, the BrdU method will underestimate the true, absolute size of the proliferating cell population. These three above stated markers have been used in combination in this thesis study.

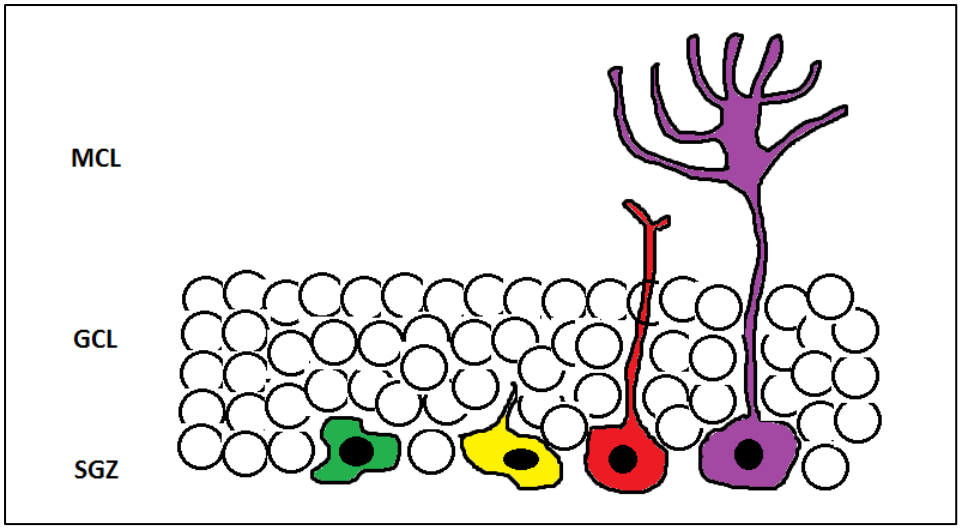

Figure 3: Overview of the adult neurogenesis in hippocampus.
Radial glia-like stem cell (green) divides and immature progenitor cells (yellow) start their differentiation towards becoming neurons. The cell maturation continues by exiting the cell cycle and the cells (red) start to stretch out their dendritic trees towards the MCL to establish functional synaptic connections. The increasing synaptic input to the cells (violet) drives the maturation process to its final phases where the cell is considered a mature neuron.

Physical activity and brain health

The health benefits of regular exercise are well documented, with an abundance of evidence pointing to a decreased risk of developing several chronic diseases, including cardiovascular disease, metabolic syndrome, type 2 diabetes and several cancers (31). Systemic effects of physical activity include increased insulin sensitivity (32), decreased blood pressure (33), improvement in blood lipid profile (34) and reduction of chronic low-grade inflammation (35). Physical activity has gathered substantial attention as a potentially effective key habit, or lifestyle, for elevating cognitive function and improving brain health throughout the lifespan. Studies have shown a direct correlation between increased levels of physical activity and improved cognition, with increases in hippocampal volume following exercise (36). The beneficial effects of physical exercise on cognition have also been seen in middle school students (37).

Thus, the benefits of physical activity on cognition appear to be widespread across all age groups. Further, physical exercise is also associated with increased general cerebral blood flow and nutrition supply (38), angiogenesis (39), neural activation (40) and reduced age-related degeneration of brain tissue (41). Moreover, it has been demonstrated that physical activity can ameliorate the effect of neurological diseases characterized by cognitive deficits, particularly in Alzheimer's disease, Parkinson's disease, and psychiatric illnesses such as depression and schizophrenia (42). Some of the potential, biological mechanisms behind the positive effects of physical activity on cognition includes the optimizing effects on hormonal stress response systems (such as the hypothalamic-pituitary-adrenal axis), the anti-inflammatory action through microglial activation and improved trophic factor signaling with attention centering on the neurotrophins (43, 44). The later are comprised of a group of closely related polypeptides that regulate a variety of neuronal functions including proliferation, survival, migration and differentiation. Among these, brain-derived neurotrophic factor (BDNF) is regarded as perhaps the most important (44), and could be a mediating factor between an exercise-related transcriptional factor and adult neurogenesis, an hypothesis introduced in more detail in a later paragraph below. Other important signaling molecules with neurotrophic properties, and has been shown to be regulated by exercise and influence adult neurogenesis include vascular endothelial growth factor (VEGF), insulin like growth factor 1 (IGF-1), fibroblast growth factor 2 and nerve growth factor (45).

Brain tumors in children

One patient group that potentially could benefit from research unraveling the molecular mechanisms behind the positive effects from exercise on adult neurogenesis and

hippocampal function is children treated with IR because of CNS tumors, sadly often suffering from long-term neurocognitive sequelae in aftermath. The mean annual incidence rate of cancer in children under 15 years is 16.0 per 100,000 in Sweden (46). Leukemia, CNS-tumors and Lymphomas constitute about 70% of the childhood cancer diagnoses. Leukemia represents approximately 30% of all these malignancies being the most common childhood cancer overall, where 85% of these cases consists of acute lymphatic leukemia. The second most common malignancy and the most common solid cancer in children is brain tumor, representing approximately 28% of all childhood cancers. The age distribution in CNS-tumors has a relatively even distribution without evident incidence peaks. In Sweden, the most common types of brain tumors are astrocytoma (originating from astrocytes, ~45%), medulloblastoma (originating from the neuroectodermal precursor cells, ~20%) and ependymoma (originating from ependymomal cells, ~10%).

Over the past four, five decades, there has been increase in the incidence of children diagnosed with leukemia and brain tumors. The increase is partly due to improved diagnostic techniques and routines of detection. However, there has been a gradual improvement of the outcome for all diagnoses of childhood cancer since the 1970s. The combination of surgery, IR and chemotherapy was a prominent breakthrough in the 70s and 80s, most pronounced in acute lymphatic leukemia and Non-Hodgkin lymphomas. This, so called, 'total therapy' is also very important in efficient brain tumor treatment. Typical treatment standard for malignant brain tumors is surgery followed by postoperative radiation and chemotherapy (47). Application of this therapy strategy these past decades, in combination with improved diagnostic measures have led to approximately 80% survival rate for children suffering from cancer in Sweden. Now, one the greatest challenge for the future will be the preparation and implementation of

new treatment methods to cure even more children while at the same time reducing the toxic long-term side effects of the treatment. Research like in this paper hopefully contributes with some knowledge, about the later subject, and adds clues about how we can make use of exercise-related mechanisms to treat, or curb neurocognitive sequelae due to cranial IR in the future.

Neurocognitive deficits due to cranial irradiation

Recent, therapeutic advancements in the pediatric treatment of CNS associated tumors has brought with it radical increase in survival rate, but also new concerns about the neurocognitive deficits seen in surviving patients many years after cranial IR , as stated above. These long term side effect, also called sequelae, affects as many as half of the children treated for brain tumors with RT. The two most common forms of sequelae is neuropsychological and neuroendocrinological damage. These sequelae comprise intellectual deterioration such as reduced attention span, memory, learning and information processing speed, as well as perturbed growth and puberty (48-50). There is evidence that even low doses of ionizing radiation can bring about these deleterious effects on cognition (51). These iatrogenic symptoms tend to be more severe the younger the children (52-54). Further, cognitive complications from brain IR appear not only in children but also to some degree in adults, greatly increasing the risk of dementia in long-term survivors (55).

Radiation therapy detrimental for hippocampal function

There are several mechanisms in which hippocampal function is impaired by radiation, but some of these negative effects can be partially restored or alleviated by exercise. Firstly, in the treatment of brain tumors and other forms of cancer, cranial RT induces persistent microglial inflammation that is accompanied by nearly complete inhibition

of human hippocampus neurogenesis, resulting in long-term cognitive deficits (56). IR target tumor cells which have prominent proliferative capacity, but also affect the stem and progenitor cells in hippocampus, shown in studies on rodents (57, 58). Secondly, radiation therapy is detrimental for the neurogenic niche due to an increased inflammation that creates a neural environment that inhibits to neuronal plasticity (59). However, studies have shown that exercise reduces inflammation and that lack of exercise obstructs neurogenesis, maybe through reduced levels of circulating factors, such as growth factors and neurotrophic factors (60, 61). In mice, voluntary physical exercise after cranial IR enhances hippocampal neurogenesis and improves long-term behavior (62). Thus, as suggested in the introduction of this paragraph, exercise in the form of voluntary running can prevent irradiation-induced memory decline and aid recovery of adult hippocampus plasticity (63). The mediating mechanisms behind these effects from exercise are although largely unknown. However, one of the most important trophic factor behind brain neurogenesis, synaptic plasticity, angiogenesis, memory and learning are believed to BDNF, mentioned earlier. Peripheral and central levels of BDNF, VEGF and IGF-1 has been shown correlated with exercise (45, 64). For example, physical exercise can increase BDNF gene expression in hippocampus and there is a significant positive correlation between voluntary running distances and levels of BDNF mRNA (65). Lastly, IR decreases microglia in hippocampus that have been implicated in maintaining the neurogenic niche in (66, 67), where voluntary exercise has been shown to increase microglial population (68). To sum up, research stated above shows that RT and IR is detrimental for hippocampal function through different mechanisms, but promisingly, results from experimental research on rodents show that exercise can alleviate some of the negative effects from IR. The next paragraph introduces the protein that is central for this project, a transcriptional factor

that acts as a key mediator behind exercise-related muscle tissue adaptions and, via BDNF, possibly linked with hippocampal neurogenesis through a newly found molecular pathway.

Transcriptional factor PGC-1a and hippocampal neurogenesis

The transcription factor peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1a) is induced in skeletal muscle in short-term and endurance exercise. Because PGC-1a is a key mediator of the metabolic and structural adaptation of muscle to aerobic exercise, PGC-1a is considered to be responsible for many of the beneficial exercise-related effects in muscle. PGC-1a is a potent regulator of gluconeogenesis, a powerful inducer of mitochondrial biogenesis, angiogenesis, muscle fiber-type switching and protects against atrophy (69-72). PGC-1a is believed to be one of the key regulators of the stimulation of mitochondrial biogenesis by exercise (73), and one isomer of PGC-1a has been found to regulate and coordinate factors involved in skeletal muscle hypertrophy (73, 74).

PGC-1a is widely expressed in the rodent brain and the level of expression is stable throughout different physiological states, e.g. states of caloric deficiency, obesity or cold exposure (75). Because PGC-1a expression in the brain is widely distributed, it suggests that it may have a fundamental physiologic role in the CNS. Later studies have shown that lack of PGC-1a in the brain is associated with neurodegeneration (76, 77). Further, PGC-1a might play an important role in the formation and maintenance of neuronal dendritic spines (78). Interestingly, long-term forced treadmill running over 12 weeks increases mRNA expression of PGC1-a in various areas of the brain, and are generally similar in magnitude as those in skeletal muscle. Exercise also increases similar molecular pathways in the brain as in the muscle (79).

Exercise-induced PGC-1a increases reactive oxygen species-detoxifying enzymes, which might be the reason for PGC-1a protective function against oxidative stress (80), which suggest that PGC-1a may also have a beneficial impact on irradiation-induced oxidative stress (81). A recently identified PGC-1a-dependent myokine, FNDC5, is cleaved and secreted from muscle during exercise (82). Interestingly, elevated peripheral levels of FNDC5 by exercise induce expression of BDNF in hippocampus (83), suggesting that there is a potential link between exercise and hippocampal neurogenesis through a PGC-1a/FNDC5 pathway.

A transgenic mouse model with skeletal muscle-specific upregulation of PGC1-alpha through the muscle creatinine kinase promoter (MCK-PGC1a) has an endurance exercise phenotype, and shows resistance to metabolic diseases by preventing age-associated insulin resistance and improving muscular insulin signaling (84), as well as against stress-induced depression (85). This transgenic mouse model is used in this thesis study.

AIM

By understanding the cellular and molecular consequences of wheel running in the rodent brain, and finding molecular targets, it may be possible to develop novel therapeutic interventions that mimic the positive effects of exercise. These potential pharmaceuticals will particularly be good for those who are unable to engage in vigorous physical activity. The purpose of this study is thus to find means to improve recovery and protect from neurological conditions such as irradiation-injury to the brain, stroke and age-related cognitive decline. The two main objectives in this study are as follows;

1) Determine if forced expression of PGC-1a in muscle results in affected baseline or exercise-induced levels of hippocampal neurogenesis

2) Determine if forced expression of PGC-1a in muscle ameliorates irradiation-reduced levels of proliferation in SGZ

MATERIAL AND METHODS

Animals

Transgenic MCK-PGC-1a animals on C57BL/6J background (The Jackson Laboratory; Stock no. 008231), a kind gift from Dr. Bruce Spiegelman (Harvard Medical School, Boston, MA), have been previously described (65). Female C57BL/6J (Charles River, Germany) mice were used for breeding purposes. 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 with ad libitum access to food and water. All the experiments were approved by the Gothenburg ethical committee on animal research (#317- 2012).

Voluntary Exercise

At 2 months of age female mice were single housed in cages with free access to running wheels. After 5 days of acclimatization, running wheels were unlocked and monitored for activity, or as a control environment kept locked for the sedentary group. For 3 consecutive days following unlocking, animals were given daily intraperitoneal injections with 50 mg/kg BrdU (Roche Diagnostics)

Irradiation Procedure

At 4 months of age male animals were anesthetized with an intraperitoneal injection of 50 mg/kg tribromoethanol (Avertin, Sigma-Aldrich) and placed on a polystyrene bed.

The head was covered with a 1 cm tissue-equivalent material to ensure an even IR dose into the underlying tissue. Whole brain IR was administered as a single dose of 4 Gy using a linear accelerator (Varian Clinac 600 CD, Radiation Oncology Systems LLC) with 4 MV nominal photon energy and a dose rate of 2.3 Gy/min. The sham-irradiated control mice were anesthetized but not subjected to IR. After IR the animals were returned to their cages. Animals were given daily intraperitoneal injections with 50 mg/kg BrdU (Roche Diagnostics) from day 3 to 8 post IR.

Sample Processing

Four weeks following unlocking of running wheels or IR mice were deeply anesthetized with a peritoneal injection of 50 mg/kg sodium thiopenthal (Pentothal, Sigma-Aldrich) and transcardially perfused with cold saline solution (0.9% NaCl) followed by 4% paraformaldehyde (PFA) in phosphate-buffered solution (PBS). The brains were immersion-fixed in PFA and subsequently cryoprotected in 30% sucrose in 0.1 M PBS after 24h. Left hemispheres were sectioned sagitally (25 mm thickness), and collected in series of 12 for immunohistochemistry using a sliding microtome (SM2000R, Leica Microsystems) modified for frozen sectioning. Sections were stored at 4°C in cryoprotectant solution (TCS) containing 25% glycerol and 25% ethylene glycol in 0.1 M PBS.

Immunohistochemistry

Staining with BrdU and NeuN

Free-floating immunohistochemistry sections were rinsed in Tris-buffered saline (TBS). An antigen retrieval step in 10mM sodium citrate (pH 6) for 20 minutes at 80°C was used for immunohistochemistry with antibodies against the neuronal nuclear

antigen NeuN. For BrdU stainings, sections were incubated in 2M HCl for 30 minutes at 37°C and neutralized in 0.1 M borate buffer (pH 8.5) for 10 minutes at room temperature. For all immunostainings for, sections were incubated in blocking solution containing 0.1% Triton X-100, 3% donkey serum (Jackson ImmunoResearch Laboratories) in TBS for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight in both rat anti-BrdU (1:250, AbDSerotec, OBT0030) and mouse anti-NeuN (1:5000, Millipore, MAB377). After rinsing in TBS, sections were incubated at room temperature with secondary antibody in blocking solution as follows: donkey anti-mouse 555 IgG (1:1000, Molecular Probes, A21202) for NeuN and donkey anti-rat 488 IgG (1:1000, Molecular Probes, A21208) for BrdU**.** For immunofluorescence TO-PRO-3 (1:1000, Molecular Probes, T3605) was used as nuclear stain. Sections for immunofluorescence were rinsed and mounted from 0.1 M PBS onto glass slides, using ProLong Gold with DAPI (Molecular Probes) for coverslipping. Two core dyes were used for practical reasons for detection compatibility with available wide field and confocal microscopy; we could only visualize DAPI in stereology and we could only visualize TO-PRO-3 in the confocal microscope.

Staining with Ki67

For Ki67 stainings, sections were incubated in 0,01M NaCi (pH 6.0) for 20 minutes in 80°C water bath (10 minutes to cool off). After rinsing in TBS, sections were incubated in blocking solution containing 0,6% H_2O2 for 30 minutes and subsequently, after another rinsing in TBS, incubated in blocking solution containing 0.1% Triton X-100, 3% donkey serum (Jackson ImmunoResearch Laboratories) in TBS for 30 minutes at room temperature. The Primary antibody, rabbit anti-Ki67 NCL-Ki67p, were diluted

(1:500) in blocking solution (0,1% TX100/3% donkey serum/TBS, as above) at 4°C overnight. After rinsing in TBS, sections were incubated at room temperature with secondary antibody, donkey-anti rabbit-BT (1:1000, 711-065-152 Jackson ImmunoResearch), in blocking solution (as above) for 2 hours in room temperature. After rinsing with TBS, sections were treated with Avidin-Biotin-Peroxidase for 1 hour. Then sections were treated with a 3,3'-diaminobenzidine (DAB) solution (per 1 ml TBS; 10 µl DAB 25 mg/ml, 0,3 µl H_2O_2 30%, 5 µl of 8% $NiCl_2$) for 5-10 minutes. Finally, the sections were washed in tap water and TBS, and mounted from 0.1 M PBS on glass slides using X-TRA-kitt® (Medite) for coverslipping.

Imaging and Quantification

Investigator blinded stereological quantification was performed using a Leica DM6000B microscope and software (StereoInvestigator v10.40, MBF Bioscience). Ki67-positive cells were quantified at 40x optical magnification within the different subregions of the dentate gyrus. The SGZ was outlined as three cell layers into the GCL and three cell layers into the hilus. For analysis of NeuN/BrdU co-labeling a Leica SP2 confocal microscope was used. Co-localization was determined at 20x optical magnification and 2.5x digital zoom using sequential scanning mode.

Statistical Analysis

Data was processed in Microsoft Excel 2010 (Microsoft Corp.) and IBM SPSS v22 (IBM Corp.). For stereological analysis, data from lost or damaged sections were estimated as the average value of the two anatomically adjacent sections. Statistical analysis was performed using a statistical software (GraphPad Prism 6, GraphPad Software). Appropriate tests were chosen as specified in the text based on homogeneity

of variance. For multiple comparisons one-way analysis of variance (ANOVA) was used in conjunction with Tukey's post-hoc test. For repeated measures comparisons two-way ANOVA was used (RMANOVA).

RESULTS

Baseline and Exercise-Induced Levels of Neurogenesis under PGC-1a Overexpression in Skeletal Muscle

In order to investigate the baseline and exercise-induced neurogenesis in the MCK-PGC1a animals, we housed animals individually for 4 weeks with free access to running wheels. Daily running distances ranged from approximately 5-8 km per day, with tendency towards slightly less running activity in the MCK-PGC1a mice (Two-way ANOVA; n.s, n=7, Fig 4B). Net neurogenesis was calculated by the ratio of NeuN-labeled $BrdU^+$ cells multiplied by $BrdU^+$ cells in the GCL. Analysis with Two-way ANOVA showed a significant increase of net neurogenesis induced by voluntary running for both animal groups (***, p<0,001; n=4-9, Fig. 4A), by approximately 2000 respectively 1750 cells for wild type and MCK-PGC1a mice. However, no difference was observed between in baseline or exercise-induced levels of neurogenesis between MCK-PGC1a and wild type mice (Turkey's multiple comparisons test, n.s., Fig. 4A).

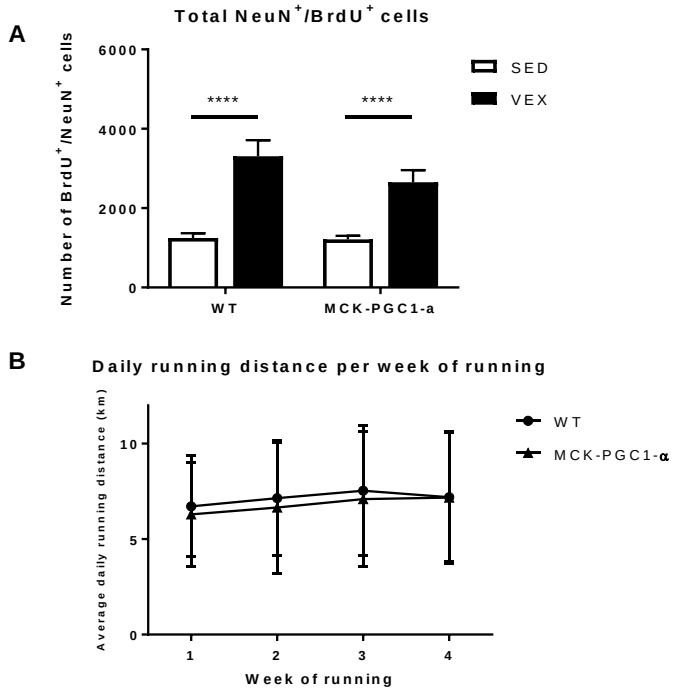

Figure 4: Characterization of Baseline and Exercise-Induced Levels of Neurogenesis under PGC-1a Overexpression in Skeletal Muscle.

A) Number of newborn neurons calculated as ratio of $NeuN^+/BrdU^+$cells in the GCL 4 weeks after unlocked running wheels, expressed as mean ± SEM for sham and irradiated animals. Significant increase after voluntary exercise in both WT and MCK-PGC1a animals (Two-way ANOVA; ****; $p<0.0001$); n=4-9), but no significant difference between groups (Turkey's multiple comparisons test; n.s.). B) Daily running distance per weeks of running (Two-way ANOVA; n.s., n=7).

Effects of Muscle PGC-1a Overexpression on Irradiation-Induced Inhibition of Neurogenesis

Using a model of cranial IR, we investigated the potential protective or regenerative effects from chronic muscular overexpression of PGC-1a on neurogenesis after IR. Net neurogenesis was calculated by the ratio of NeuN-labeled $BrdU^+$ cells multiplied by $BrdU^+$ cells in the GCL, with a significant decline in the relative number of new neurons to other cell types after radiation therapy in both groups (Two-way ANOVA; *, $p<0,05$, n=7-8, Fig. 5A). To analyze the neuronal lineage, DCX immunoreactivity

was used as marker for immature neuron (work done by another student in the research group), 4 weeks after radiation treatment, a significant decline in the number of DCX positive cells was observed in the GCL in both groups (Two-way ANOVA; *, p<0,05, Fig. 5B). The number of newborn cells in the neurogenic regions of the DG, SGZ and GCL in both groups showed a significant decline after IR (Two-way ANOVA; *, p<0,05, Fig. 5C). Total numbers of newborn, Ki67 positive cells in the GCL was not statistically significant before or after IR between the groups (Two-way ANOVA, n.s.; n=7-8, Fig. 5D).

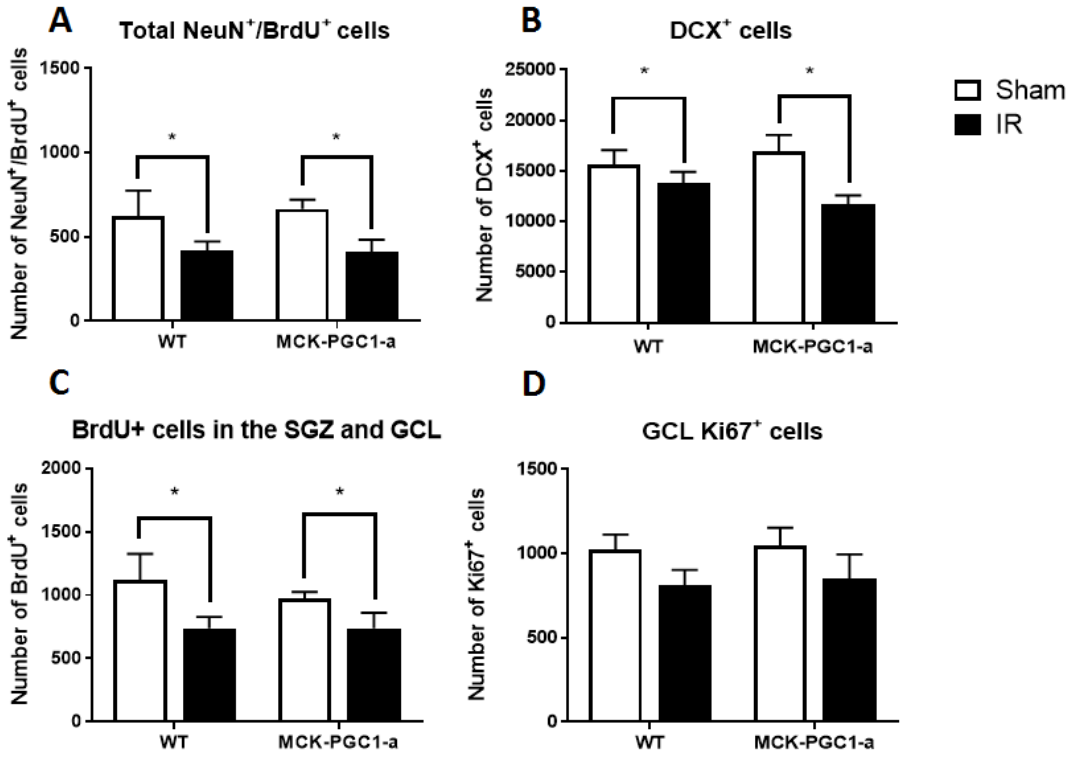

Figure 5: Effects of Muscle PGC-1a Overexpression on Irradiation-Induced Inhibition of Neurogenesis. A) Number of newborn neurons calculated as ratio of $NeuN^+/BrdU^+$cells in the GCL 4 weeks after IR, expressed as mean ± SEM for sham and irradiatied animals. Significant reduction after IR in both the WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05; n=7-8). B) Total DCX^+ cells in the GCL 4 weeks after IR (work done by another student in the research group). Significant reduction after IR in both the WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05). C) Number of newborn cells in the SGZ and GCL 4 weeks after IR. Significant reduction after IR in both WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05). D) Number of cells in cell cycle in the GCL 4 weeks after IR (Two-way ANOVA; n.s; n=7-8).

DISCUSSION

The objective of this study was to use a transgenic mouse model to observe the contribution of PGC1-a in exercise-induced recovery of neurogenesis following brain IR. Muscular overexpression of PGC-1a did not change baseline or exercise-induced levels of neurogenesis and did not ameliorate the negative effects on neurogenesis after IR in mice, thereby suggesting that PGC-1a overexpression in muscle is not sufficient to phenocopy the exercise-induced effects on neurogenesis that have previously been described.

Some earlier studies have reported upregulation of neurotrophic factors in these transgenic animals (82, 83), therefore we hypothesized that a constitutive PGC-1a overexpression could result in increased levels of baseline neurogenesis, with possible additive effect on exercise-induced neurogenesis. However, we did not detect any effect of chronic muscular overexpression of PGC-1a on physiological levels of baseline and exercise-induced neurogenesis in a running-wheel paradigm (Fig. 4). Other studies have reported that the MCK-PGC1a animals have unchanged baseline levels of gene expression for a number of neurotrophic factors in the hippocampus, including BDNF (85, 86). This, together with our findings, suggest that the FNDC5/BDNF pathway might not play a major role in exercise-mediated induction of hippocampal neurogenesis.

Although this study did not detect differences at baseline between PGC-1a mice and controls, we hypothesized that a different effect could emerge in a pathophysiological model where exercise has beneficial effects, in this case after IR to the brain. We observed a significant reduction of cytogenesis (BrdU) 4 weeks after IR in the GCL for

both MCK-PGC1a and wild type mice (Fig 5C), which disaffirm our hypothesis that muscular overexpression of PGC-1a could protect hippocampal cytogenesis from IR damage. The reduction of immature neurons (DCX) after radiation therapy was statistically significant in both (Fig. 5B), which could indicate that there is no difference between animals regarding survival rate of immature neurons or maturation rate after IR. We did observe a significant difference in the number of newborn mature neurons between the groups (Fig. 5A), indicating that the net neurogenesis in both animals is negatively affected following brain IR as was to be expected from previous studies (87). However, we did not observe any difference in number of cells in cell cycle 4 weeks after IR between the groups (Fig. 5D), suggesting that the dosage of IR used was not sufficient to elicit significant difference in rate of proliferation after treatment with IR.

Differences in dosage of IR could affect the level of effect on neurogenesis; using a lower or higher IR dose, as well as older or younger animals at the point of IR could lead to different results. However, the IR dose used was chosen based on the expected age-dependent decline of neurogenesis at 4 months of age in mice (88). In some species, for example rats and voles, there are sex differences in hippocampal neurogenesis. In laboratory mice however, research is equivocal as some studies have found higher cell proliferation and survival in female mice, while other studies have shown no such differences between male and female mice (89). In the running experiment we only used female mice. Because of the aforementioned equivocal results concerning sex difference on hippocampal neurogenesis in mice, we cannot draw certain conclusions on baseline and exercise-induced levels of neurogenesis in MCK-PGC1a mice based only on this data. However, in the larger PhD project, the running experiment includes both sexes and animals of different ages, and data from those experiments allows for more certain generalizations concerning neurogenesis in mice. In the IR experiment,

we only used male animals. It would be better to include female animals also. However, the decision to only include male animals was merely a matter of practicality, an issue of breeding and housing the animals, and not of methodological choice.

Here, we use a transgenic murine model with chronic upregulation of PGC-1a in skeletal muscle. Acute overexpression of FNDC5, a PGC-1a-inducible protein, resulted in increased BDNF expression in the hippocampus (83), at the same time Agudelo et. al did not see a difference in hippocampal BDNF gene expression in the chronic muscle activation model (85). In our study, the MCK-PGC1a mice showed a trend towards slightly less elevated levels of neurogenesis after exercise in comparison to wild type (n.s.; Fig. 4A). Since the transcriptional factor has been expressed constitutively during the development phase, this could have led to compensatory adaptations of molecular pathways. Hence, chronic expression of PGC-1a, seen as an adaptive response to long-term regular physical exercise, might not have the same effects on hippocampal neurogenesis as an acute expression of the same factor. It needs to be clarified if MCK-PGC-1a mice can upregulate downstream signaling factors released into the blood stream in the same physiologically relevant levels as exercise, and what other pathways in muscle also could be important for mediating exercise-inducing changes in the brain. Lastly, it also remains unclear if exercise-induced signaling from muscle alone is sufficient to produce changes in hippocampal neurogenesis.

The research on PGC-1a and its related molecular pathways, and downstream targets, adds to our understanding of exercise-related benefits on the brain. Further studies are needed to investigate the actions of PGC-1a and other molecular mechanisms by which muscle and other tissue could mediate exercise-induced effects after IR. Hopefully, this could lead to the creation of new drugs that can ameliorate irradiation-induced neurocognitive impairments and increase quality of life in cancer survivors.

CONCLUSIONS

Before any conclusions can be drawn, the study should be repeated. However, this study found no effects of muscular PGC-1a overexpression for baseline and exercise-induced neurogenesis, or on irradiation-reduced proliferation in SGZ. This suggest that PGC-1a overexpression might not be sufficient to phenocopy the exercise-induced effects on neurogenesis. As for future studies, using an acute model of PGC-1a expression, that mimics the acute physiological adaptions by exercise, could perhaps elucidate if PGC-1a plays a role in mediating the exercise-induced neurogenesis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Molekylära mekanismer bakom träningens positiva effekter på hjärnans återhämtning efter strålning

Introduktion

Hjärntumörer utgör cirka en tredjedel av alla årliga fall av barncancer i Sverige. Strålningsbehandling är en mycket viktig hörnsten i den aggressiva behandlingen av dessa tumörer, men detta medför också skador på friska delar av hjärnan. Strålning förgör i första hand ”nyfödda” celler i hjärnan vilket kraftigt minskar nybildningen av hjärnceller, s.k. neurogenes. Vi tror att neurogenes är viktigt för normal hjärnfunktion och att strålningens negativa effekt på den för övrigt friska hjärnan, åtminstone delvis, kan förklara varför unga patienter nästan uteslutande upplever livslång fortskridande försämring av kognitiva funktioner, t ex minnet. En möjlighet är att använda sig av behandlingsstrategier för att stimulera hjärnans egna möjligheter till läkning genom ökning av neurogenes. Studier har visat en stark korrelation mellan fysisk aktivitet,

exempelvis löpning, och en ökad neurogenes. Tidigare studier gjorda på gnagare har visat att fysisk aktivitet delvis kan återställa nivåerna av nybildning av hjärnceller efter strålning. Det är dock i stort sett okänt hur fysisk aktivitet stimulerar neurogenes. En studie har visat en möjlig koppling mellan fysisk aktivitet och neurogenes via ett äggviteämne kallat PGC1-alfa. I denna studie har vi använt möss som via genmodifiering producerar onaturligt mycket av detta äggviteämne för att studera om det kan skydda hjärnan från skada av strålning.

Syfte

Syftet är att undersöka om en överproduktion av äggviteämnet PGC-1-alfa som är kopplat till fysisk aktivitet kan öka nybildningen av hjärnceller och därmed öka hjärnans återhämtningsförmåga efter skadande strålning i samband med behandling mot hjärntumörer. Genom att undersöka och förstå de bakomliggande mekanismerna som orsaker träningens positiva effekter på hjärnans återhämtning efter skada, kanske man i framtiden kan skapa läkemedel som kan hjälpa patienter.

Metod

För strålningsexperimentet sövde man både genmodifierade möss med onaturligt mycket av äggviteämnet PGC1-alfa för att sedan stråla de över huvudet. Som kontrolldjur sövdes normala möss också, men de utsattes aldrig för strålning. För springexperimentet, lät man genmodifierande möss och kontrollmöss hållas med och utan tillgång till springhjul. I båda experimenten genomfördes dagliga injektioner av en celldelningsmarkör i mössens bukhåla, i antingen 3 eller 5 dagar under experimentens första vecka. Efter 4 veckor avlivades djuren och hjärnorna fixerades i formalinlösning för att senare klyvas i mycket tunna hjärnsnitt för cellräkning. Analysen utgjordes av

en kvantifiering och karaktärisering av nybildningen av hjärnceller med hjälp av olika cellmarkörer och mikroskop som tillåter väldigt hög upplösning för räkning.

Resultat

I springexperimentet såg man att mössen som tilläts springa i springhjul hade en statistiskt säkerställd högre nivå av nybildning av hjärnceller, jämfört med de möss som inte fick springa. Däremot fann man ingen skillnad mellan de genmodifierade mössen med en överproduktion av äggviteämnet PGC-1-alfa i jämförelse med normala möss. I strålningsexperimentet såg man ingen skillnad i totala antalet nybildade hjärnceller, varken före eller efter strålning, mellan de genetiskt modifierade mössen och de normala mössen.

Slutsats

Överproduktion av äggviteämnet PGC-1-alfa som är kopplat till fysisk aktivitet hos möss påverkade inte nybildning av hjärnceller, sk neurogenes. Överproduktion av äggviteämnet PGC-1-alfa skyddade inte heller nybildningen av hjärnceller från den skadliga effekten från strålning. Denna studien kunde inte visa att springande, genmodifierade möss med onaturligt hög produktion av äggviteämnet PGC-1-alfa hade mer nybildning av hjärnceller jämfört med normala springande möss, eller att genmodifierade möss tog mindre skada av strålning jämfört med normala möss.

ACKNOWLEDGEMENTS

I would like to thank Lars Karlsson, MD, PhD student, University of Gothenburg/Sahlgrenska Academy and Professor Georg Kuhn for their support and supervision during my work with this masters thesis.

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FIGURES

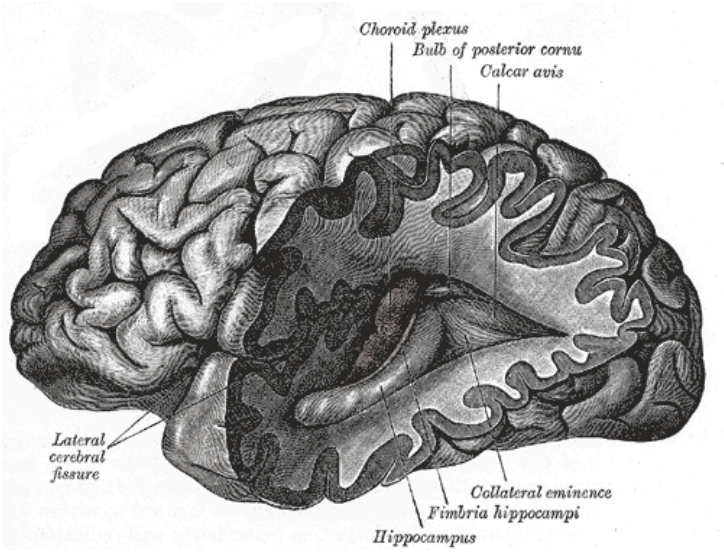

Figure 1: The hippocampus is located within the medial temporal lobe of the human brain hemispheres (7)

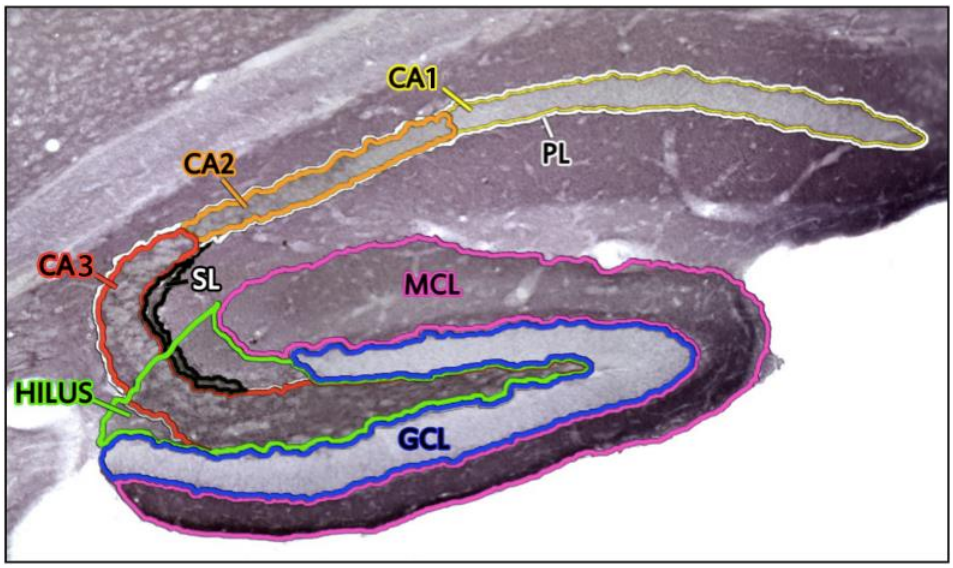

Figure 2: Anatomical description of the different hippocampal layers in mice. The pyramidal layer (PL) is divided into three subareas (CA1-3). The stratum lucidum (SL) is a separate area alongside the PL. The SGZ is outlined by the border between the GCL and hilus. Colors outline the areas: GCL (blue), molecular cell layer (MCL, magenta), Hilus (green), PL (white), CA1 (red), CA2 (orange), CA3 (yellow) and SL (black). Used and edited by permission from Lars Karlsson.

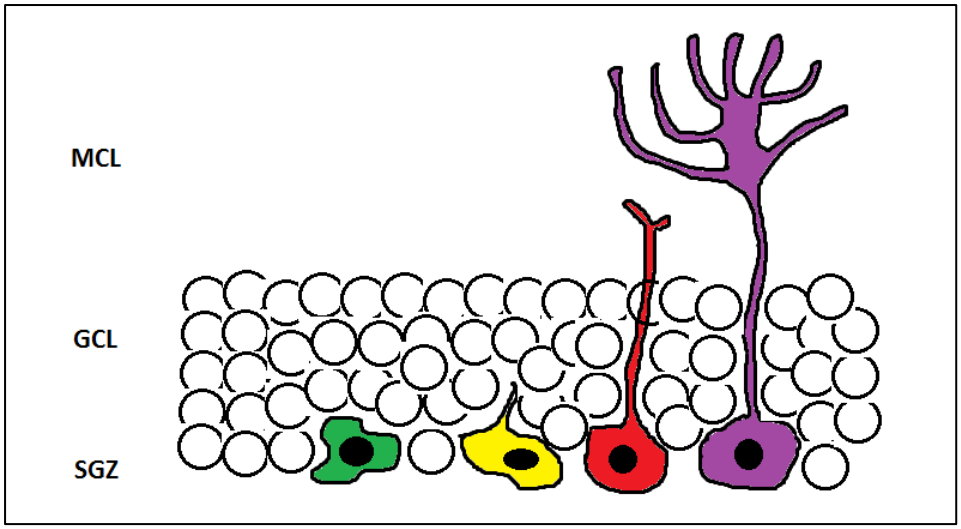

Figure 3: Overview of the adult neurogenesis in hippocampus.
Radial glia-like stem cell (green) divides and immature progenitor cells (yellow) start their differentiation towards becoming neurons. The cell maturation continues by exiting the cell cycle and the cells (red) start to stretch out their dendritic trees towards the molecular cell layer (MCL) to establish functional synaptic connections. The increasing synaptic input to the cells (violet) drives the maturation process to its final phases where the cell is considered a mature neuron.

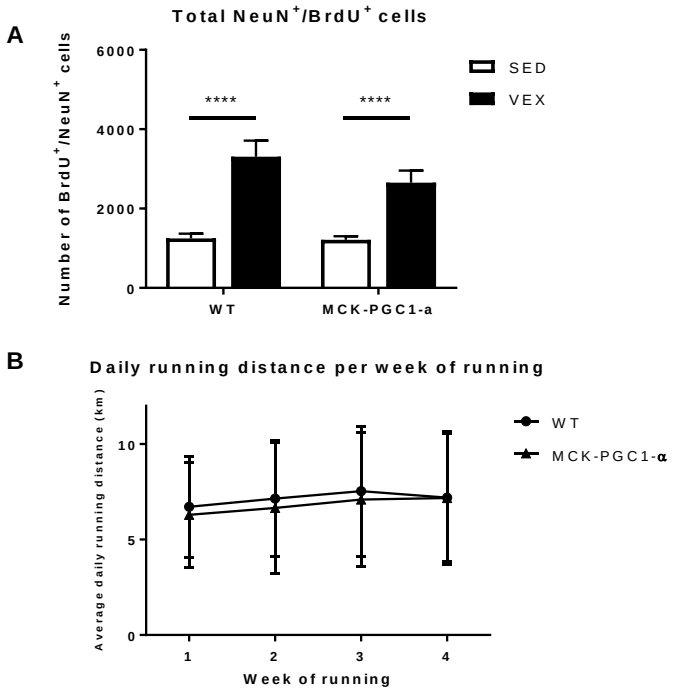

Figure 4: Characterization of Baseline and Exercise-Induced Levels of Neurogenesis under PGC-1a Overexpression in Skeletal Muscle.
A) Number of newborn neurons calculated as ratio of $NeuN^{+}/BrdU^{+}$cells in the GCL

4 weeks after unlocked running wheels, expressed as mean ± SEM for sham and irradiated animals. Significant increase after voluntary exercise in both WT and MCK-PGC1a animals (Two-way ANOVA; ****; p<0.0001); n=4-9), but no significant difference between groups (Turkey's multiple comparisons test; n.s.). B) Daily running distance per weeks of running (Two-way ANOVA; n.s., n=7).

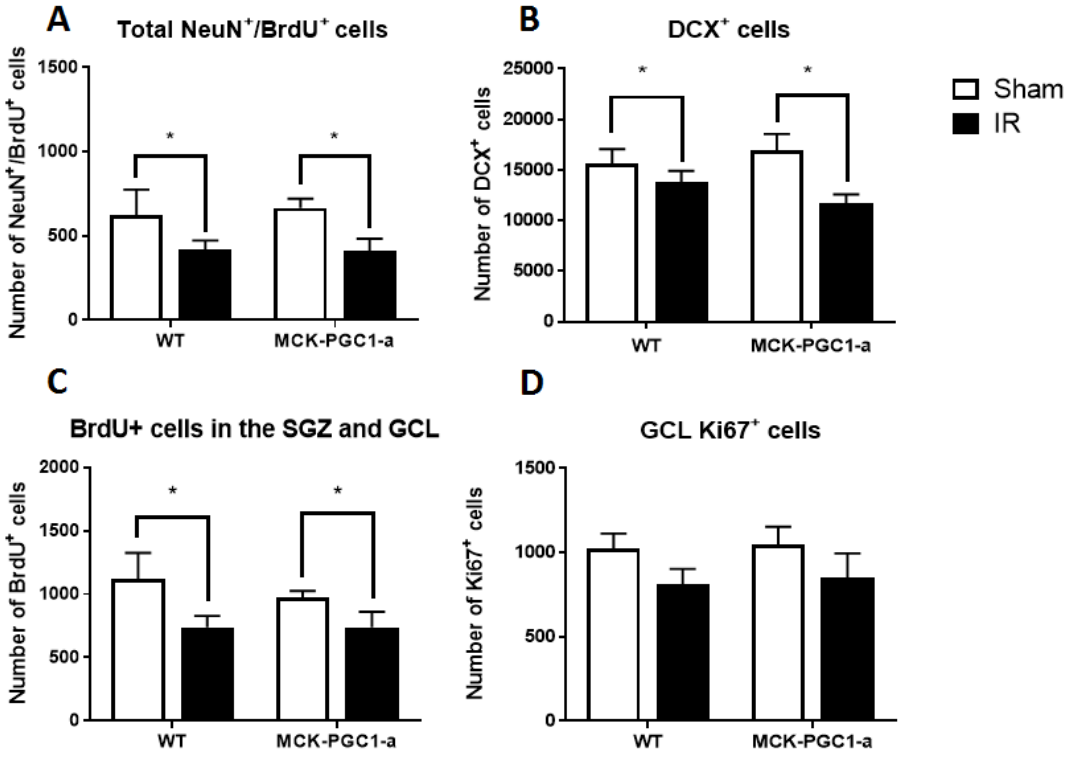

Figure 5: Effects of Muscle PGC-1a Overexpression on Irradiation-Induced Inhibition of Neurogenesis. A) Number of newborn neurons calculated as ratio of $NeuN^+/BrdU^+$cells in the GCL 4 weeks after IR, expressed as mean ± SEM for sham and irradiatied animals. Significant reduction after IR in both the WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05; n=7-8). B) Total DCX^+ cells in the GCL 4 weeks after IR (work done by another student in the research group). Significant reduction after IR in both the WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05). C) Number of newborn cells in the SGZ and GCL 4 weeks after IR. Significant reduction after IR in both WT and MCK-PGC1a animals (Two-way ANOVA; *, p<0,05). D) Number of cells in cell cycle in the GCL 4 weeks after IR (Two-way ANOVA; n.s; n=7-8).