EFFECT OF INHERITED AEROBIC CAPACITY ON NEURAL PLASTICITY IN HIPPOCAMPUS

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ABSTRACT

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Neural plasticity in hippocampus is a prerequisite for cognitive functions such as memory and learning. Different forms of neural plasticity include changes in the working of synapse functions, production of new neurons *i. e.* neurogenesis, and changes in microglia, that are cells of the immune system. Aging and neurodegenerative diseases result in cognitive decline and can impair hippocampal functions. Aerobic exercise has shown to improve cognitive functions and to increase plasticity markers in hippocampus. However, the role of intrinsic aerobic capacity is not as well known. The purpose of this thesis was to study the effect of intrinsic aerobic capacity and age on different forms of neural plasticity: synaptic plasticity and neuronal activation, neurogenesis and microglia activation. The study used an animal model with heterogenic rats that were selectively bred to have high aerobic capacity (HCR) or low aerobic capacity (LCR). The animals were kept sedentary and divided to four groups: young HCR (n = 9), young LCR (n = 10), old HCR (n = 12) and old LCR (n = 10). The young animals were euthanized at ~8 weeks and old animals at ~40 weeks of age. Four different regions of the hippocampus were analyzed: CA1, CA3, dentate gyrus (DG) and granule cell layer (GCL). Antibodies for microglia, neuronal activation, and neurogenesis were used for immunohistological analysis of hippocampus. Western blotting was used to determine expression of synaptic plasticity proteins and microglia in hippocampal homogenate.

The present results demonstrate that expression of synaptic plasticity proteins SYN-1 and SYP was higher in younger animals, but the results were inconsistent between the HCR and LCR lines, and there were no differences in neuronal activation between groups. The number of newborn neurons was significantly higher in younger compared to older rats, and in the HCR compared to LCR animals. The number of Iba-1 positive cells was higher in older animals. Density of activated microglia was significantly higher in the inner part of GCL of DG compared to other analyzed hippocampal regions. Moreover, activated microglia in that region were negatively associated with the number of newborn cells in hippocampus, which are located roughly in the same region.

Rats with high aerobic capacity showed increased neurogenesis compared to those with low aerobic capacity. This may be accompanied by increased synaptic plasticity, but those results are inconclusive. Older animals tended to have higher numbers of activated microglia, and the negative correlation between microglia of inner parts of GCL in DG and newborn cells suggests that activated microglia may downregulate neurogenesis. Thus, better intrinsic aerobic capacity seems to promote hippocampal neurogenesis, while aging and activation of microglia seem to be associated with impaired hippocampal neural plasticity.

Key words: hippocampus, aerobic capacity, microglia, neurogenesis, synaptic plasticity, rat

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
BDNF	brain-derived neurotrophic factor
BrdU	5’-Bromo-2-deoxyuridine
CNS	central nervous system
CREB	cAMP response element binding protein
DCX	doublecortin
DG	dentate gyrus
EC	entorhinal cortex
HCR	high-capacity runners
IBA-1	ionized calcium-binding adapter molecule 1
IHC	immunohistochemistry
LCR	low-capacity runners
LPS	lipopolysaccharide
LTP	long-term potentiation
MHC II	major histocompatibility complex II
NMDA	N-methyl-D-aspartate
PND	postnatal day
SUB	subiculum
SYN-1	synapsin 1
SYP	synaptophysin
WB	Western blotting

CONTENTS

1 INTRODUCTION

Cognitive decline is common among aging population, especially when accompanied with neurodegenerative diseases, such as Alzheimer's- and Parkinson's disease. In addition to traditional medicine treatment, aerobic exercise seems to be a potential strategy in preventing cognitive decline. (Yau et al. 2014.) Exercise is shown to indeed improve cognitive functions like memory, both in older adults and in non-human animals (Swain et al. 2012). A central brain structure benefiting from exercise is hippocampus, which is responsible for declarative memory (Kandel et al. 2014). Aging-related cognitive decline correlates with changes in several neural plasticity processes like neurogenesis, synaptic plasticity and inflammatory balance in hippocampus. All these forms of plasticity can be positively affected by physical exercise. (Barak et al. 2015.) However, these phenomena are difficult to study in humans since the methods in cellular level are invasive. Additionally, it is hard to determine whether, for example, better school performance of physically active children is because of the physical exercise alone or if other factors like inherited cardiovascular fitness modulate the effect (Haapala et al. 2017; Haapala et al. 2019). Therefore, neural plasticity studies usually utilize animal models.

The effect of physical exercise and fitness on neural plasticity can be studied with couple of different methods. Traditional exercise interventions in rodents have looked at whether animals doing the exercise, usually aerobic, show improvements in neural plasticity, such as neurogenesis (van Praag et al. 1999; van Praag et al. 2005; Pereira et al. 2007; Nokia et al. 2016). Since the benefits seem to follow especially interventions with aerobic exercise, the role of cardiovascular fitness raises in interest. By comparing animals with different intrinsic aerobic capacity, exercise can be removed from the equation, leaving cardiovascular fitness as the differentiating variable. This has been done by studying rats that are bred to have high- and low intrinsic aerobic capacity (Hussain et al. 2001; Koch & Britton 2001). These so-called high capacity runners (HCR) and low capacity runners (LCR) end up differing much more in their

running capacity than what could be achieved in standard training interventions (Koch et al. 2013).

The purpose of this study was to investigate the effect of intrinsic aerobic capacity and age on neural plasticity in hippocampus. The possible differences between the two rat lines in hippocampal plasticity markers would indicate an important role for cardiovascular and genetic background in determining hippocampal health. Moreover, as the differences in aerobic capacity between the rat lines are inherited, and do not come from differences in physical exercise, the study design does not create differences in social factors and environment between the animals. Thus, the study design truly allows to investigate if differences in genetic background related to endurance performance affect hippocampal health.

2 NEURAL BASIS OF LEARNING AND MEMORY

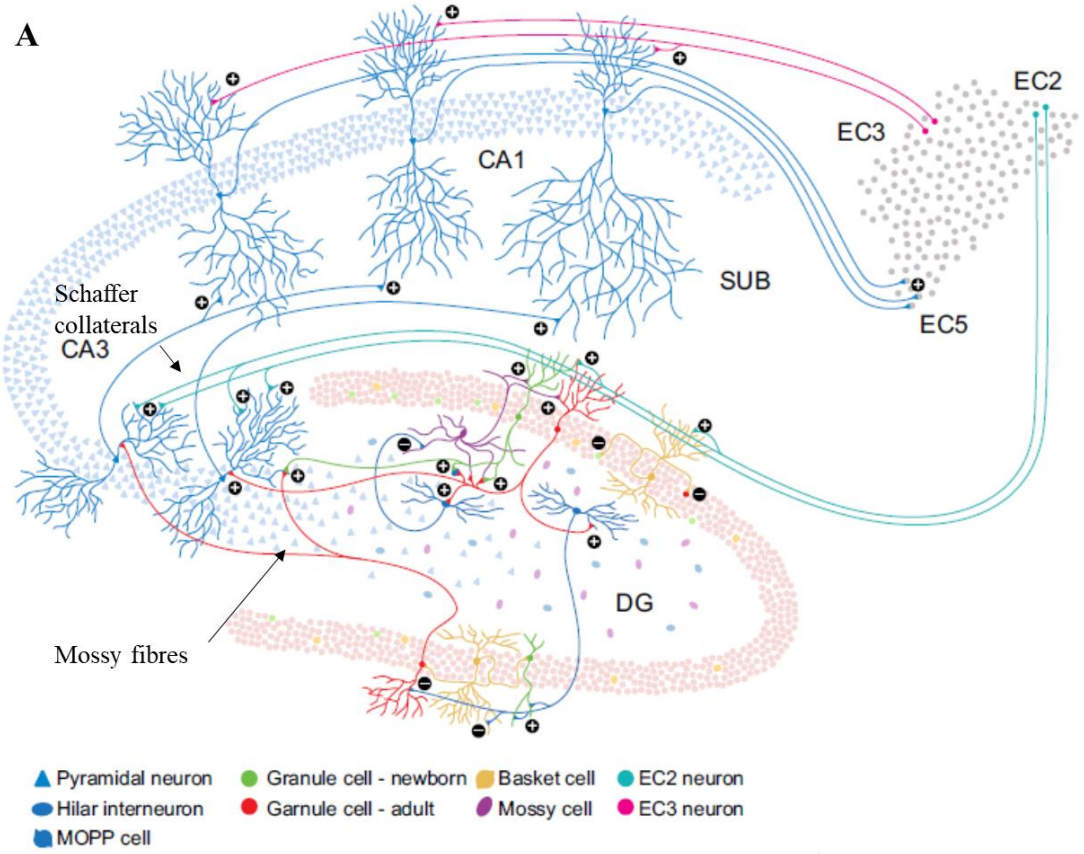
Memory in the brain can be divided into two major types: implicit- and explicit memory. Implicit (nondeclarative) memory is for perceptual and motor skills, while explicit (declarative) memory is storage for facts, events and places (Baddeley et al. 2007, 9-12; Kandel et al. 2014). The main brain areas required for explicit memory are hippocampus and adjacent cortex areas (Kandel et al. 2014), of which the former is the focus of this thesis.

Neural plasticity is the basis of memory and learning in the brain. Classen (2013) defines neural plasticity as the capacity of the brain to change in response to experience, use, environmental changes or injury. Neural plasticity is a general term and includes, in addition to neurons, glial cells and it occurs in multiple timescales from seconds to years. (Classen 2013.) These different forms of neural plasticity are discussed later in chapters: 2.2.1. Synaptic plasticity: LTP and LTD; 3.1. Synaptogenesis; 3.2. Neurogenesis; 3.3. Microglia and neural plasticity. Neuronal plasticity, not to be confused with neural plasticity, refers to the ability of neurons to respond to changes and experiences. Neuronal plasticity includes several different processes such as long-term potentiation - LTP (Bliss & Lomo 1973), synaptogenesis (Black et al. 1990) and neurogenesis (Altman & Das 1965). Neuronal changes have usually been divided to functional or structural but the distinction between the two is arbitrary since functional changes usually also lead to detectable changes in the neuronal structure and vice versa.

2.1 Anatomy of hippocampus

The role of hippocampus in memory system was reported as early as 1957, when Scoville & Milner, showed that the degree of memory loss was dependent on the extent of hippocampus removal in patients with schizophrenia and epilepsy. Next, an overview of the anatomy and main hippocampal circuits will be presented. It should be noted, that the following anatomy is from rat hippocampus, which has some differences to nonhuman primate and human hippocampus (Amaral et al. 2007).

Hippocampus is located in the caudate part of the brain and it consists of three subregions: the dentate gyrus (DG), the hippocampus proper – *Cornu Ammonis* areas (which consists of CA1, CA2 and CA3) and the subiculum (SUB) (Figure 1A). Hippocampal cortex is organized in three layers of cells. The bottom layer, known as polymorphic layer, consists of both efferent and afferent fibers and interneurons. In DG the bottom layer is often referred to as hilus and in CA regions as stratum oriens. The second layer, superficial to the first, is cell layer. In DG cell layer is also known as granule (cell) layer, and in CA regions and subiculum as pyramidal (cell) layer. (van Strien et al. 2009.) DG has also inhibitory interneurons located along the interface between granule layer and polymorphic layer, called basket cells (Amaral et al. 2007). The third layer, which is the most superficial, is called molecular layer in DG and subiculum. The third layer of CA regions is further divided into sublayers, which are not described here. (van Strien et al. 2009.)



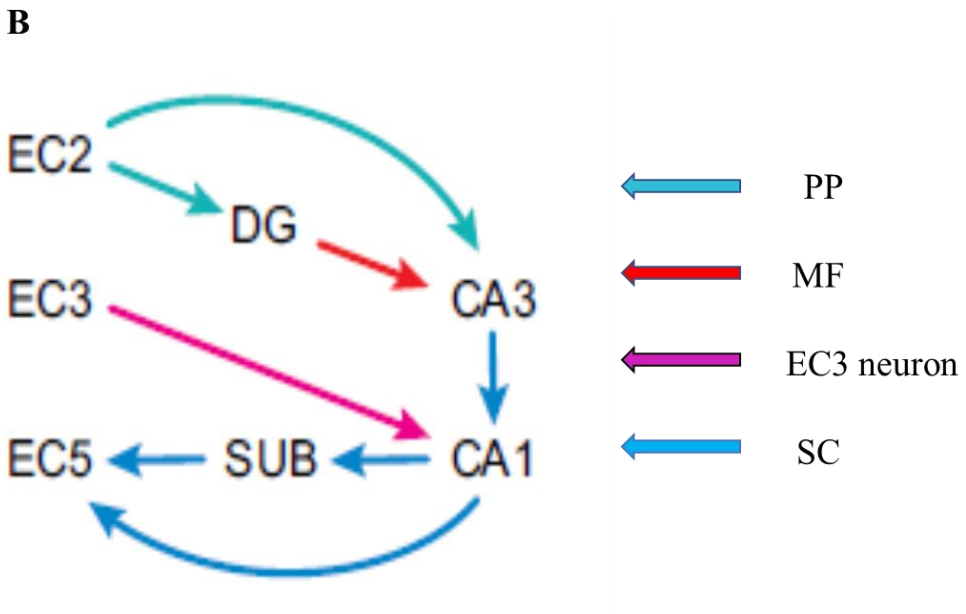


Figure 1. Schematic drawing showing the anatomy of rat hippocampus (A) and hippocampal circuits (B). Abbreviations: DG = dentate gyrus, EC = entorhinal cortex, SUB = subiculum, PP = perforant pathway, MF = mossy fibers, EC3 neuron = entorhinal cortex layer 3 neuron, SC = Schaffer collaterals. (modified from Aimone et al. 2014.)

Hippocampus receives information input through its connection with entorhinal cortex (EC), called perforant pathway. EC acts as a source of perforant pathway and makes projections to all hippocampal subregions, while EC itself receives input from neocortex. EC layer II cells (EC2) make projections to DG and CA1, while EC layer III cells (EC3) make projections to CA1. Connectivity within hippocampus is usually described with polysynaptic pathway consisting of three 'steps'. (van Strien et al. 2009.) The 'first step' of the pathway is mossy fibres, which are unidirectional projections from DG to CA3 (Amaral et al. 2007; van Strien et al. 2009). The next part of the loop are the Schaffer collaterals from CA3 to CA1. The last step is from CA1 to SUB. Output from hippocampus arises from CA1 and SUB, and it is projected to the deeper layers of EC (for perforant- and polysynaptic pathway, see Figure 1B). (van Strien et al. 2009.) There are also several backprojections within the hippocampal loop that are not described in this thesis.

2.2 Cellular mechanisms of memory

2.2.1 Synaptic plasticity: LTP and LTD

As previously mentioned, plasticity in the brain is the basis for both memory and learning, which meant the ability of the brain to change in response to experience (Classen 2013). An important cellular mechanism accounting for the overall plasticity is synaptic plasticity which refers to the activity dependent modification of synaptic transmission, resulting from changes in strength or efficacy at pre-existing synapses (Citri & Malenka 2008). Synaptic plasticity represents classical Hebbian learning, which states that neurons firing together, wire together – strengthen their synaptic connections. Classic example of synaptic plasticity is long-term potentiation (LTP), first found by Bliss & Lomo (1973). They demonstrated that repetitive simulation of the perforant path fibers resulted in potentiated response in granule cells in the dentate area in hippocampus (for pictures of hippocampal circuits, see 2.2.2. hippocampus). They suggested that LTP was due to increased efficacy of synaptic transmission and increased excitability of postsynaptic neurons (Bliss & Lomo 1973). LTP in hippocampus was then used as a model for the synaptic basis of learning and memory in vertebrates (Bliss & Collingridge 1993).

The most studied form of LTP is N-methyl-D-aspartate (NMDA) receptor dependent LTP (Malenka & Bear 2004; Lüscher & Malenka 2012). In short, it consists of neurotransmitter glutamine being released from presynaptic terminal and binding to two major types of glutamate receptors in postsynaptic terminal: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and the afore mentioned NMDA receptors. AMPA receptors are responsible for rapid synaptic signaling causing depolarization of the postsynaptic membrane. NMDA receptors are responsible for the influx of calcium which activates intracellular signaling cascades ultimately leading to altered synaptic efficacy. In order to open NMDA receptors both binding of glutamate and depolarization of membrane (to remove magnesium-ion from the receptor) are needed. Strong stimulation leads to increased trafficking of AMPA receptors to postsynaptic membrane via exocytosis, that enables more efficient

response for future stimulus – LTP (Figure 2). (Malenka & Bear 2004; Lüscher & Malenka 2012.) If the NMDA receptor activation is only modest, that is with the low frequency stimulation, the intracellular signaling results in long-term depression (LTD), where AMPA receptors are removed from the postsynaptic membrane via endocytosis (Figure 2) (Malenka & Bear 2004; Lüscher & Malenka 2012).

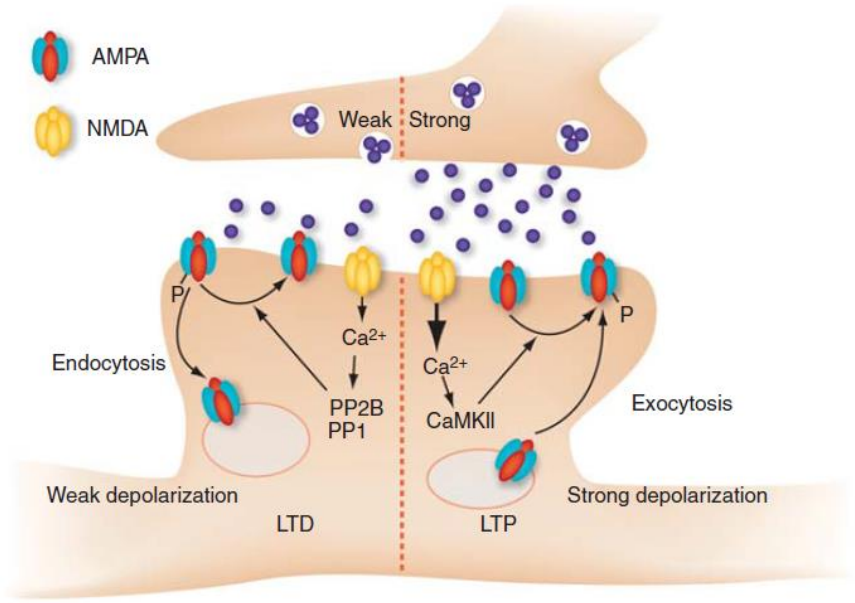


Figure 2. Schematic drawing that shows postsynaptic mechanisms for long-term potentiation (LTP) and long-term depression (LTD). Weak presynaptic activity leads to modest calcium influx into cell via NMDA receptors, leading to receptor endocytosis. Strong activity paired with strong depolarization leads to exocytosis and LTP. (Adapted from Lüscher & Malenka 2012.)

However, LTP is not always dependent on postsynaptic NMDA receptors. For example, at the mossy fiber synapse in CA3 region of hippocampus, LTP is achieved with presynaptic release of transmitters, independently from NMDA receptors (Mellor et al. 2002; Malenka & Bear 2004). LTP and LTD show the basic synaptic manipulation that is thought to be responsible for learning and memory. There are also other forms of synaptic plasticity aside from LTP and

LTD, namely homeostatic plasticity and metaplasticity. An example of homeostatic plasticity is synaptic scaling, where the strength of the synapses of a given neuron are adjusted accordingly to prolonged changes in activity by changing the quantity of AMPA-receptors at postsynaptic terminal. Metaplasticity on the other hand refers to how synaptic plasticity influences direction or magnitude of synaptic plasticity in the future without directly affecting synaptic efficacy. In other words, it is 'plasticity of plasticity'. (Citri & Malenka 2008.) These different forms of synaptic plasticity are the basis for the simple learning and short-term memory (Kandel et al. 2014).

2.2.2 Protein synthesis

What is it about long-term memory that makes it last, compared to short-term memory? Short-term memory is based on synaptic strengthening (LTP) that lasts from minutes to maybe hours, whereas long-term memory lasts from days to weeks (Kandel et al. 2014). The major cellular difference between short- and long-term memory is that long-term memory requires also synthesis of new proteins (Sutton & Schuman 2006; Kandel et al. 2014). The requirement of protein synthesis for functioning memory has been known for a long time. Several studies have shown that protein synthesis is required for memory retention after learning (Davis & Squire 1984; Bekinschtein et al. 2007) as well as for restabilization after retrieval (Nader et al. 2000).

The activation of protein synthesis happens via activation of transcription factors like cAMP response element binding protein, CREB, or epigenetic regulation by micro- and non-coding RNA molecules (Rajasethupathy et al. 2012; Kandel et al. 2014). Transcription refers to encoding of DNA information into RNA. Basically, intracellular signaling cascades activate transcription in the cell nucleus which produces messenger RNA, mRNA, which is then sent out of the nucleus to act as a script for new proteins. In the case of memory formation and maintenance, the new mRNA then travels to specific synapses (Kandel et al. 2014). In the cytoplasm mRNA binds to a ribosome which allows transfer RNA (tRNA) to recognize different section by base-paired codons and anticodons. According to the base-pairing t-RNA brings amino acids to ribosomes until the whole mRNA is 'scanned', creating an amino acid

chain. (Jackson et al. 2010.) In neurons, translation of the new proteins can take place either in the cell body or in the dendrites (Sutton & Schuman 2006). This initial phase of protein synthesis after learning is essential for the long-term memory formation and synaptic plasticity. Therefore, measuring the expression of proteins in hippocampal cells can give an idea about changes in synaptic and/or neural plasticity in that region of the brain.

3 NEURAL PLASTICITY IN THE BRAIN

3.1 Synaptogenesis

Synaptogenesis is a modulation of the number of synapses of a neuron. Synaptogenesis is not only dependent on pre- and postsynaptic neurons but also on the surrounding immune cells like microglia and astrocytes (Christopherson et al. 2005). The amount of synaptogenesis can be calculated by estimating neuron density and number of synapses per unit volume (Kleim et al. 2004). Synaptogenesis can also be indirectly estimated with IHC or western blot -analyses by measuring the expression of synaptic proteins. There are many synaptic proteins but here the focus is specifically on two synaptic vesicle proteins: synapsin-1 and synaptophysin.

Synapsins are a family of vesicle associated proteins which have been used in estimating plastic changes in the synapses. Increases in the amount of synapsins has been shown to also correlate with synaptogenesis (Lohmann et al. 1978; Bogen et al. 2009). Synapsins have several subtypes, but here the focus is mainly on synapsin-1 (SYN-1). The function of SYN-1 is to regulate the neurotransmitter release in synapses (Jovanovic et al. 2000). Using synapsin-1 and -2 double knockout mice, Bogen et al. (2009) demonstrated that synapsins 1 and 2 modulate postnatal synaptic vesicle number and functioning in excitatory glutamatergic synapses. These synaptic changes were synapsin independent for the first postnatal weeks but synapsin dependent in adolescent and adult brain (Bogen et al. 2009). Another protein found in synaptic vesicles is synaptophysin (SYP) (Jahn et al. 1985). SYP is exclusively localized in pre-synaptic vesicles, making it a widely used marker for pre-synaptic terminals (Kwon & Chapman 2011). SYP seems to be required for efficient synaptic vesicle trafficking and endocytosis, demonstrated by deficits in synaptic vesicle endocytosis in cultured hippocampal SYP -knock out neurons (Kwon & Chapman 2011). Both SYN-1 and SYP are universal presynaptic proteins used as general synaptic markers as they are expressed in both excitatory and inhibitory synapses (Micheva et al. 2010) and changes in their expression represents changes in synaptic plasticity.

In early observations, it was found that synapsin-1 (SYN-1) and synaptophysin (SYP) are both expressed in developing hippocampal neurons in a cell culture (Fletcher et al. 1991). Synaptogenesis was also studied early in response to habituation and sensitization (Bailey & Chen 1988) as well as in response to motor learning and -activity (Black et al. 1990). Bailey & Chen (1988) showed that long-term memory in *Aplysia* was accompanied by morphological changes in membrane specialization of synapses and in modulation of the total number of synapses. Black et al. (1990) observed synaptogenesis in cerebellar cortex with rats doing skill training but not on those which did running. Since synaptogenesis is usually studied in relation to some form of learning, it can be paired with measuring c-Fos expression which tells about neuron activation (Bullit 1990). C-Fos is an immediate early gene, which encodes nuclear phosphoproteins (Bullit 1990). It has been shown that Fos expression is elevated in association with motor skill learning and specifically in the skill acquisition phase (Kleim et al. 1996). Tamakoshi et al. (2014) demonstrated that motor skill training increased the number of Fos -positive cells during skill acquisition in the motor cortex and striatum. They also showed that synaptogenesis, measured with the amount of synaptic scaffolding proteins, increased in motor cortex in the later phases of learning (Tamakoshi et al. 2014). This happens probably because synaptogenesis requires protein synthesis that is seen in the later phases of learning (Kleim et al. 2004).

However, synaptogenesis can occur also in response to stimuli other than motor skill training. Ambrogini et al. (2013) demonstrated that physical exercise, as well as environment exploration, increased number of primary dendrites in immature adult-born granule neurons in dentate gyrus of hippocampus. These early connections could be important in the survival of the newborn neurons, thus being a possible mechanism how physical exercise may promote cell survival in hippocampus. (Ambrogini et al. 2013.)

3.2 Neurogenesis

Neurogenesis refers to generation of new neurons. Neurogenesis is not only an ability of developing brain, although it was long thought so, but it can be detected also in adults. However,

the rate at which adult born neurons generate is substantially slower than that of developing brain. In mammals, adult neurogenesis seems to occur in two brain areas: in dentate gyrus (DG) of the hippocampus and in the lateral ventricles (Aimone et al. 2014.), of which the latter is not in the focus of this thesis. Postnatal neurogenesis was shown first with rats by Altman et al. (1967) and later also in humans (Eriksson et al. 1998; Spalding et al. 2013).

In adult hippocampus, new neurons arise from subgranular zone (SGZ) of DG, from neural stem cells (NSC), further developing into two types of progenitor cells (Gage 2000; Aimone et al. 2014). SGZ is the thin lamina between hilus and granular cell layer in the DG (Palmer et al. 2000). The first type of progenitor cells is radial glial cells, which are multipotent stem cells in the DG and are able to self-renew (Bonaguidi et al. 2011). The second type is neural progenitor cells, which can differentiate into neurons or glia and amplify faster than radial glial cells. The newly generated immature neurons can migrate from subgranular zone to olfactory bulb along rostral migratory stream or to granular cell layer of DG, where they can integrate to existing hippocampal circuitry. Neurogenesis can be considered to consist of three processes: cell proliferation, neuronal differentiation and cell survival (Figure 3). (Aimone et al. 2014.) For neurogenesis to occur, right microenvironment – also called neurogenic niche – in SGZ is required (Palmer et al. 2000). Microglia in the DG also participate in regulating neurogenesis via phagocytosis of apoptotic cells, and by secreting different cytokines and growth factors together with astrocytes (for more about microglia and neurogenesis, see 3.3 Microglia and neuronal plasticity) (Zhao et al. 2008; Aimone et al. 2014).

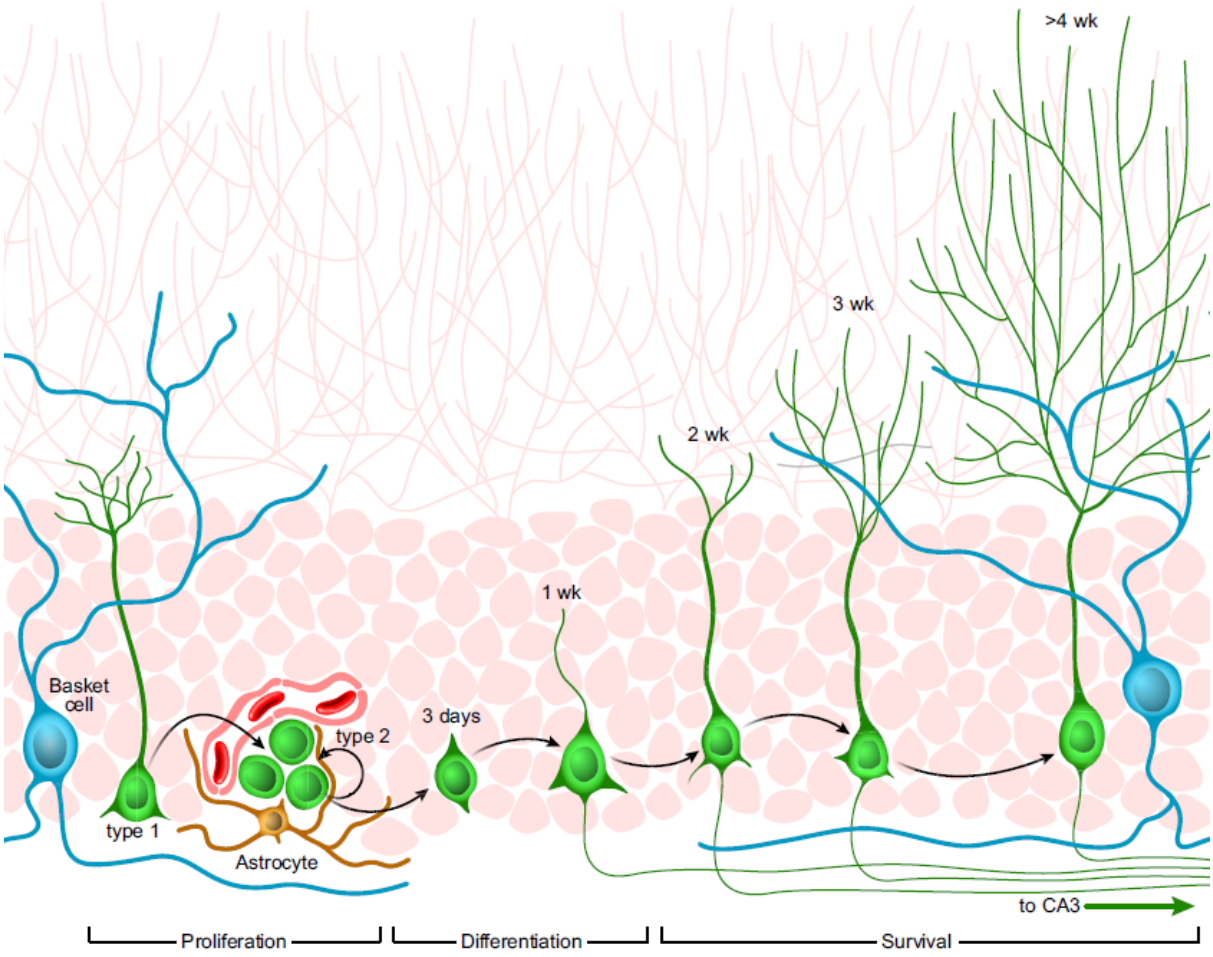


Figure 3. Development of DG granule cells from stem cells to mature neurons. New neurons can arise from either the slowly dividing type 1 cells – radial glial cells or from more rapidly amplifying type 2 cells, neural progenitor cells. (Adapted from Aimone et al. 2014.)

There are numerous different factors, that regulate neurogenesis: from local neurotransmitters like GABA and glutamate to extrinsic factors like stress, learning and physical exercise. (Zhao et al. 2008; Aimone et al. 2014.) Enriched environment (EE) leads to improvements in survival of the new neurons and increased synaptogenesis, measured with synapsin-1 and synaptophysin (Birch et al. 2013). Kirschen et al. (2017) found also that novel environmental experiences increased the number of newborn granule cells in dentate gyrus. This increase was shown to be at least partly dependent on enhanced hippocampal activity and neuron firing, since silencing

dentate gyrus granule cells during environment exploration resulted in no addition of newborn hippocampal neurons (Kirschen et al. 2017). Physical exercise can also regulate hippocampal neurogenesis (see Chapter 4.3 Cardiovascular fitness and neurogenesis in hippocampus). There are also several different growth factors contributing to adult neurogenesis, like brain-derived neurotrophic factor (BDNF) (Zhao et al. 2008) and nerve growth factor (NGF) (Birch et al. 2013).

The newborn neurons show enhanced synaptic plasticity, demonstrated by lower LTP threshold and larger LTP amplitude, during a critical period of 1-1.5 months of cell age (Ge et al. 2008). In mice neurogenesis is shown to have cognitive relevance in spatial pattern separation, that is the ability to form discrete, non-overlapping representations from similar mnemonic information (Clelland et al. 2009; Sahay et al. 2011). These studies demonstrate that ablation of neurogenesis in mice leads to impairments in pattern separations tasks (Clelland et al. 2009; Sahay et al. 2011) and increasing neurogenesis leads to improvements in pattern separation – discrimination learning - but not in other forms of memory (Sahay et al. 2011). However, when it comes to humans, one cannot expect exactly similar results as found in animal studies. The functional contribution of adult neurogenesis in humans is still under debate and therefore, the topic is under deep investigation. (Kempermann et al. 2018.)

The golden standard method in studying neurogenesis has been injection of 5'-Bromo-2-deoxyuridine (BrdU) (Gratzner 1982), which substitutes an endogenous DNA base thymidine with the BrdU analogue, which can be visualized later. This happens during the s-phase of dividing cells, which is why it can be used as a marker for proliferating cells (Taupin 2007). Another mitotic marker that is used in labeling newborn cells is Ki-67, which is expressed during most phases of cell cycle and thus labels more cells than BrdU (Taupin 2007). A different way to measure neurogenesis is to use doublecortin (DCX) as label. DCX is a microtubule - associated protein, required in migration of the cells (Gleeson et al. 1999). As such DCX is expressed in the migrating neuroblasts (Couillard-Despres et al. 2005). Therefore, there is a time window for DCX expression in newborn neurons, since it does not measure NSCs and

mature neurons older than approximately 30 days (i.e. in Figure 3 the cells that have finished proliferation but are under four weeks old).

3.3 Microglia and neural plasticity

Microglia are innate immune system cells of central nervous system (CNS) with functions from phagocytosis to neuroprotection (Kettenmann et al. 2011; Aimone et al. 2014). Microglia have two major phenotypes: ramified and unramified. The ‘resting state’ has a ramified morphology and a small soma. Unramified morphology is the activated state of microglia. If there is some sort of danger such as infection, microglia are activated and their phenotype changes into an amoeboid appearance (Figure 4). While activated, microglia act as macrophages, moving towards infectious invaders by following chemotactic gradients. Microglia are also able to increase their number locally through proliferation and release different proinflammatory and immunoregulatory cytokines. When microglia move to target location they can phagocytose - that is eat - damaged tissue, cells or microbes. The activation of microglia is complex and heavily regulated. (Kettenmann et al. 2011.) When not in their active form, the resting ramified microglia actively scan their environment for threats against CNS (Aimone et al. 2014). Thus, it is important to note that ramified microglia are not necessarily inactive even though it is said that they are ‘activated’ while transforming to amoeboid state (Kettenmann et al. 2011).

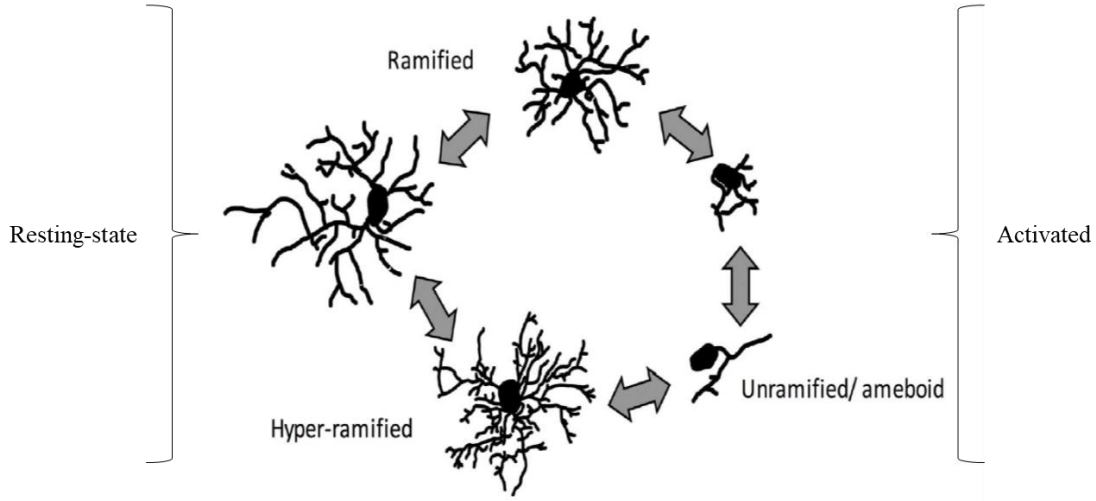


Figure 4. Microglial morphology. The ramified microglia are in a 'resting state', whereas unramified microglia are activated. (modified from Sominsky et al. 2018.)

Microglia have been typically studied in cultured cells that are activated using lipopolysaccharides (LPS). Once activated, microglia release proinflammatory substances such as TNF-α, IL-6 and nitric oxide. Microglia have a load of different receptors for neurotransmitters, neurohormones, cytokines as well as pattern-recognition (toll-like receptors, TLR) and other receptor systems which allow microglia to interact with both surrounding CNS tissue and possible threats to it (Wake et al. 2009).

There are several different markers for detecting microglia (see Kettenmann et al. 2011), one of which is ionized calcium-binding adaptor molecule 1 (Iba-1) (Imai et al. 1996; Imai & Koshaka 2002). Iba-1 is a protein that is involved in Rac signaling, regulating underlying cellular events of microglial activation, like reorganization of actin cytoskeleton (Imai & Koshaka 2002). This is seen in a rise of Iba-1 expression in response to trauma such as ischemia (Ito et al. 2001). In brain tissue Iba-1 is expressed on the protein level in microglia, but not in neurons, astroglia or oligodendroglia (Ito et al. 1998). However, it is important to note that Iba-1, like many other macrophage markers, will most likely label all macrophages in CNS, meaning not only the resident microglia of CNS but also the macrophages entering CNS form elsewhere (Carson et al. 2007).

In addition to their essential role in immune system, microglia have several responsibilities in neuronal plasticity. It has been shown *in vivo* that 'resting' microglia make specific and direct contacts to synapses, in an activity-dependent manner (Wake et al. 2009). One of the functions of microglia-synapse interactions is synaptic pruning during the development of young animals, where microglia engulf synaptic material and eliminate unnecessary synapses (Paolicelli et al. 2011). Mature mice depleted of microglia show deficits in learning tasks and impairments in motor-learning induced dendritic spine remodeling (Parkhurst et al. 2013). Furthermore, when Parkhurst et al. (2013) removed brain-derived neurotrophic factor (BDNF) from microglia, it resulted in decrease in motor-learning induced spine formation, but not spine elimination. These results suggest that microglia have an important role in learning-based synaptic formation regulated by microglial BDNF (Parkhurst et al. 2013). However, when Elmore et al. (2014) eliminated microglia in adult mice with colony stimulating factor 1 receptor (CSF1R) inhibitors the mice did not show any deficits in cognitive tests such as Barnes maze. These differences between studies highlight the complexity of microglia, when it comes to neural plasticity. Depending on the state and chemical environment, microglia can also inhibit neuronal plasticity. Peripheral inflammation can activate inflammation response also in the brain through microglia and their cytokines like TNF-α (Riazi et al. 2015). This leads to decrease in synaptic plasticity, shown by diminished LTP and LTD in CA1 of hippocampus during inflammation, that was reversible by inhibiting microglial and macrophage activation (Riazi et al. 2015).

Microglia seem to play also a role in regulation of neurogenesis. Immune-deficient mice lacking T- and B-cell populations show impairment in neurogenesis (Ziv et al. 2006). The decrease in neurogenesis was suggested to be due to impairment in activation of microglia by T-cells, that was supported by decrease in neurogenesis after blocking microglial activity of T_{MBP}-transgenic mice (Ziv et al. 2006). Manipulating microglial function directly also shows effects on neurogenesis. Blocking a critical retromer protein (VPS35), essential for microglial function, leads to regionally increased microglial density and activity in subgranular zone of dentate gyrus (DG) in hippocampus (Appel et al. 2018). Interestingly this led to increase in neuronal progenitor proliferation but decrease in neuronal differentiation, suggesting a disruption in adult hippocampal neurogenesis by increasing the number of neural stem cells and/or neural

progenitor cells, but reducing neuronal differentiation (Appel et al. 2018). The effect of microglia activation on neurogenesis depends on the form of microglial activation - the balance between secreted molecules with pro-and anti-inflammatory properties. These seem to determine whether microglia promote or impair neurogenesis (Ekdal et al. 2009). In summary, microglia are important for neuronal plasticity, but the effect depends on the morphology and subpopulation of microglia and on the surrounding chemical environment.

3.4 Neural plasticity and age

The number of newborn neurons/neurogenesis is substantially higher in younger mammals compared to older ones (Altman & Das 1965; Drapeau et al. 2003; Bizon et al. 2004; Driscoll et al. 2006). The age in early development is usually expressed in relation to birth, referring to prenatal and postnatal time periods. Adolescence in rats can be considered to last until postnatal day (PND) 60, when they can reproduce. Chronological age is a strong predictor for cell proliferation in DG across mammal species (Figure 5). (Amrein et al. 2011.) Neurogenesis declines exponentially in rodents with age shortly after birth. The shift from postnatal to adult neurogenesis happens probably between one and two months of age. (Amrein et al. 2011). In rodents, neurogenesis is reduced to low but rather stable levels at middle-age (Amrein et al. 2011). The same is true for humans, as only modest decline in neurogenesis is seen during aging and the median turnover rate of neurons in human adulthood is ~1.75 % per year (Spalding et al. 2013).

Decline in spatial memory and associative learning are behavioral age-related changes that can be seen across species (Burke & Barnes 2006). Performance in Morris water maze spatial memory task was positively correlated with the level of neurogenesis in aged rats (Drapeau et al. 2003; Driscoll et al. 2006). However, this correlation is not found in all studies (Bizon et al. 2004). Thus, changes in neurogenesis alone do not explain age-related cognitive decline. According to Burke & Barnes (2006) other age-related impairments in hippocampus include Ca^{2+} dysregulation and changes in synaptic connectivity. Aged rats have deficits in both LTP induction and maintenance, but they are complex, protocol-dependent and region-specific.

Aged animals also show upregulation of genes associated with inflammation and intracellular Ca^{2+} release pathways, and downregulation of genes associated with energy metabolism, biosynthesis and activity-regulated synaptogenesis. (Burke & Barnes 2006.)

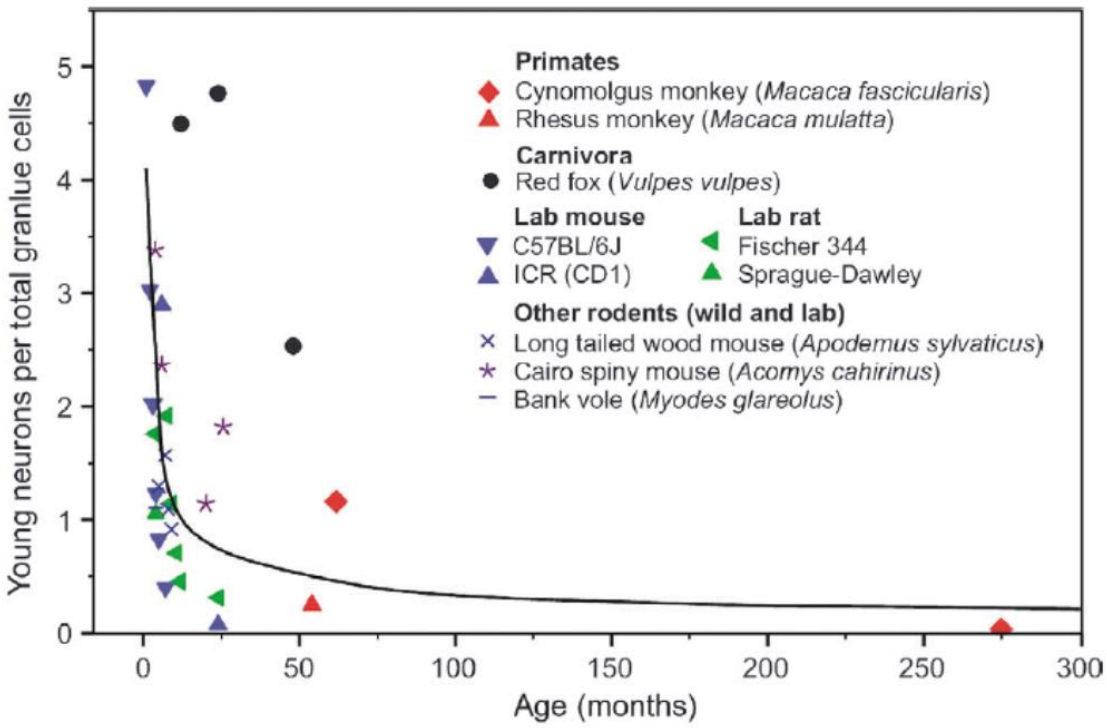


Figure 5. Number of young differentiating neurons declines with age across species (DCX- or PSA-NCAM-positive cells as a percentage of total granule cell number). Age in the figure is normalized to life stage. (modified from Amrein et al. 2011.)

Changes in microglia differ from neurons especially in the early development. Microglia development in mice has been shown to have three distinct temporal phases: early microglia (until embryo day 14), pre-microglia (embryo day 14 to few weeks after birth) and adult microglia (from few weeks after birth onward) (Matcovitch-Natan et al. 2016). After second postnatal week mice reach adult levels of transcriptional activity, shown by equal number of highly specific adult microglia between PND (14) and PND (60), $TMEM119^{+}$ being a highly specific marker for microglia cells (Bennett et al. 2016). Number of microglia peaks in PND

14 and is reduced to the density of adults by six weeks of age (Nikodemova et al. 2015). After the early development the density of microglia in mice, measured by Iba-1, seems to remain relatively stable from the young (4-6 months) to the aged (18-24 months) (Askew et al. 2017). However, Mouton et al. (2002) found that female middle aged (13-14 months) and aged (20-24 months) mice had on average 20% more microglia and astrocyte in DG and CA1 than young (3-4 months) mice. Females also had on average 20 to 38% higher glial cell numbers than their age-matched male counterparts in each age group, while males did not show significant differences in microglia numbers between age groups (Mouton et al. 2002).

4 AEROBIC CAPACITY AND ITS EFFECT ON NEURAL PLASTICITY

The possible beneficial effects of physical exercise/fitness on cognitive functions have been studied a lot. For example, in humans aerobic exercise has been shown to increase hippocampal perfusion, and hippocampal volume is larger in higher-fit adults (Erickson et al. 2011). The increase in hippocampal size after aerobic exercise is associated with improvements in spatial memory tests and increases in multiple different growth factors, such as BDNF (Erickson et al. 2011). The focus of this thesis is on, how cardiovascular fitness and aerobic capacity might affect or regulate hippocampal-related learning and memory. In humans this has been studied especially in relation to neurodegenerative diseases (Voss et al. 2013) and aging (Voss et al. 2013; Barak et al. 2015). However, animal studies are required for actual cellular and mechanistic information, even though the direct extrapolation of those results to humans needs to be done with caution (Voss et al. 2013; Barak et al. 2015).

4.1 Exercise and synaptic plasticity in hippocampus

Voluntary exercise leads to increased neurogenesis and LTP compared to inactive controls. This is accompanied with increased BDNF and mRNA of a NMDA receptor subtype in DG. (Farmer et al. 2004.) Voluntary exercise has also been shown to increase the level of SYN-1 in CA1, DG and CA3. In DG and CA3 the increase in SYN-1 was modulated by increase of BDNF. (Vaynman et al. 2004.) O'Callaghan et al. (2007) showed that forced exercise also resulted in increase of BDNF in DG as well as increased LTP. Together these results suggest that exercise enhances synaptic plasticity in the form of LTP and that BDNF probably has some sort of modulatory role in this.

4.2 Cardiovascular fitness and neurogenesis in hippocampus

The beneficial effect of aerobic exercise on hippocampal neurogenesis has been studied a lot in the past few decades. Voluntary exercise training increases neurogenesis in DG compared to

control or forced exercise (van Praag et al. 1999). The increase in neurogenesis in physically active animals is true for both young and old animals (van Praag et al. 2005). One growth factor regulating the effects of exercise on neurogenesis is vascular endothelial growth factor (VEGF): blocking VEGF in running animals returns the neurogenesis to baseline, but not below it, in running or non-running animals (Fabel et al. 2003). Indeed, exercise selectively increases cerebral blood volume in mice DG, that correlates with neurogenesis, shown by *in vivo* imaging of blood flow and comparing that to postmortem analysis of neurogenesis (Pereira et al. 2007). The same selective increase in blood flow of DG was also observed in humans, and it correlated to aerobic fitness and cognitive performance (Pereira et al. 2007).

Genetic variation also contributes to exercise-induced neurogenesis (Clark et al. 2011; Nokia et al. 2016). Clark et al. (2011) showed that exercise induced neurogenesis in twelve different mouse strains, but the magnitude of effect was dependent on the genotype. Moreover, the differences in exercise-induced neurogenesis between strains were not the same as the differences in sedentary conditions, suggesting different pathways and genes controlling the two conditions of neurogenesis (Clark et al. 2011). In rats, those with higher acquired cardiovascular fitness, so-called high-response trainers, demonstrate greater exercise-induced neurogenesis than low-response trainers (Nokia et al. 2016). However, the effect was dependent on the mode of exercise: sustained aerobic exercise resulted in differences but high intensity interval training or strength training did not (Nokia et al. 2016). On a behavioral level rats with high intrinsic aerobic capacity display better spatial memory (Sarga et al. 2013). There are also differences in neuronal activation in DG between active and sedentary, and in different lines of animals. Runners express more cFos in DG and entorhinal cortex than sedentary animals, and among the runners those selectively bred for high wheel running activity demonstrate higher cFos expression in entorhinal cortex compared to controls (Rhodes et al. 2003). More specifically, Clark et al. (2010) showed that mice in the running group display more c-Fos positive cells in the granular layer of DG compared to their sedentary counterparts. The running induced increase in c-Fos expression was in parallel with proliferation and survival of new cells in DG, possibly related to ‘activity-sensing’ properties of neuronal progenitor cells (Clark et al. 2010).

4.3 Exercise and microglia in hippocampus

Inflammation is shown to decrease hippocampal neurogenesis. In their study, Wu et al. (2007) inhibited neurogenesis with inflammation by peripheral lipopolysaccharide (LPS) injections. This resulted in decreased number of differentiating cells measured with DCX, but in no change in proliferating cells, measured with BrdU. Interestingly, treadmill running restored the LPS-inhibited neurogenesis without reducing inflammation in hippocampus, measured by microglial activation. Exercise also restored the performance in spatial learning and memory test as well as prevented LPS-induced decline of BDNF expression in hippocampus (Wu et al. 2007). Similarly, Littlefield et al. (2015) showed that exercise increased the proportion of microglia co-labeled with BDNF, which correlated with the number of surviving new neurons in aged mice. In their study, LPS decreased the number of new neurons in aging sedentary animals, which was preventable with exercise (Littlefield et al. 2015). Selectively removing GFP^+ microglia from runner mice abolishes the exercise-induced neurogenesis, and addition of microglia from active to sedentary animals increased neurogenesis (Vukovic et al. 2012). Positive modulation of neurogenesis was associated with more neuroprotective phenotype of microglia, in hippocampus after voluntary exercise (Vukovic et al. 2012). However, if microglia were removed from old mice, the result was increase in neurogenesis, which is thought to be cause of more proinflammatory role of microglia in aging brain. Interestingly, depletion of major histocompatibility complex II positive ($MHCII^{pos}$) microglia subpopulation from runners resulted in increase of neurogenesis. (Vukovic et al. 2012.) This subpopulation of microglia has usually been studied in inflammatory conditions and its expression is often elevated in older mice (Kohman et al. 2013), together with increase in the proportion of dividing microglia (Kohman et al. 2012).

Exercise seems to alter microglial activation in aged animals by changes in expression of MHCII, but the nature of these changes seems to be dependent on the sex and brain region (Kohman et al. 2013). In aged mice, exercise can also increase expression of insulin-like growth factor 1 (IGF-1) of microglia and thus promote the neuroprotective phenotype, as well as decrease the proportion of dividing microglia in DG (Kohman et al. 2012). These studies

highlight that different subpopulations of microglia, being either neuroprotective or proinflammatory, have very different effects on neurogenesis.

4.4 Rat model of high and low aerobic capacity

When looking at how physical exercise affects neuroplasticity, studies usually utilize either animals with inherited low versus high aerobic capacity or control versus exercise training groups. In the latter method, animals are simply randomized into control and training groups, which shows if exercise leads to differences between groups. Studying the effect of inherited aerobic capacity without exercise has the advantage that the exercise itself should not contribute to possible differences. This is important since exercise itself might lead to improvements in learning and memory not only because of improved physical fitness, but also due to more stimulated nervous system in active animals (Wikgren et al. 2012).

The effects of inherited (intrinsic) aerobic capacity can be studied with animals bred to high-capacity runners (HCR) and low-capacity runners (LCR) (Koch & Britton 2001). In their study Koch & Britton (2001) showed that the difference between rats selectively bred for low- and high-capacity in distance run to exhaustion was 171% already at generation six, most of the change coming from HCR. The difference between HCR and LCR rats in running capacity results to be much bigger than that achieved with 8 eight weeks of exercise in high and low responder animals (Koch et al. 2013). HCR rats show improved skeletal muscle O_2 utilization through increased capillary density and changes in enzyme activity (Howlett et al. 2003). It should be noted that this form of selection leads, in addition to differences in maximal oxygen uptake, to increase in cardiovascular risk factors in animals with low aerobic capacity (Wisløff et al. 2005), and significant differences in weight (Koch & Britton 2001). Thyfault et al. (2009) showed that LCR rats have reduced mitochondrial content and oxidative capacity in their liver, increasing the risk to hepatic steatosis and liver injury. Thus, LCR does not represent exactly a control group but rather animals with increased risk of health problems. The HCR rats have also showed better performance than LCR rats in tasks requiring flexible cognition, like in discrimination-reversal -task (Wikgren et al. 2012).

5 RESEARCH QUESTIONS AND HYPOTHESES

1. Does high inherited aerobic capacity (HCR) and/or age decrease the number of activated microglia (Iba-1) in hippocampus compared to low aerobic capacity (LCR)- and/or younger animals? Does this vary between specific hippocampal compartments?

Yes. Exercise can change microglia phenotype to more anti-inflammatory (Kohman et al. 2012; Vukovic et al. 2012; Littlefield et al. 2015), especially, in the older animals (Kohman et al. 2013). It is hard to say if this would also mean decrease in the number of activated microglia in those with higher cardiovascular fitness. However, since LCR rats in general have cardiovascular risk factors (Wisløff et al. 2005), one could hypothesize that it could result also in more inflammatory type of microglia in hippocampus in them compared to HCR. When it comes to specific compartments in hippocampus, it is hard to predict results, since there are several microglial subpopulations. Kohman et al. (2012) showed that exercise in aging animals reduced the proportion of dividing microglia. As the effect of exercise on microglia seems to be more established in aged animals (Kohman et al. 2012; Vukovic et al. 2012; Littlefield; 2015), one could expect bigger differences between LCR and HCR in aged, rather than between younger animals.

2. Does greater inherited aerobic capacity (HCR) increase the number of newborn neurons (DCX) in young and/or old animals?

Yes. The positive effect of aerobic exercise on neurogenesis seems to be well established (van Praag et al. 1999; van Praag et al. 2005; Nokia et al. 2016). However, differences in sedentary conditions may differ from those observed in exercise (Clark et al. 2011). The younger animals are expected to have higher rate of neurogenesis and therefore more newborn neurons (Altman & Das 1965; Drapeau et al. 2003; Bizon et al. 2004; Driscoll et al. 2006; Amrein et al. 2011). The term old is used here for group comparison and it refers to adult animals, comparable to middle age in humans.

3. Is the number of newborn neurons (DCX) associated with microglial activation (Iba-1)?

Yes. Although microglia may be necessary for neurogenesis to some degree (Ziv et al. 2006; Appel et al. 2018) and exercise may shift microglia phenotype to more anti-inflammatory (Kohman et al. 2012; Vukovic et al. 2012; Littlefield et al. 2015), these rats did not do any exercise. Increased number of activated microglia is shown to inhibit neurogenesis (Wu et al. 2007) and, especially, in older animals microglia tend to be more proinflammatory (Kohman et al. 2013). Therefore, it is expected that the number of activated microglia is negatively associated with the number of newborn neurons.

4. Does the number of newborn neurons (DCX) positively correlate with neuronal activation (p-cFos) and expression of synaptic plasticity markers (SYN-1, SYP)?

Yes. The newborn neurons demonstrate enhanced synaptic plasticity (Ge et al. 2008). Therefore, it could be expected that the newborn neurons demonstrate increased expression of synaptic plasticity markers. Additionally, aerobic exercise is shown to increase SYN-1 in DG and CA3 (Vaynman et al. 2004) and synaptogenesis in DG (Ambrogini et al. 2013). However, it is important to remember that aerobic exercise and innate aerobic capacity are different from one another, and this could result in different outcome in this model compared to exercise models. Running is also shown to increase neuronal activation in DG (Rhodes et al. 2003; Clark et al. 2010), while the HCR animals are shown to be more spontaneously active than LCR (Karvinen et al. 2016). Thus, HCR are expected to have more neuronal activation than LCR.

6 METHODS

This study is based on an animal model developed by Koch and Britton (Koch et. al. (2011) and Koch & Britton (2017)), in which rats are selectively bred to high- and low capacity runners. The neurogenesis data is from Active Fit and Smart (AFIS) -project (Lensu et al. 2016) and the western blotting data is from Honkanen (2019). Figure 6 shows the overview of the study design.

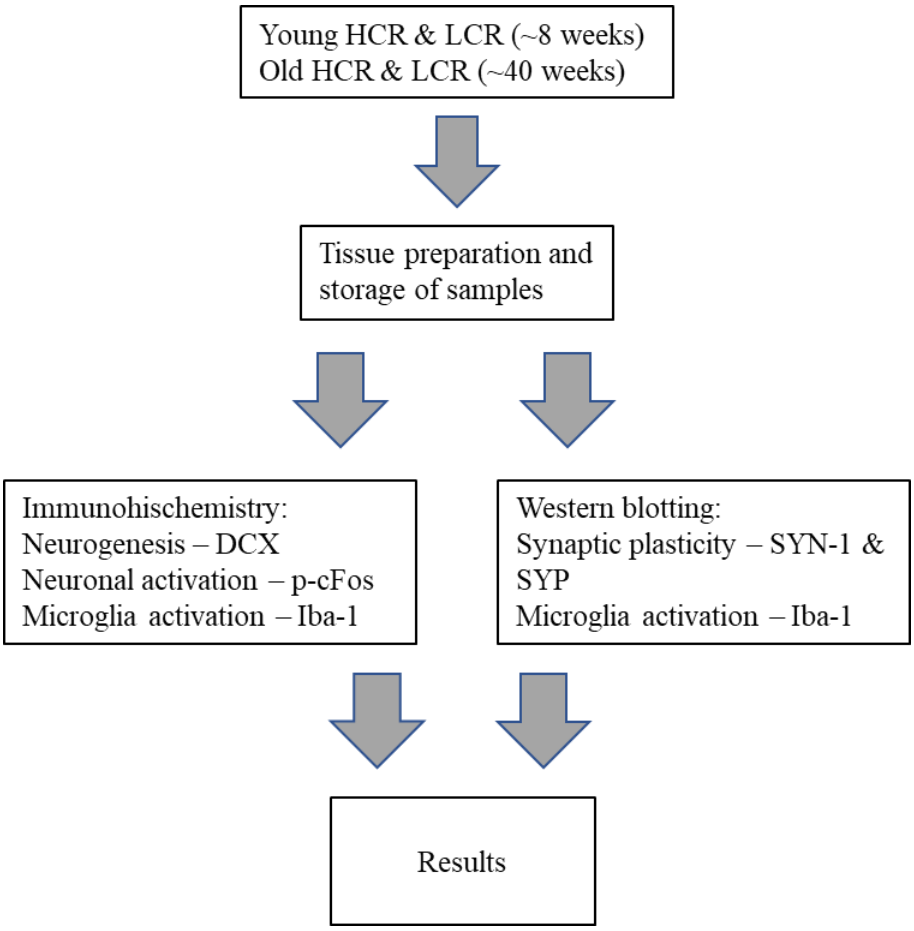


Figure 6. Overview of the study design. All animals in the study were kept sedentary.

6.1 Animals

The samples for this thesis were from a study 'Active, Fit and Smart' (AFIS) and the animals and experiments are previously described by Pekkala et al. (2017). The rats were selectively bred high (HCR) and low aerobic capacity rats (LCR) originating from the University of Michigan, USA. These animals were born in University of Jyväskylä, Finland and were the 36th generation and their parents were phenotyped in University Michigan. The animals were divided into four groups: young HCR (mean age 7.90 ± 0.27 wk), young LCR (mean age 7.77 ± 0.29 wk), old HCR (mean age 39.98 ± 0.33 wk) and old LCR (mean age 39.81 ± 0.34 wk). The term old is used here for group comparison and it refers to adult. All animals were kept sedentary meaning that no group had any physical training. Only males were used for the study and siblings were divided as equal as possible to young and old groups. For more detailed description of the animals see Pekkala et al (2017) and Mäkinen (2018).

The experiments were done in accordance with the Guidelines of the European Community Council directives 86/ 609/EEC, and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985). Ethical permission (ESAVI/7647/04.10.07/2014) was given by the ethical board of regional state administrative agency of Southern Finland (ESAVI).

6.2 Tissue preparation

After the animals were euthanized, their brains were collected, and the right hemispheres were post fixed with 4% paraformaldehyde solution. The hemispheres were cryoprotected and cut into 40 µm thick coronal serial sections with sliding microtome. Every 12th section throughout the brain was collected within the same Eppendorf tube containing cryoprotectant solution. Tubes were then stored at -20 °C until staining. For more detailed description see Mäkinen (2018).

6.3 Immunohistochemical staining

Immunohistochemistry is done by using antibodies that recognize specific molecules called antigens in the target tissue by binding to a specific structure. Antibodies are produced by injecting an antigen into a mammal, such as rabbit or goat, which causes the immune system to produce polyclonal antibodies in their serum. These antibodies then attach to their target antigens in the tissue that will be stained. After that, the stained structures/cells can later be visualized by attaching secondary or tertiary antibody with fluorescein compound or coloring substance to the primary antibody. Thus, immunohistochemistry provides information about the localization, distribution and number of antigens in the target tissue.

6.3.1 Staining for reactive microglial cells using Iba-1 antibody

Immunohistochemistry was used to measure the number of reactive microglial cells in the hippocampal brain sections using Iba-1 antibody. Staining was done using free floating staining technique. One tube of sections contained 9-14 sections per brain, with 480 µm between each section. Sections from each rat were transferred into small bottles that were placed on shaker plate during all washes and incubations. First, cryoprotectant was washed from sections with 0.1 M PBS (3 x 15min, pH 7.6). Antigen retrieval was done by heating samples in 90°C 0.01 M Na-citrate + 0.05% Triton x-100 for 30 minutes. This is done because a long time in fixative (like paraformaldehyde) may create some chemical crosslinking and thus mask the epitopes which are the parts of the antigen in which the antibody would attach. Then the samples were cooled for ~ 10 min in PBS. To block endogenous peroxidases, (such as heme in the blood), the samples were pretreated with 3% H_2O_2 PBS for 30 min. After peroxidase blocking samples were washed with PBS (2 x 10 min) before serum blocking with 2% goat serum for 1h (PBS + 0.3% Triton X-100 + 2% normal goat serum (Biowest, France, Cat#S200H-500, Lot#S114525200H) to block nonspecific binding sites in which primary antibody could otherwise bind. Rabbit anti-human Iba-1 (ThermoFischer, USA, Cat#PA5-27436, Lot#SI2446351E, rabbit) was used as a primary antibody, diluted at 1:500 in PBS-T with 2% goat serum. Primary incubation was done for overnight in room temperature (RT) on a shaker.

On the second day, the samples were first washed with PBS-T (3 x 5 min). Secondary antibody staining was done using BA-1000 (Biotinylated goat anti-rabbit IgG (H+L), Vector Laboratories, Cat# BA-1000, Lot# 2A0324), 1:500 dilution in PBS-T for 2h. The samples were washed with PBS-T (3 x 5 min) followed by incubation in tertiary antibody for 2h, using Streptavidin-Horseradish Peroxidase Conjugate diluted in 1:1000 in PBS-T (RPN1231, GE healthcare/VWR, Cat# RPN1231V, Lot#9644148).

The visualization of the staining was done with metal -intensified 3'3 -Diaminobenzidine (DAB, D4293, Sigma) solution (Tris 0.05M; pH 7.6 + DAB + nickel ammonium sulphate solution). In the reaction, DAB is oxidized in the presence of previously added peroxidases by 30% H_2O_2, that was added to solution just before staining. As a staining result, a metal-intensified DAB precipitate with black color can be detected with light-microscope. The reaction time of the staining visualization was three and half minutes after which the reaction was stopped by washing the sections with PB (0.1M Na phosphate buffer; pH 7.6). After the staining protocol samples were mounted on an objective glass in gelatin solution. Finally, dry samples were cleared with xylene and covered with cover glasses using Depex (VWR).

6.3.2 Staining for neural activation using p-cFos

P-cFos staining was done to measure neuronal activation in the hippocampal sections. The staining protocol was the same as the protocol for microglia staining. The primary antibody p-cFos (ser32, rabbit mAb, Lot1, 5348S, Cell signaling Netherlands, Cat# 4511S, Lot# 10) was diluted 1:800 in PBS-T. The staining incubation with DAB was four minutes in the p-cFos protocol. Complete staining protocols for both Iba-1 and p-cFos can be seen in appendixes.

6.3.3 Staining for newborn neurons using DCX

Doublecortin (DCX) staining was done to measure the number of newborn neurons in the hippocampal sections. The primary antibody DCX (#sc-8066, Santa Cruz, USA) was diluted 1:1200 in TBS-T. The staining incubation with DAB was 3.5 minutes. The sections were also

counterstained with Cresyl Violet. The complete staining protocol for DCX can be seen in appendixes. DCX measurements were already done before this thesis as part of Active Fit and Smart (AFIS) -project.

6.3.4 Imaging and image analysis

The samples were scanned in the Central Finland Central Hospital with NanoZoomer microscope (Hamamatsu, Japan; 40x resolution). The taken pictures were then analyzed with Qpath -software to count the number of stained cells. Automatic cell detection was used to count the cells from four middle hippocampal sections of each brain. The used threshold for cell counting was determined by calculating the mean optical density (OD mean) of the four hippocampal sections, which was then used as an intensity threshold for the first brain. For the following samples, OD mean of background was compared to the background of the first sample to adjust the threshold for each brain. If the background between sections was uneven then intensity threshold was calculated for each section separately. The areas of interest in each hippocampus were CA1, CA3, dentate gyrus (DG) and granule cell layer (GCL) (see Figure 9). For CA1 and CA3 OD was measured from an area of 200 x 200 µm rectangle and for DG from 100 x 100 µm rectangle, approximately from the same spots in all sections. Granule cell layer was selected by the experimenter from the image, using brush tool of the Qpath -software. The chosen areas do not represent the whole area but are representative samples of the regions (for data analysis, see Kärkkäinen et al. 2015). As for GCL, the whole area was included by experimenter. For Iba-1 the number of stained cells right next to the granule cell layer were also measured (see Figure 13). This was done to ensure that the whole GCL was analyzed, since without counterstaining the exact cellular layers of the GCL were undetectable in Iba-1 staining. The data was then transferred to Excel 2016 (Microsoft, Redmond, WA, USA) for further analyses. The average cell counts and cell per μm^2 from each area of the four hippocampal sections were calculated for each animal and used in statistical analyses.

6.4 Western blotting for Iba-1, SYN-1 and SYP

Western blotting is a method to identify and quantify proteins. First, samples are homogenated and prepared for the analysis, and protein concentrations are measured before the actual procedure. The main procedure begins by separating proteins with electrophoresis, where proteins of different molecular weight travel a weight-determined distance driven by electric current. After that proteins are transferred to nitrocellulose membrane, where they are labeled with antibodies. The labeled proteins are visualized and finally quantified. (for more detailed description see Honkanen 2019.)

The antibodies used were SYN-1(#ANR-014, Alomone labs, Jerusalem, Israel), SYP (#ANR-013, Alomone labs, Jerusalem, Israel) and Iba-1 (#PA5-27436, ThermoFisher Scientific, Rockford, IL, USA). Synaptic proteins SYN-1 and SYP were quantified to represent synaptic plasticity and synaptogenesis and IBA-1 was quantified to represent changes in microglia. SYN-1, SYP and IBA-1 Western blotting data used here is from Honkanen (2019) and they were quantified with Image Lab -software (version 6.0, Bio-Rad, Hercules, CA, USA).

The total protein quantification was measured from protein of molecular weight between 10 to 250 kDa from stain-free image. Automatically detected background noise was taken from total lane protein volumes. The total lane protein volume was used as a correction factor to reduce variance in total protein. Pictures of western blots were taken with ChemiDoc^{TM} MP and the areas and optical densities of the blots were quantified with Image Lab -software.

6.5 Statistical analyses

The number of cases for each antibody is presented in table 1 with both original number of cases and the final number of cases after removing failed stainings and outliers. The differences in the original number of cases (n) between antibodies was due to differences in the number of usable samples for those measurements. Both immunohistochemistry- and western blotting data was analyzed using Excel 2016 (Microsoft, Redmond, WA, USA) and IBM SPSS Statistics 24

for Windows (Chicago, IL, USA). The normality was tested with Shapiro-Wilk normality test in SPSS. Since the data were not entirely normally distributed, nonparametric tests were chosen. The group differences were tested with Mann-Whitney U -test of independent samples and correlations between variables with Spearman's rank correlation. Wilcoxon signed-rank test was used to compare whether the number of Iba-1 positive cells per μm^2 varied in different regions. The chosen statistical significance level was $p < 0.05$.

Table 1. Number of cases (n) for each antibody: final (n) / original (n). *the missing case is missing results from CA1, CA3 and DG. **one of the missing cases is missing only GCL.

Antibody	Young HCR	Young LCR	Old HCR	Old LCR
Iba-1 (IHC)	8* / 9	10 / 10	11 / 12	7** / 10
p-cFos (IHC)	9 / 9	8 / 10	11 / 12	10 / 10
DCX (IHC)	9 / 9	10 / 10	11 / 11	10 / 10
Iba-1 (western)	8 / 8	10 / 10	10 / 10	10 / 10
SYN-1 (western)	8 / 8	10 / 10	9 / 10	10 / 10
SYP (western)	8 / 8	10 / 10	10 /10	10 /10

7 RESULTS

7.1 Synaptic plasticity and neuronal activation

Synapsin 1 expression was significantly higher in the young LCR group compared to young HCR ($p = 0.004$) and old LCR ($p < 0.001$) (Figure 7). The expression of synaptophysin was significantly higher in HCR groups compared to the LCR groups in both young ($p = 0.009$) and old animals ($p < 0.001$) (Figure 8). Additionally, SYP expression was significantly higher in young HCR compared to old HCR ($p = 0.002$) and in young LCR compared to old LCR ($p < 0.001$).

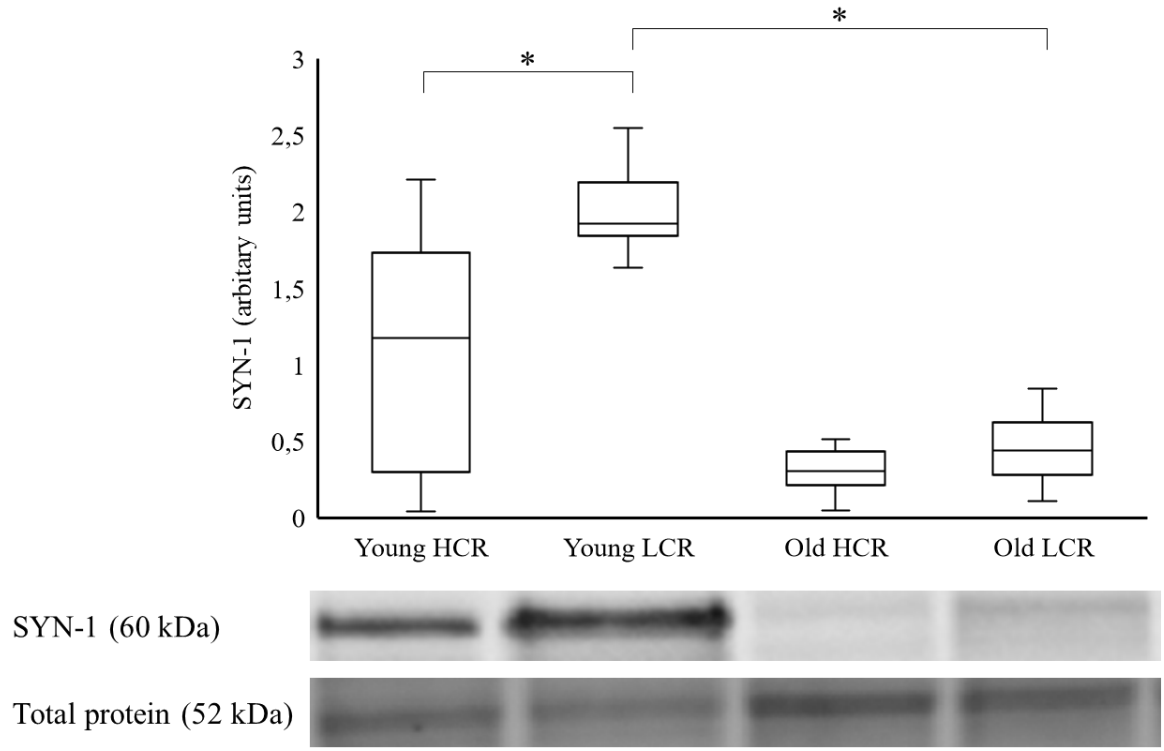


Figure 7. Expression of synapsin-1 (SYN-1) in hippocampus measured by western blotting. * $p < 0.05$

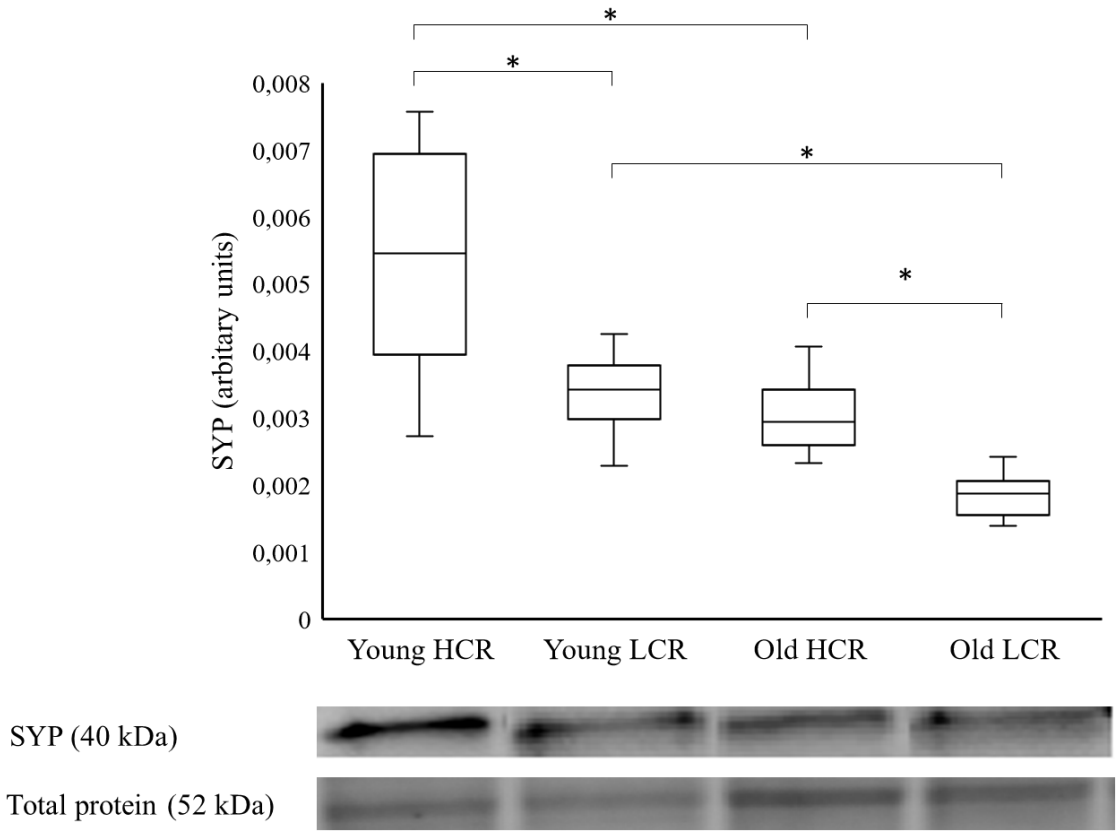


Figure 8. Expression of synaptophysin (SYP) in hippocampus measured by western blotting. * $p < 0.05$

P-cFos positive cells were detected almost exclusively in granule cell layer as one can see in Figure 9. Therefore, the number of p-cFos positive cells is presented only regarding GCL and the combined number of positive cells, adding CA1, CA3, DG and GCL results together (Figure 10). There were no statistically significant differences between the groups.

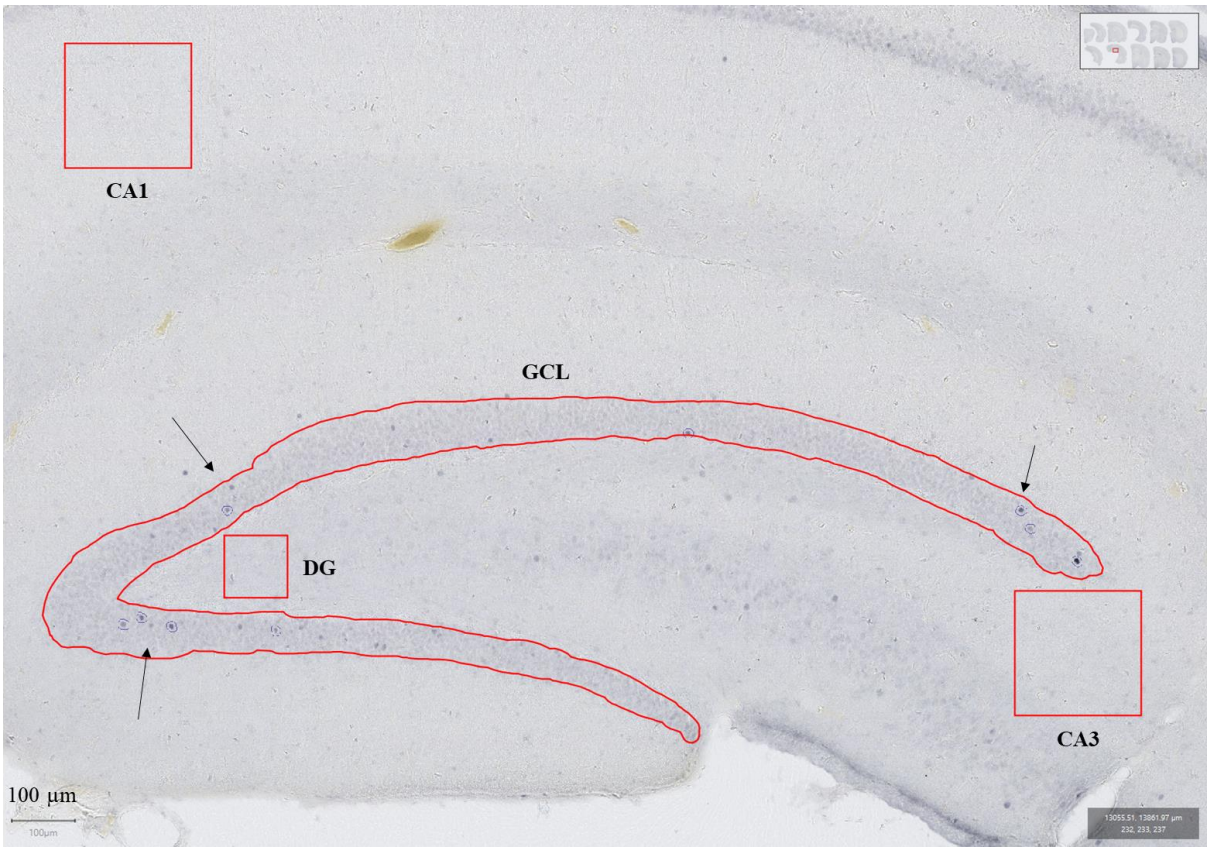


Figure 9. Image of p-cFos staining in hippocampus. The number of positive cells was counted using Qpath –software. Cells were counted from CA1, CA3, dentate gyrus (DG) and from granule cell layer of DG (GCL).

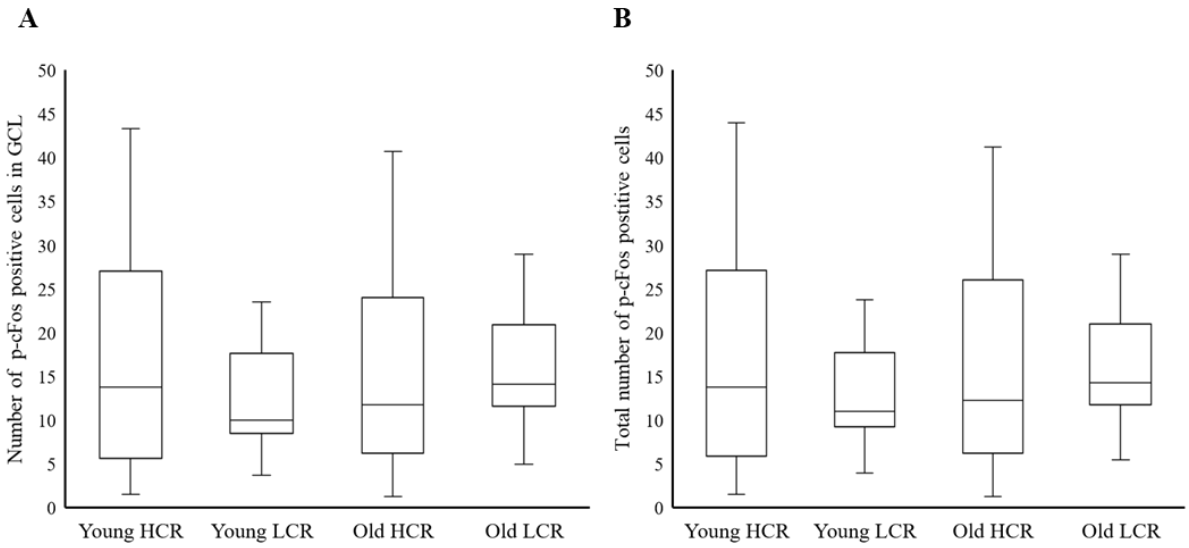


Figure 10. A) Number of p-cFos positive cells in granule cell layer (GCL) in hippocampus. B) number of p-cFos positive cells is the sum of the p-cFos positive cells in the analyzed areas of hippocampus. The areas were CA1, CA3, dentate gyrus and granule cell layer.

7.2 Newborn neurons

The number of doublecortin positive neurons (DCX) in dentate gyrus was significantly higher in in HCR compared to LCR in both young ($p = 0.043$) and old animals ($p < 0.001$). Younger animals also had significantly more DCX positive neurons compared to older animals in both HCR ($p < 0.001$) and LCR ($p < 0.001$) groups. The number of migrating neurons (DCX) in hippocampus was almost identical to that observed in dentate gyrus (Figure 11). The number of DCX positive cells was again significantly higher in HCR compared to LCR in both young ($p = 0.035$) and old animals ($p < 0.001$), and younger animals had significantly more migrating neurons compared to older, in both HCR ($p < 0.001$) and LCR ($p < 0.001$) groups.

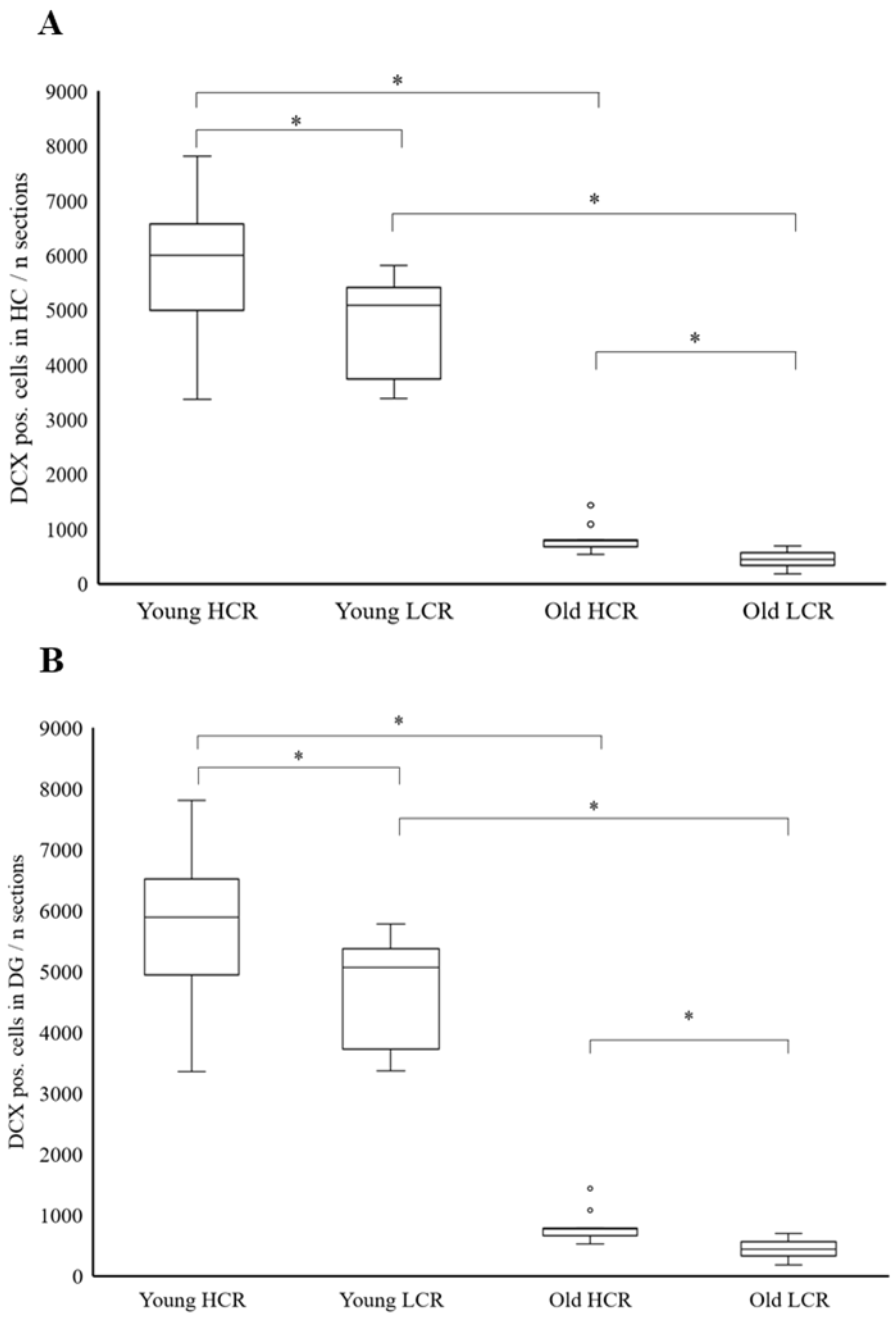


Figure 11. A) Number of doublecortin (DCX) positive cells in hippocampus (HC), corrected with n sections. B) Number of doublecortin (DCX) positive cells in dentate gyrus (DG), corrected with n sections. The correction with n sections was made to control for the differences between hippocampal volumes. * $p < 0.05$

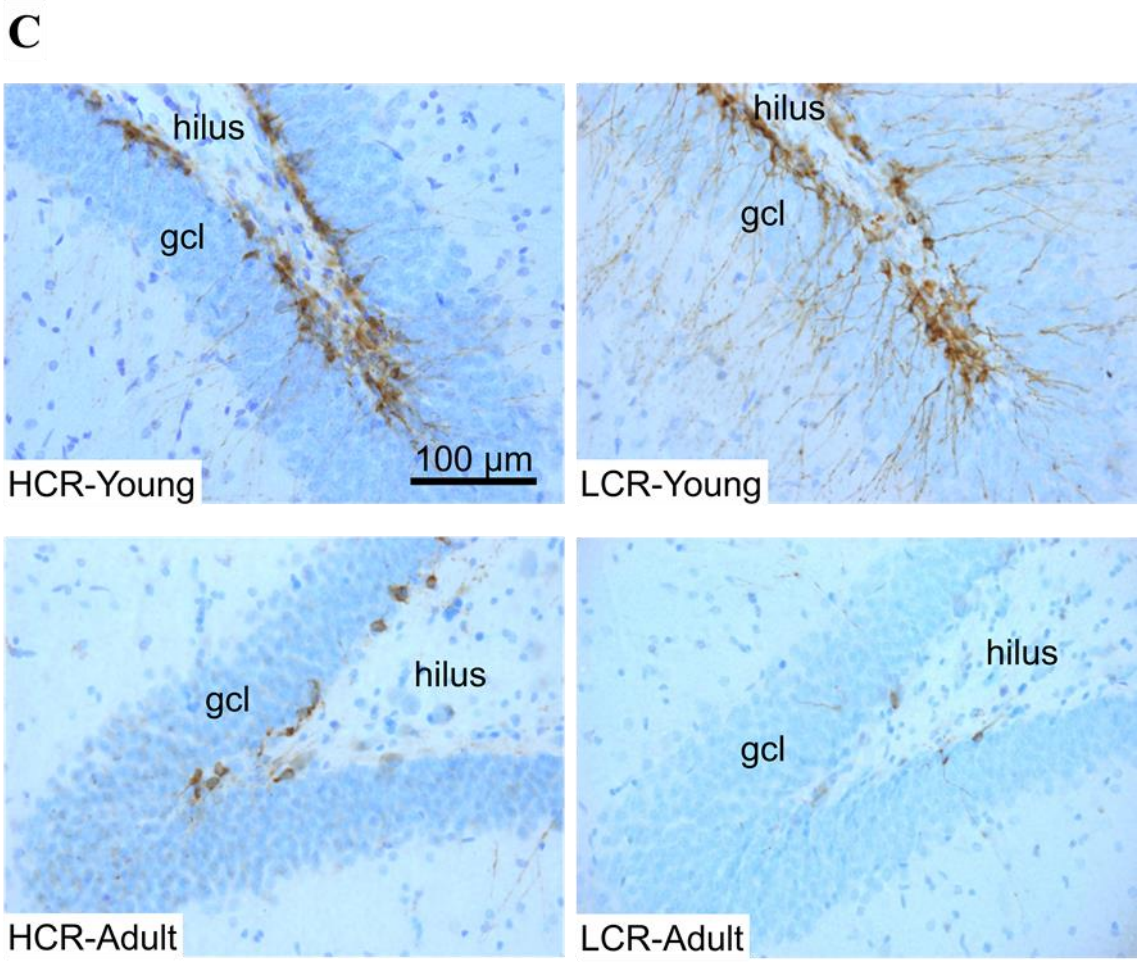


Figure 11. C) Images of DCX staining in hippocampus, newborn neurons can be seen in the granule cell layer (GCL) of DG.

7.3 Microglia in hippocampus

The expression of ionized calcium-binding adapter molecule 1 (Iba-1) in hippocampus is shown in Figure 12. There were no statistically significant differences between the groups.

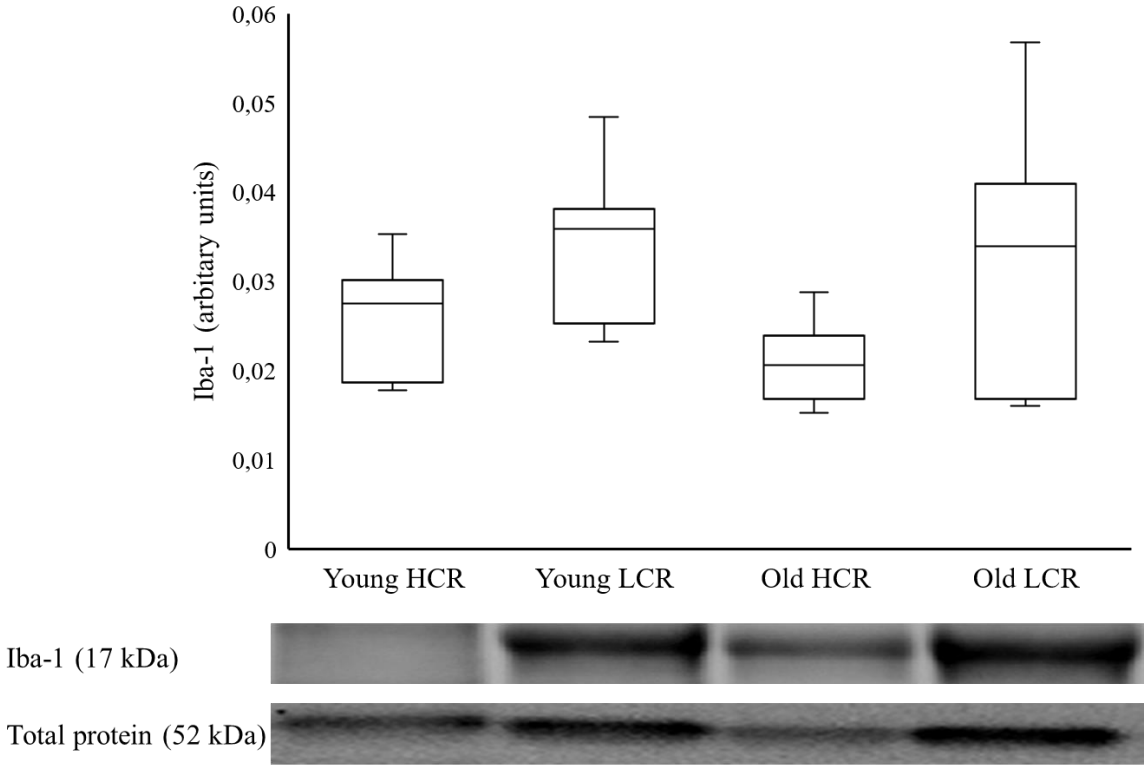


Figure 12. Expression of ionized calcium-binding adaptor molecule 1 (Iba-1) in hippocampus measured by western blotting.

Immunohistochemistry results are presented in Figures 13 and 14. The number of Iba-1 positive cells were measured in CA1, CA3, DG, GCL and in the area right next to GCL. Additionally, the total number of positive cells in all these areas was counted. In CA3 the old animals had significantly more Iba-1 positive cells in both HCR ($p = 0.026$) and LCR ($p = 0.034$). The old HCR animals had also significantly more Iba-1 positive cells in DG compared to young HCR ($p = 0.009$). In other areas there were no statistically significant differences between the groups. Figure 15 shows the number of Iba-1 positive cells / μm^2, which allows the comparison of the number of microglia between regions. The inner part of GCL had significantly more cells than other regions per μm^2 in all four groups. In young HCR, old HCR and old LCR CA3 and DG had significantly more positive cells than CA1 or GCL.

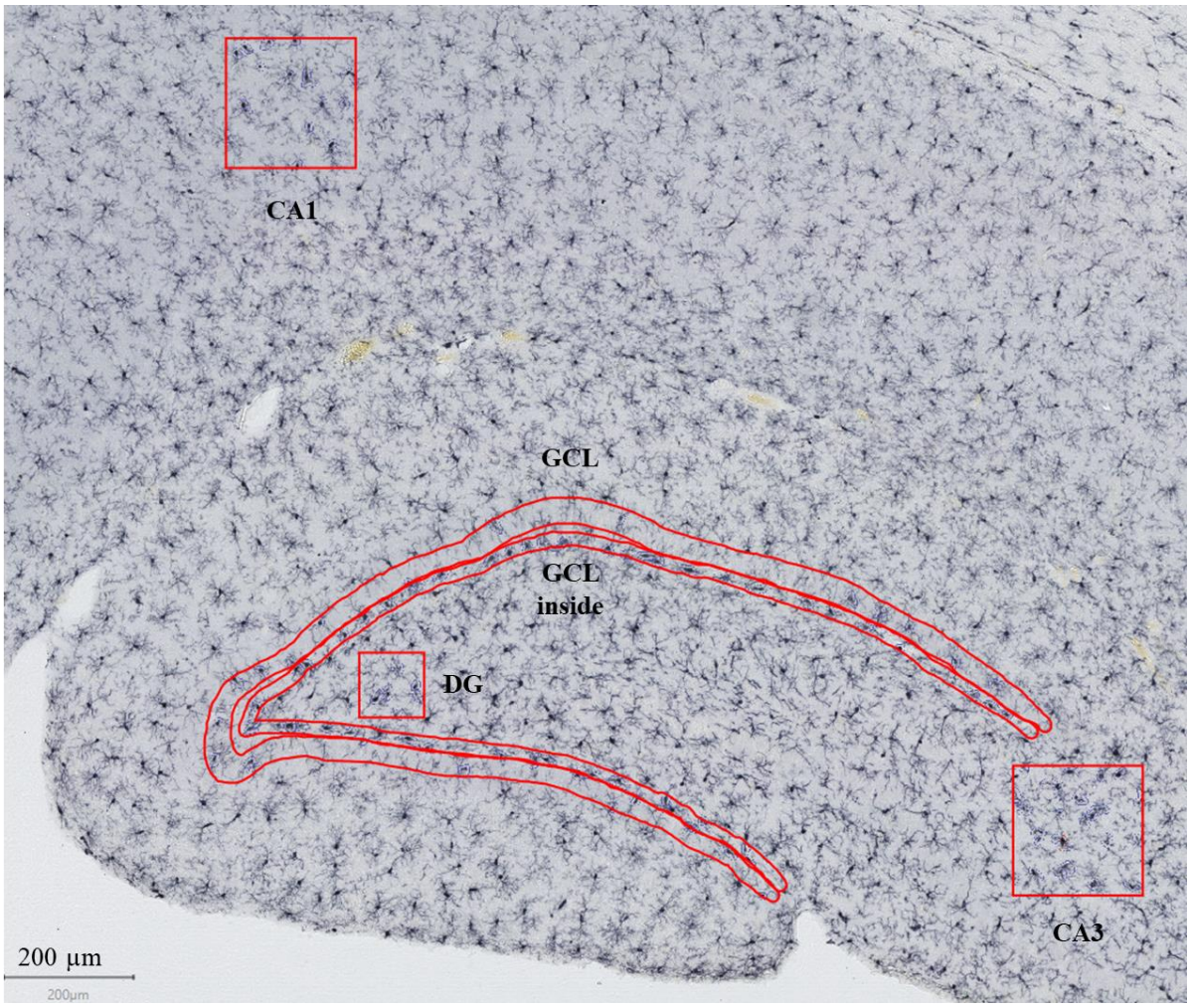


Figure 13. Image of Iba-1 staining in hippocampus. The number of positive cells was counted using Qpath –software and they are circled in the image. Cells were counted from CA1, CA3, dentate gyrus (DG), granule cell layer (GCL) and from the inner part of GCL (GCL inside).

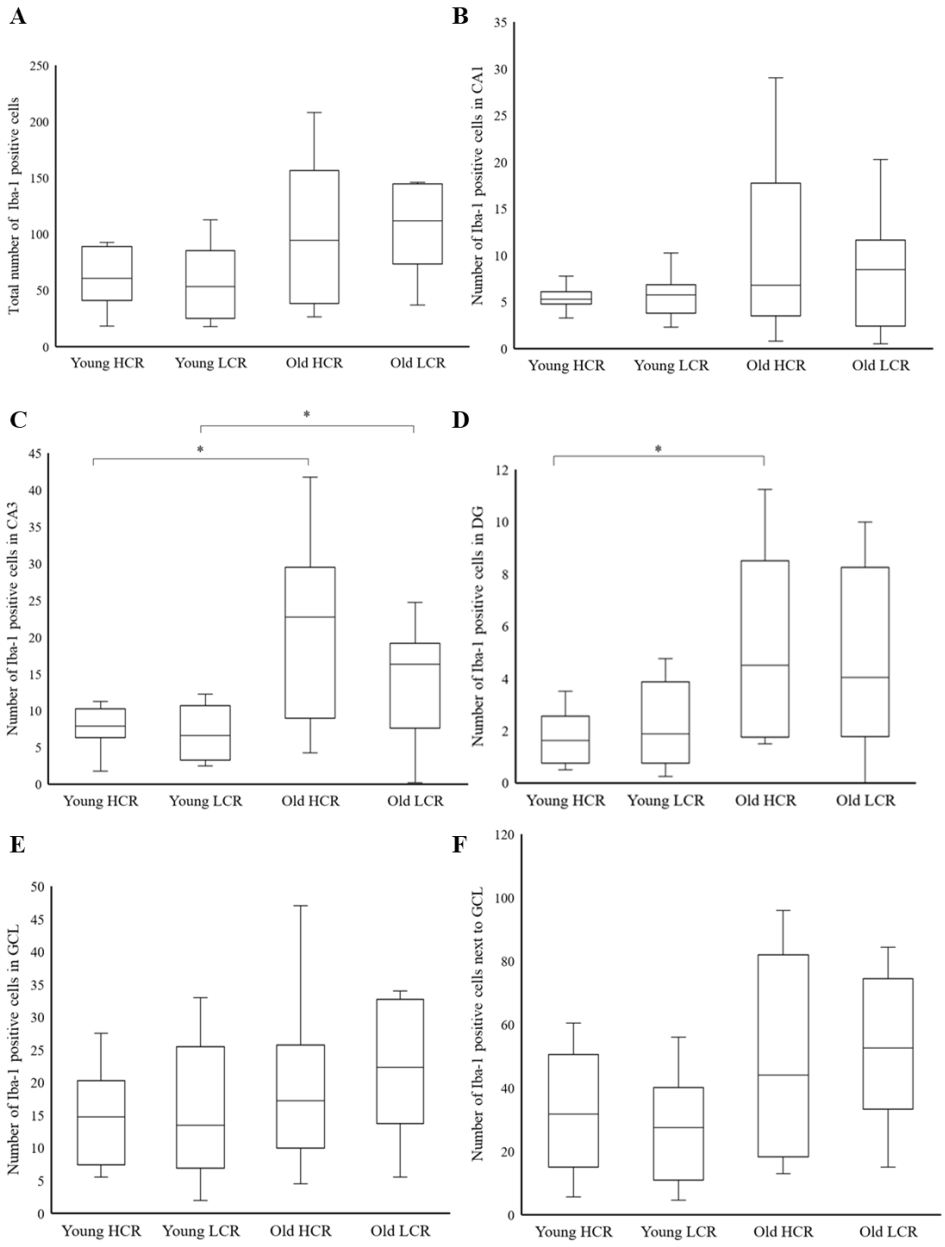


Figure 14. Results of ionized calcium-binding adaptor molecule 1 (Iba-1) positive cells in different regions of hippocampus. A) total number of Iba-1 positive cells in the analyzed areas, B) Iba-1 positive cells in CA1, C) Iba-1 positive cells in CA3, D) Iba-1 positive cells in dentate gyrus, E) Iba-1 positive cells in granule cell layer, F) Iba-1 positive cells right next to granule cell layer in the dentate gyrus. The absolute numbers of microglia between regions are not comparable because the regions differ in their size. * $p < 0.05$

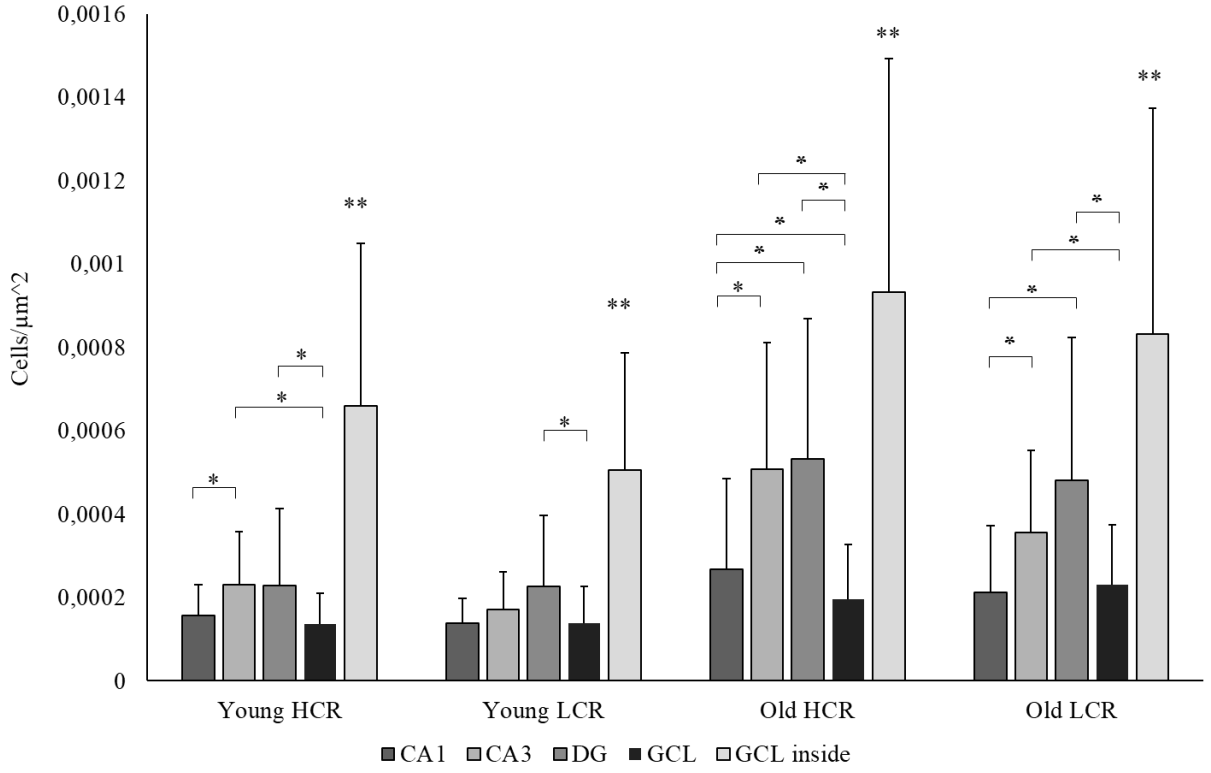


Figure 15. Density of activated microglia was achieved by dividing the number of Iba-1 positive cells with the surface area of the region. The figure presents the number of Iba-1 positive cells divided by the volume of the selected regions. ** inner part of granule cell layer (GCL) had significantly more cells / μm^2 than any other region in all four groups. * $p < 0.05$

7.4 Associations between different forms of neuronal plasticity

Several correlations between different plasticity variables were calculated. Expression of SYN-1 positively associated with the expression SYP ($r = 0.376$; $p = 0.02$). Synaptic plasticity markers were associated with the number of DCX positive cells in HC as the number of DCX positive cells in HC was positively correlated with both SYN-1 ($r = 0,487$; $p = 0.02$) and SYP ($r = 0,748$; $p < 0.001$).

The associations between activated microglia in DG, GCL and cells next to GCL with newborn neurons in DG were measured for each analyzed hippocampal compartment. The number of Iba-1 positive cells right next to GCL in DG was negatively correlated with the number of DCX positive cells in DG ($r = -0,345$; $p = 0.039$). Microglia in DG or GCL were not significantly correlated with the number of DCX positive cells in DG. Additionally, the expression of Iba-1 in western blotting positively correlated with expression of SYN-1 ($r = 0.441$; $p = 0.006$) but not with SYP. Expression of Iba-1 in western blotting was not correlated with the total number of Iba-1 positive cells in the analyzed areas.

8 DISCUSSION

The purpose of this thesis was to study if inherited aerobic capacity and/or age influences different forms of plasticity markers in hippocampus such as synaptic plasticity, neurogenesis and alterations in microglia. Findings of possible differences would indicate differences in brain plasticity between the fit and unfit animals even without physical exercise. This would indicate that there are factors other than exercise driving the difference, such as differences in genetic background related to endurance capacity. The positive effect of aerobic exercise on neural plasticity is well documented (Vaynman et al. 2004; van Praag et al. 2005; Kohman et al. 2012; Ambrogini et al. 2013; Nokia et al. 2016) but intrinsic aerobic capacity may possibly show different results since the exercise component is removed. Differences in markers of brain plasticity were evaluated by comparing expression of markers of synaptic vesicles, neuronal activation, microglia activation and neurogenesis between the old (that is middle aged) and young HCR and LCR rats. The main finding of the study was that HCR animals demonstrate higher numbers of newborn neurons in hippocampus compared to LCR animals, independent of age. This was associated with increased expression of synaptic plasticity markers, from which especially synaptophysin positively correlated with the number of newborn neurons and was expressed more in HCR compared to LCR animals. In contrast to synaptic plasticity markers, the number of activated microglia in the inner part of granule cell layer was negatively correlated with the number newborn neurons in dentate gyrus. Old animals tended to have more microglia than young in several hippocampal compartments.

8.1 Synaptic plasticity and neuronal activation

Synaptic plasticity was measured by western blot expression of synaptic vesicle proteins SYN-1 and SYP. SYN-1 expression was significantly higher in young LCR animals compared to the old LCR and young HCR animals. The fact that SYN-1 was expressed more in LCR was surprising since aerobic exercise is shown to increase SYN-1 in some hippocampal regions (Vaynman et al. 2004). However, one should note that young HCR present great variation in their results, even though it does not explain the differences. SYN-1 expression was higher in

young animals compared to old animals as one would expect. SYP expression was the higher in young animals compared to old ones and HCR expressed more SYP than LCR in both age groups, which was the initial hypothesis. Here again, young HCR showed more variation in their results than the other groups. The correlation between the two synaptic plasticity markers was significant, though surprisingly only 0.376. Put together, younger animals demonstrated greater expression of SYN-1 and SYP, but the difference between HCR and LCR was only clear with SYP, where HCR expressed more SYP. This indicates that one should be cautious about interpreting the results about synaptic plasticity, taking also into account the seen variation in young HCR with both antibodies.

There were no significant differences in neuronal activation between the groups in favor of HCR animals, that was against what was expected. Even though some HCR animals had considerably more p-cFos positive cells than the highest cell counts seen in LCR, the group averages were equal. Notably, GCL was the only region of considerable amounts of p-cFos positive cells, while the other regions had next to no p-cFos positive cells. It is important to note, however, that the whole GCL was analyzed while from the other regions only a representative sample was analyzed since they are not as 'clear cut'. On the other hand, it is possible that neurons in GCL are indeed more active than in other regions. This could be supported by the role of granule cells of DG in pattern separation theory, given their central role of GCL of DG as a segregator of upstream information, in receiving input from EC and its output to other regions (McNaughton & Morris 1987; Knierim & Neunuebel 2016; Senzai & Buzsáki 2017). Previous studies have shown that running increases neuronal activation in DG compared to sedentary animals (Rhodes et al. 2003; Clark et al. 2010). However, all the groups in the present study were kept sedentary, which could suggest that intrinsic aerobic capacity without exercise does not lead to differences in basal neuronal activation in hippocampus.

8.2 Aerobic capacity improves neurogenesis

Neurogenesis was measured by DCX, which stains migrating cells. In line with previous literature (Altman & Das 1965; van Praag et al. 1999; Nokia et al. 2016), DCX positive cells in

hippocampus were almost exclusively located in dentate gyrus, which is why there are almost no differences in cell counts between DG and HC in (Figure 11 A and B). As expected, young animals produce considerably more new neurons compared to old animals, that is over eight times more DCX positive cells. This is in line with the previous literature and has been well established in rodents (Altman & Das 1965; Drapeau et al. 2003; Bizon et al. 2004; Driscoll et al. 2006) and across mammal species (Amrein et al. 2011).

The HCR animals had significantly more new neurons in both age groups compared to the LCR animals. These results would suggest that inherited aerobic capacity affects the basal rate of neurogenesis since all animals were kept sedentary. Other studies provide support for this on a behavioral level: high intrinsic aerobic capacity has been associated with better spatial memory (Sarga et al. 2013) and better performance in tasks requiring flexible cognition (Wikgren et al. 2012). This is relevant since neurogenesis in mice is shown to possibly contribute to spatial pattern separation (Clelland et al. 2009; Sahay et al. 2011).

Regulation of cell metabolism contributes also to neural stem and progenitor cell (NSPC) proliferation. Knobloch et al. (2013) showed that adult neurogenesis requires fatty acid synthase dependent lipogenesis for proliferation, which is regulated by Spot14 -gene. Another study highlighting importance of cell metabolism on neurogenesis is by Steib et al. (2014). They showed that the development of adult-born neurons in DG is accompanied by extensive mitochondrial biogenesis. Additionally, voluntary exercise accelerated maturation of these adult-born neurons by remodeling the mitochondrial compartment. By manipulating the activity of specific mitochondrial fission factor dynamin-related protein 1, which controls mitochondrial morphology and distribution, they showed that loss of that protein function interfered neuronal survival, differentiation and dendritogenesis both in sedentary and exercise conditions. (Steib et al. 2014.) This is interesting since the HCR rats are shown to have better mitochondrial oxidative capacity than the LCR rats in liver (Thyfault et al. 2009), skeletal muscle (Howlett et al. 2003) and in hippocampus (Choi et al. 2014). These studies indicate overall differences in cell metabolism between HCR and LCR, that could also contribute to the

seen differences in the basal neurogenesis. Indeed, Choi et al. (2014) found that hippocampal volume and neuronal number were reduced in the LCR animals.

8.3 Old animals demonstrate more activated microglia than young animals

Microglia were measured with the number of Iba-1 positive cells by immunohistochemistry in specific regions and the overall expression of Iba-1 by western blotting (Figures 12-15). The chosen areas in immunohistochemistry were CA1, CA3, DG, granule cell layer (GCL) and the region inside dentate gyrus right next to GCL. The hypothesis was that HCR and young animals would have less activated microglia than the LCR animals and old animals, respectively. Indeed, the immunohistochemistry results showed that number of Iba-1 positive cells tended to be the same or higher in old animals compared to young animals, in all hippocampal compartments (Figure 14). However, these differences were statistically significant only with the HCR animals in DG and with both HCR and LCR in CA3. This was somewhat to be expected since other studies have reported that microglia number remains relatively stable throughout adulthood (Askew et al. 2017) and male show smaller differences between age groups than their female counterparts (Mouton et al. 2002). However, most of these studies are done in mice and not in rats. There were no significant differences between the HCR and LCR animals, but there was also considerable variation in the results. In western blotting, the LCR animals seemed to have higher expression of Iba-1 but the differences between lines were not statistically significant. Previous studies suggest that aerobic exercise may shift the phenotype of microglia towards more neuroprotective, inflammatory phenotype being the activated microglia (Kohman et al. 2012; Vukovic et al. 2012; Kohman et al. 2013; Littlefield et al. 2015). However, differences in intrinsic aerobic capacity did not result in major differences in microglia activation in the current study. However, the available data in this study does not allow to confirm the exact phenotype of microglia and intrinsic aerobic capacity is different from aerobic exercise. The present immunohistochemical results suggest that younger animals might have less activated microglia, at least in some regions, in hippocampus.

The hypothesis was also that there would be differences in microglia number between hippocampal regions, and it was measured comparing number of Iba-1 positive cells per μm^2. In all four groups the inner part of GCL had significantly more cells per μm^2 than other hippocampal regions. Additionally, young HCR, old HCR and old LCR had more microglia in CA3 and DG compared to CA1 or GCL. In the young LCR animals the differences in microglia number per μm^2 between CA1, CA3, DG and GCL were not as drastic as in other groups. Microglia are known to have several subpopulations, which can vary in phenotype and across brain regions (Kohman et al. 2013). Thus, it can be expected that microglia numbers could also vary across brain regions, as was seen in the present study.

8.4 Associations between plasticity markers

Consistent with hypothesis, expression of synaptic plasticity markers was correlated with the number of newborn neurons. For SYN-1 correlation was 0.487 and for SYP 0.748. This was to be expected since newborn neurons have increased synaptic plasticity (Ge et al. 2008). However, it is worth remembering that the correlation between SYN-1 and SYP was only 0.376 indicating that these results should be interpreted with caution. The number of newborn neurons did not correlate with neuronal activation as was hypothesized. Clark et al. (2010) had previously reported that neuronal activation in granular layer of DG was elevated in runners and was parallel to proliferation of new neurons in DG. The major differences between the study by Clark et al. (2010) and the present study that could explain the different findings were running versus sedentary conditions, as well as mice versus rats with differences in intrinsic aerobic capacity. A probable explanation could also be that newborn neurons do not contribute very much to overall neuronal activation since their relative number to pre-existing cells is very low and thus changes in newborn neurons do not affect overall neuronal activation. However, neuronal activation may represent the activity level or overall brain activity of the animals as it is high in active compared to sedentary animals (Rhodes et al. 2003; Clark et al. 2010) as well as in skill training (Kleim et al. 1996; Tamakoshi et al. 2014). This could indicate that there were indeed no significant differences in the basal brain activity between the groups in the present study.

Activation of microglia was hypothesized to be negatively associated with the number of newborn neurons. Microglia were not correlated as strongly with the number of newborn cells as was expected. Only Iba-1 cells inside DG next to GCL were inversely associated with the number of newborn neurons (r = -0.345). Those microglia in GCL are, however, the cells that are arguably the closest to the newborn neurons from the analyzed hippocampal regions. Given the negative correlation between Iba-1 and DCX, Iba-1 may have acted as an inflammation marker in this study, but further analyses would be needed to verify the phenotype of microglia. The findings that older animals indeed usually express more of the proinflammatory phenotype of microglia (Kohman et al. 2013) supports the proinflammatory phenotype consideration, as older animals also had more Iba-1 positive cells. If the microglia phenotype was more on the proinflammatory side, they could inhibit neurogenesis, known their dual role (Ekdal et al. 2009), explaining the negative correlation between the newborn neurons and microglia in the border of DG and GCL.

Surprisingly, expression of Iba-1 western blot did not correlate with the total number of Iba-1 positive cells in the histologically analyzed regions, but it did correlate with expression of SYN-1 (r = 0.441). These results could suggest couple of things. Immunohistochemistry and western blotting are different methods and although Iba-1 was more present in some areas, the overall expression in hippocampus might still be different. However, the finding that the number of microglia did not considerably vary across the analyzed hippocampal regions does not support this argument. A possible contributor is also the big variation seen in Iba-1 results with both western blotting and immunohistochemistry.

8.5 Strengths and limitations

The HCR-LCR model has some notable advantages in measuring the effects of aerobic capacity on different variables. The HCR rats are known to demonstrate significantly higher intrinsic aerobic capacity and fitness than their LCR counterparts (Hussain et al. 2001; Koch & Britton 2001; Koch et al. 2013), and the difference is greater than what could be achieved in standard training interventions (Koch et al. 2013). This makes it possible to study the effect of aerobic

capacity without exercise intervention, narrowing the variables that could possibly confound the results. However, this comes with the fact that the LCR animals are not purely a healthy control group as they have increased cardiovascular risk factors (Wisløff et al. 2005).

Another strength was combining IHC and western blotting in measuring microglia. The two methods are complimentary for each other and can cover some of each other's weaknesses since both can use same antibodies. Western blotting allows to check if the molecular weight of the target protein is correct, ensuring that the antibody binds specifically and to the right target (Hewitt et al. 2014). IHC complements western blotting as it shows the localization of the proteins in the tissue, which was also of interest in this study. However, the same antibodies do not always behave equally in both methods. This was the case, for example, with SYN-1 which did not work as well in IHC as in western blot and thus, it was not used for the immunohistochemistry. But seen the results, the immunohistochemical data would have been interesting. The fact that antibodies do not work in similar manner in both methods might be partly due to the fact that in western blotting proteins are denatured and thus differ from their native conformation seen in IHC (Bordeaux et al. 2010). The specific phenotype of microglia detected in this study is also unknown.

When it comes to limitations, there were some differences in the number of usable samples for different antibody measurements as well as failed stainings in immunohistochemistry and removal of outliers from the data (Table 1). This was partly because the samples used in this study were collected already between 2015 and 2016. The long storage time and freezing and unfreezing may have had an impact on the quality of samples in western blotting, perhaps explaining some of the curiosities in the results. There is also the possibility of human error in both IHC and western blotting especially since the experimenters were relatively unexperienced with the methodology. Lastly, one should note that correlations do not allow to suggest causal relationships based on these results, as the causality was not the focus in this study. A different study design would be required to determine possible mediating or moderating factors between the variables.

8.6 Conclusions

Aerobic exercise has been shown previously to have several positive effects on neural plasticity including changes in synaptic plasticity, neurogenesis and microglia regulation (Vaynman et al. 2004; van Praag et al. 2005; Kohman et al. 2012; Ambrogini et al. 2013; Nokia et al. 2016). The purpose of this study was to see if differences in intrinsic aerobic capacity without exercise would also influence neural plasticity in hippocampus. Intrinsic aerobic capacity was hypothesized to affect neural plasticity in HCR and LCR animals.

Based on these results, it seems that high cardiac fitness improves neurogenesis in both young and old animals resulting in increased number of migrating newborn neurons. This was also accompanied by increased expression of synaptic plasticity markers in hippocampus, although these results are not conclusive since SYN-1 and SYP expression were the opposite in young HCR and LCR. Increased number of the Iba-1 positive cells right next to granule cell layer was negatively associated with the number of DCX positive cells in DG supporting the hypothesis that in LCR inflammation might be downregulating neurogenesis. However, based on these results it is impossible to say if there is a causal relationship between microglia and neurogenesis since these are only correlations. Moreover, the number of Iba-1 positive cells in the selected hippocampal regions tended to be higher in older animals compared to younger animals, but there were only few statistical differences between groups. Taken together, the HCR and LCR animals show some differences in neural plasticity, especially in neurogenesis, and these differences are present already in the young animals.

Age was also an important factor contributing to differences in brain plasticity so that older animals have less neurogenesis and more activated microglia. Compared with LCR, the higher intrinsic cardiovascular fitness of the HCR seems to result in higher rate of neurogenesis in older age, which might explain some of the differences seen in previous studies in flexible cognition between HCR and LCR (Wikgren et al. 2012). There are also differences between the lines in their microbiome as the LCR animals have more taxa related to obesity as well as differences in their metabolites (Pekkala et al. 2017), which might also have an effect on top of

other metabolic differences. When it comes to brain activity, sedentary conditions might be stressful for the HCR animals as they are more spontaneously active (Karvinen et al 2016). However, the present results show that under basal conditions, there were no differences in hippocampal activity between the rats. In relation to humans, these results would support the idea that differences in genetic background may affect, for example, the positive relationship between physically active children and school performance (Haapala et al. 2017; Haapala et al. 2019), as intrinsic cardiovascular fitness can indeed promote brain plasticity.

For future perspectives, it would be interesting to study whether exercise could mitigate the seen differences in neural plasticity between the HCR and LCR animals or are the differences something that are determined by one's genes. Additionally, one could study if there are differences in microglia phenotypes between the HCR and LCR animals, and if they it would have mediating or moderating effect on neurogenesis and/or synaptic plasticity.

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APPENDICES

APPENDIX 1. Immunohistochemistry staining protocols

Staining protocol for Iba-1.

DAY 1

1. 0.1 M PBS (pH = 7.6) washes 3 x 15 min
2. 90℃ 0.01 M Na-citrate + 0.05% Triton x-100 solution for 30 min
3. Cooling the samples for ~ 10 min in PBS
4. Peroxidase blocking 3% H_2O_2 PBS for 30 min
5. PBS washes 2 x 10 min
6. Serum blocking: PBS + 0.3% Triton X-100 + 2% normal goat serum (Biowest, France, Cat#S200H-500, Lot#S114525200H) for 1h
7. Primary antibody staining over night: PBS + 0.3% Triton X-100 + 2% normal goat serum + 1:500 rabbit anti-human Iba-1(ThermoFischer, USA, Cat#PA5-27436, Lot#SI2446351E, rabbit)

DAY 2

1. PBS-T washes 3 x 5 min
2. Secondary antibody staining 2h: PBS-T + 1:500 goat anti-rabbit BA-1000 (Biotinylated goat anti-rabbit IgG (H+L), Vector Laboratories, Cat# BA-1000, Lot# 2A0324)
3. PBS-T washes 3 x 5 min
4. Tertiary antibody staining 2h: PBS-T + 1:1000 Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE healthcare/VWR, Cat# RPN1231V, Lot#9644148)
5. 1 x 5 min PBS-T wash and 2 x 5 min Tris
6. DAB (D4293, Sigma) staining 3min 30s:
    - 20 ml Tris (0.05M; pH = 7.6) + 1/2 DAB tablet + 1 ml Nickel ammonium sulphate-solution
    - 5 min sonication, 20s vortexing
    - 4 µl 30% H_2O_2 per 5 ml DAB just before staining
    - DAB staining
    - wash 3 x 1 min with PB (0.1M Na phosphate buffer, pH = 7.6)
7. Mounting of samples to glass in gelatin
    - 100 ml MilliQ H_2O + 0.4g gelatin + 0.05g potassium chrome sulphate
    - mix water and gelatin with magnet mixer and add potassium chrome sulphate when all gelatin is dissolved, +60 ℃, 30 min

Staining protocol for p-cFos

DAY 1

1 0.1 M PBS (pH = 7.6) washes 3 x 15 min
2. 90°C 0.01 M Na-citrate + 0.05% Triton x-100 solution for 30 min
3. Cooling the samples for ~ 10 min in PBS
4. Peroxidase blocking 3% H_2O_2 PBS for 30 min
5. PBS washes 2 x 10 min
6. Serum blocking: PBS + 0.3% Triton X-100 + 2% normal goat serum (Biowest, France, Cat#S200H-500, Lot#S114525200H) for 1h
7. Primary antibody staining over night: PBS + 0.3% Triton X-100 + 2% normal goat serum + 1:800 p-cFos (ser32, rabbit mAb, Lot1, 5348S, Cell signaling Netherlands, Cat# 4511S, Lot# 10)

DAY 2

1. PBS-T washes 3 x 5 min
2. Secondary antibody staining 2h: PBS-T + 1:500 goat anti-rabbit BA-1000 (vector laboratories)
3. PBS-T washes 3 x 5 min
4. Tertiary antibody staining 2h: PBS-T + 1:1000 Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE healthcare/VWR)
5. 1 x 5 min PBS-T wash and 2 x 5 min Tris
6. DAB (D4293, Sigma) staining 4min:
    - 20 ml Tris (0.05M; pH = 7.6) + 1/2 DAB tablet + 1 ml Nickel ammonium sulphate-solution
    - 5 min sonication, 20s vortexing
    - 4 µl 30% H_2O_2 per 5 ml DAB just before staining
    - DAB staining
    - wash 3 x 1 min with PB (0.1M Na phosphate buffer, pH = 7.6)
7. Mounting of samples to glass in gelatin
    - 100 ml MilliQ H_2O + 0.4g gelatin + 0.05g potassium chrome sulphate
    - mix water and gelatin with magnet mixer and add potassium chrome sulphate when all gelatin is dissolved, +60 °C, 30 min

Staining protocol for DCX

DAY 1

1. Wash with 0.1 M Phosphate Buffer (pH 7.6) for 24 hrs/overnight
2. Antigen retrieval: Boil in 85°C citrate solution (pH 6.0) for 30 min; 0.01M Na-citrate
3. Endogenous peroxidase blocking in 1% H_2O_2 for 30 min at room temperature
4. Wash with 0.5 M TBS-T (3x)
5. Serum blocking 1 h
    - 0.5 M TBS + 0,25 % Triton X-100 + 2 % normal rabbit serum (S-5000, Vector; Lot. ZA0812)
6. Prepare primary ab dilution: Doublecortin 1:2000 (sc-8066, Santa Cruz Biotechnology, USA)
7. Soak in primary antibody sol'n for 24 hrs/overnight (in +4)

DAY 2

1. Wash with TBS-T (pH = 7.6) 3 x 5 min
2. Soak in secondary antibody for 2 hrs in room temperature, IgG antibody (BA-5000, rabbit anti-goat, vector laboratories)
3. Wash with TBS-T (pH = 7.6) 3 x 5 min
4. Soak in tertiary antibody for 2 hrs in room temperature, Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE Healthcare/VWR)
5. Wash 3 times: with TBS-T (pH = 7.6) 2 x 5 min; last wash with 0.05 M Tris-buffer, pH 7.6
6. Soak in DAB in room temperature (3.5 min)
7. Wash with 0.1 M PB (pH 7.6) 3 x 1 min. Sections can be left in PB overnight (+4 in shaker), and mount them on next day.
8. Mount sections on slides using gelatin sol'n
9. Dry slides in (+37°C) for a minimum of 24 hrs (overnight) to remove liquids

DAY 3**:** COUNTERSTAINING

1. Counterstain with Cresyl Violet
    a. dH2O (2 dunks)
    b. Cresyl Violet (4-10 min)
    c. dH2O (3 dunks)
    d. 70% EtOH + 4 drops acetic acid (variable)
    e. 95% EtOH (3 dunks)
    f. 100% EtOH (2 dunks)
    g. Xylene (4 min)
    h. Xylene (1 min <)
2. Use Depex to coverslip
3. Dry in hood for at least 24 hrs

APPENDIX 2. Antibodies used in Western blots

Antibody	Dilution	Product number	Manufacturer
SYN-1	1:400	ANR-014	Alomone labs, Israel
SYP	1:800	ANR-013	Alomone labs, Israel
Iba-1	1:700	PA5-27436	ThermoFisher Scientific,Rockford, IL, USA