EFFECT OF THE INNATE AEROBIC CAPACITY OR ACROBATIC TRAINING ON THE INFLAMMATION OF THE BRAIN IN RATS

Markus Honkanen

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University of Jyväskylä
Supervisors:
Satu Pekkala, Sanna Lensu

ABSTRACT

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A mammal body reacts to the invader via an inflammation reaction. Immune system in brain is special because of the blood-brain barrier and glial cells. Hippocampal neurogenesis produces new neural cells. The rate of neurogenesis can be elevated by aerobic exercise and skill learning. Inflammation is detrimental to the neurogenesis and learning. Western blot is often used as a tool for study of inflammation and neurogenesis, but lack of validation studies and uniform protocols causes uncertainty to results. High aerobic capacity should protect from inflammation and enhance learning. The purpose of the thesis was to study a connection with an intrinsic aerobic capacity, age or acrobatic training and inflammation or synaptic plasticity in the rat brain. Also inter-user variability and difference between two western blot devices and softwares were studied.

19 high aerobic capacity (HCR) and 20 low aerobic capacity (LCR) sedentary male rats were killed at the age of 8 or 40 weeks. 24 Sprague-Dawley rats were divided to the acrobatic training (Acro) and control (Ctrl) groups for 8 weeks of acrobatic training intervention. Quantitative western blotting analysis was made for protein homogenates of left hippocampi. Antibodies for brain inflammation and synaptic plasticity were used. Two analysts quantified results of three antibodies and between two devices and softwares for methodological comparison.

The LCR rats expressed more inflammation markers than the HCR rats in overall. The age comparison resulted inconsistent outcome. Synaptic plasticity results were contradictory. There were no differences between the Acro and Ctrl groups in inflammation markers or in synaptic plasticity. In the method comparison, SYN-1 results were consistent regardless the analyst or device. IBA1 resulted moderate correlations in comparisons. SYP resulted high inter-user correlation but lower inter-device correlation.

Higher aerobic capacity of the HCR rats reduces brain inflammation compared to the LCR rats. Differences in aerobic capacity and inflammation markers are present already at young age, but results were inconsistent. Contradictories in synaptic plasticity results leads to no conclusion with effect of aerobic capacity to learning. Acrobatic training did not result marked differences in this study. Reliability of western blotting results between analysts and devices depend on the antibody used. To ensure reliable results, one analyst should execute all the tests in a study using the same device and software. Results should also be confirmed using some other method.

Key words: inflammation, aerobic capacity, acrobatic training, western blotting, synaptic plasticity, neurogenesis

ABBREVIATIONS

Acro	acrobatic-trained
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CCD	charge-coupled device
CNS	central nervous system
Ctrl	control
GFAP	glial fibrillary acidic protein
HCR	high-capacity runner
IBA1	ionized calcium-binding adaptor molecule 1
IL-1ß	interleukin-1ß
LCR	low-capacity runner
LPS	lipopolysaccharide
NaCl	sodium chloride
MyD88	myeloid differentiation primary response protein 88
NK	natural killer
Old-HCR	old HCR
Old-LCR	old LCR
PRRs	pattern-recognition receptor
RT	room temperature
SDS	sodium dodecyl sulfate
SYP	synaptophysin
TBS	Tris-buffered saline
TBS-Tween	0.1 % Tween 20 in TBS
TLR	toll-like receptor
TLR3	toll-like receptor 3
TLR5	toll-like receptor 5
$VO_{2\,max}$	maximal oxygen uptake
Yng-HCR	young HCR
Yng-LCR	young LCR

CONTENTS

1. INTRODUCTION

Positive health effects of good physical fitness and exercising have been studied for decades as a possible treatment for type II diabetes, coronary heart disease, and overall decline of health as a consequence of obesity (Powell & Paffenbarger 1985). However, regardless of the increasing knowledge of healthy habits and lifestyle as well as risk factors for type II diabetes and other obesity-related diseases, the prevalence of those conditions has escalated recently (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators 2016). Poor aerobic fitness and obesity also cause chronic inflammation overall in the body (Lumeng & Saltiel 2011), and in the brain (Palavra et al. 2016).

For the study of a role of aerobic fitness for various health aspects, two lineages of rats have been bred for high and low running capacity (Koch & Britton 2001), producing valuable data of intrinsic, non-exercised level of fitness, and its relations to health or diseases. For example, type II diabetes induces cognitive decline in humans (Strachan 2010), whereas aerobic exercising improves learning and task acquisition in rodents and has beneficial effect to cognitive abilities in humans (Hillman et al. 2008). Enhanced cognitive skills of rats due to the exercise training has been linked to hippocampal neurogenesis (Erickson et al. 2011). Also, exercising reduces inflammation in the brain, thus diminishing neurodegeneration (Cotman et al. 2007). Acrobatic training for rats has been introduced as a different approach for inducing synaptic changes in the rat brains (Gutierrez et al. 2018b).

The purpose of this thesis was to investigate the connections between an intrinsic aerobic capacity, age and acrobatic training to the inflammation and synaptic plasticity in the brain of rats. In addition, a comparison between results from different devices and analysts was made for evaluation of the reliability of the western blotting.

2. IMMUNE SYSTEM AND FUNCTION

2.1. Overview of the immune system

The mammal body recognizes possible threats and tries to defend against pathogens threatening organs or tissues. This ability is called immunity, and organization taking part in defensing the body is called immune system. The immune system can be divided into the innate and adaptive immune system. These divisions co-operate organizing the immune response in efficient manner. (Guyton & Hall 2006, 439–450; Kohman & Rhodes 2013.)

The innate immune system has an ancient origin, it evolved and developed with the life itself (Matzinger 1998; Yatim & Lakkis 2015). The innate immune system consists of general defense mechanisms of the body. It is activated immediately when an infectious agent attempts to enter the body, and thus it provides rapid but non-specific response to threat. Innate immune system includes physical barriers such as skin and mucous membranes, destruction of swallowed organisms by acidic environment of the stomach, white blood cells capable of phagocytosis of bacteria and other invaders, and finally presence of chemical compounds in blood that attach to foreign organisms or toxins and destroy them. The response of the innate immune system against one specific invader stays more or less the same from time to time, and even across different individuals. For more sophisticated protection against invaders, innate immune system co-works with acquired immune system. (Guyton & Hall 2006, 439–450; Gleeson & Bosch 2013.)

The acquired immune system has developed later to support and enhance the functions of immunity (Yatim & Lakkis 2015). While characteristic to innate immune system is similar response to one invader from time to time, acquired or adaptive immune system has a ”memory”. It makes more efficient and effective response against repeated attacks of similar invaders possible, even if successive attacks had years in between. The acquired immune system bases on pluripotent hematopoietic stem cells, which mature to different kinds of lymphocytes. These lymphocytes then take care of specific immune responses against invaders (Guyton & Hall 2006, 439–450; Gleeson & Bosch 2013).

2.2. Inflammation reaction in general

A pathogen attempting to infect the body will first have to figure out a way past body's surface barriers, like skin and mucous layers (Gleeson & Bosch 2013). If an invader succeeds in that, it may start an inflammation reaction (figure 1). Inflammation is a series of tissue responses to trauma, invasion of bacteria or any other phenomenon causing tissue injury (Graham et al. 2006; Guyton & Hall 2006, 429–438; Lumeng & Saltiel 2011). When injured, multiple substances are released by the damaged tissues causing secondary changes in surrounding healthy tissues (Guyton & Hall 2006, 429–438). Inflammatory status of a tissue is characterized by vasodilation of local blood vessels, increased permeability of the capillaries, often clotting of the fluid in interstitial spaces, migration of large number of granulocytes and monocytes into the tissue and swelling of the tissue cells (Guyton & Hall 2006, 429–438). These reactions are caused by secretion of histamine, bradykinin, serotonin, prostaglandins and other products (Guyton & Hall 2006, 429–438; Lumeng & Saltiel 2011).

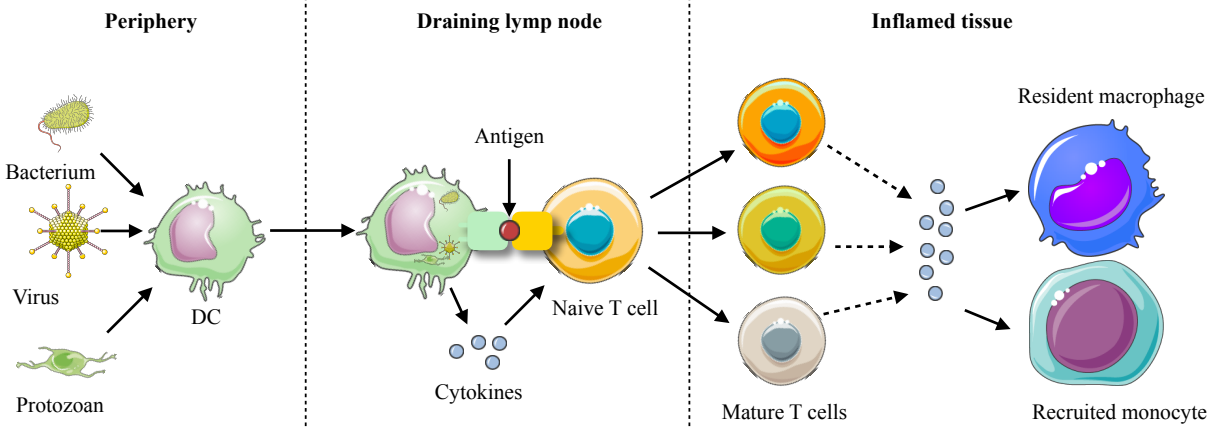

FIGURE 1. Innate immunity in the periphery. Tissue dendritic cells internalize microbial protein antigens. Then they migrate to draining lymph nodes and present antigens to naive T cells. DCs also release regulatory cytokines (interleukins) to T cells. Naive T cells then mature to subclasses and migrate to infected and inflamed tissues. Besides straight actions, mature T cells express cytokines that act on resident and recruited immune system cells, which cooperate with factors such as the complement system to clear the infection. DC, dendritic cell. (Modified from Ransohoff & Brown 2012; images from SmartServier s.a..)

The reason for tissue inflammation is to extract the traumatized area from healthy tissues by swelling and at the same time by accelerating blood flow and vessel leakage to drive as many white blood cells to the area as possible to take care of pathogens (Guyton & Hall 2006, 429–438). Acute infection or tissue damage causes immune system to trigger inflammatory processes which help to heal a wound or sickness response, even though they may cause swelling and fever as temporary discomfort (Graham et al. 2006). Innate immunity is a key element in the inflammatory response as well as in the immune response against pathogens (Akira et al. 2006).

2.3. Compartments of the immune system

2.3.1. Recognition of pathogens by toll-like receptors

The body has receptor cells (for example monocyte, macrophage or microglia) that produce pattern-recognition receptors (PRRs) able to recognize non-self particles and pathogens (Matzinger 1998; Akira et al. 2006; Gleeson & Bosch 2013). When invader has been noticed, receptor cells release cytokines (chemical messengers, for example interleukins (Gleeson et al. 2013)). Interleukins initiate immune response by producing ”acute phase proteins” from the liver and leukocytes (Gleeson & Bosch 2013; Kohman & Rhodes 2013). Acute phase proteins help to identify invaders and stimulate phagocytes to kill them and remove the debris (Gleeson & Bosch 2013).

Toll-like receptors (TLRs) were the first PRRs to be identified (Kawai & Akira 2011). TLRs and other later found PRRs co-operate in fighting infections by taking part both pathogen-specific and cell type-specific host immune responses (Kawai & Akira 2011). TLRs recognize pathogens by their specific surface molecules, and bind to their ligands (Blasius & Beutler 2010; Gleeson & Bosch 2013). This starts a cascade involving activation of innate immunity by inducing phagocytosis, production of inflammatory cytokines and generation of acquired immunity by induction of several signaling molecules (Gleeson & Bosch 2013). TLRs are expressed on various immune cells in response to pathogens, a variety of cytokines and envi-

ronmental stresses (Akira et al. 2006). TLR family includes both intercellular and cell surface receptors specialized for different tasks (Blasius & Beutler 2010).

One member of TLR family, toll-like receptor 3 (TLR3), is found almost exclusively in intracellular compartments such as endosomes (figure 2; Akira et al. 2006; Kawai & Akira 2011). TLR3 is involved especially in the recognition of viral nucleotides (Akira et al. 2006; Blasius & Beutler 2010; Kawai & Akira 2011), as are other intracellular TLRs (Blasius & Beutler 2010). TLR3 activates the toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β (TRIF) signaling cascade that results in antiviral immune response and T cell stimulation in addition to inflammasome activation in cytosol (figure 2; Kawai & Akira 2011; Ullah et al. 2016). TLR3 also promotes the production of inflammatory cytokines in macrophages and dendritic cells (Kawai & Akira 2011; Ullah et al. 2016). In the brain, TLR3 is expressed in astrocyte and glioblastoma cell lines (Akira et al. 2006; Park et al. 2006). Because TLR3 triggers inflammatory cascades and is also expressed during viral infection, it is used as a marker for inflammation in the area of interest (So et al. 2006; Vercammen et al. 2008; Henry et al. 2014).

Another receptor molecule, toll-like receptor 5 (TLR5), is mainly expressed on the cell surface (Akira et al. 2006). TLR5 is specialized in recognizing flagellin of the bacteria (figure 2; Takeda & Akira 2004; Akira et al. 2006; Kawai & Akira 2011). TLR5, like all the other TLRs with the exception of TLR3, uses myeloid differentiation primary response protein 88 (MyD88) signaling pathway (figure 2; Kawai & Akira 2011; Ullah et al. 2016). When TLR5 recognizes flagellin, it recruits MyD88. This activates a signaling cascade resulting in induction of various cytokines and chemokines, thus promoting inflammatory reaction (Kawai & Akira 2011). TLR5 responses are regulated by TRIF (Choi et al. 2010). TLR5 is used as a marker for flagelling-induced proinflammatory signaling (Tallant et al. 2004).

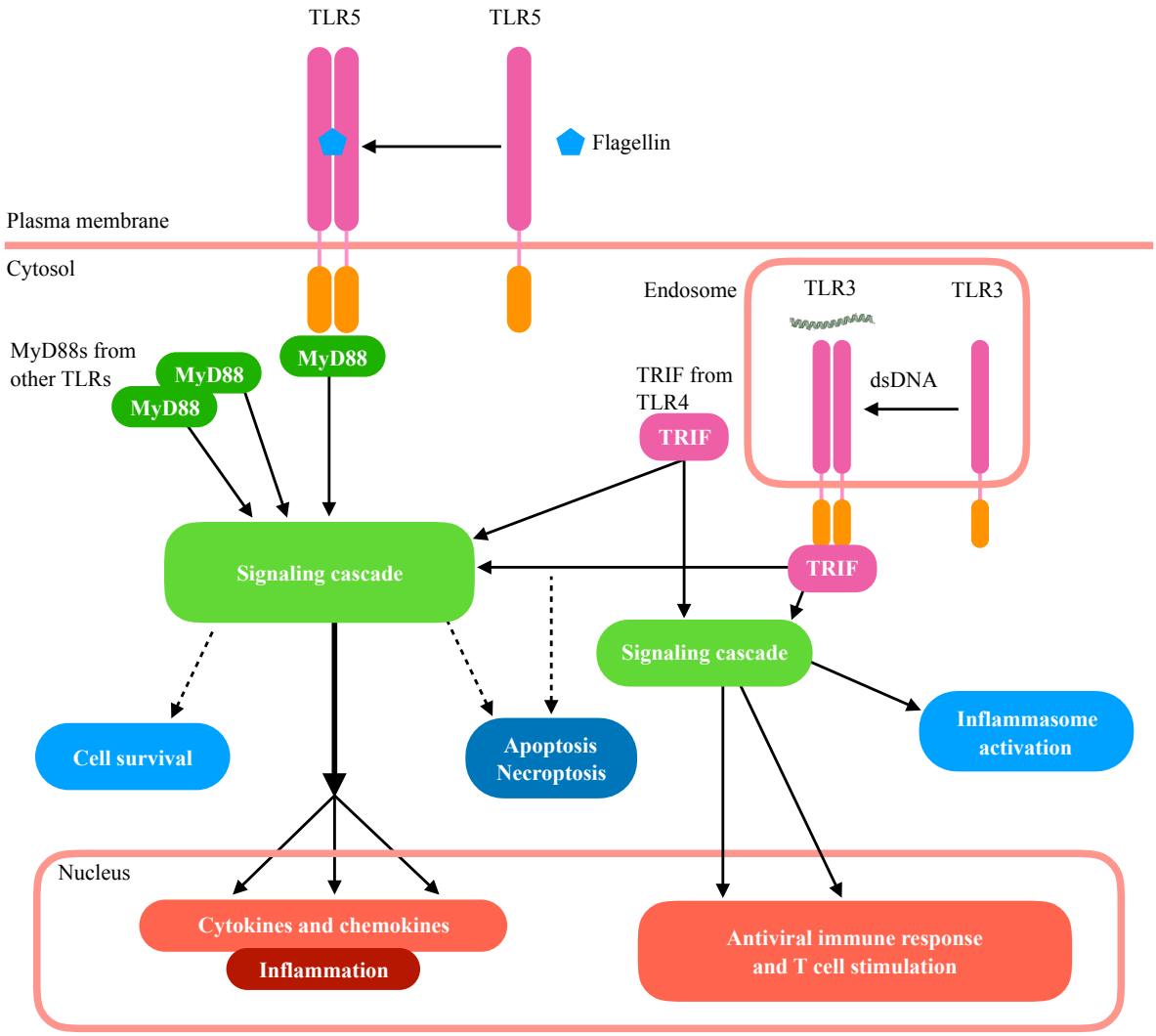

FIGURE 2. Signaling pathways for TLR5 and TLR3. MyD88-dependent pathway is responsible for producing inflammatory cytokines. Flagellin is recognized on the cell surface by TLR5, which recruits MyD88. MyD88s from various TLRs then activate a highly regulated signaling cascade, which ends up in induction of inflammatory cytokines and chemokines. Also, cell survival or cell death can be promoted in the signaling pathway. TRIF-dependent signaling is responsible for the antiviral response and T cell stimulation. Viral dsDNA in endosome is recognized by TLR3, which recruits TRIF. TRIFs activate a signaling cascade, that leads to transcription of antiviral immune response and T cell stimulation genes in the nucleus, and inflammasome activation in the cytosol. TRIFs also regulate the MyD88-dependent signaling cascade and can induce apoptosis or necrosis if needed. dsDNA, double-stranded deoxyribonucleic acid; MyD88, myeloid differentiation primary response protein 88; TLR, toll-like receptor; TRIF, T toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β. (Modified from Ullah et al. 2016.)

2.3.2. Cytokines and interleukins

Cytokines are, by one definition, ”proteins secreted by white blood cells and a variety of other cells in the body” and their actions ”include numerous effects on cells of the immune system and modulation of inflammatory responses” (Vilček 2003). Thus, cytokines are immune response mediating molecules produced and released mainly by the cells of immune system (Graham et al. 2006; Gleeson 2013). Cytokines usually act as autocrines or paracrines rather than rather than as endocrines (Vilček 2003). They can be either pro-inflammatory or anti-inflammatory (Henry et al. 2009; Gleeson & Bosch 2013).

In general, interleukins function as cytokines mediating communication between cells during immune responses. Some of interleukins have pro-inflammatory properties while others play anti-inflammatory roles (Afonina et al. 2015). Interleukin-1’s induce inflammation, and their dysregulation can lead to tissue damage (Rosenzweig et al. 2014). Interleukin-1’s are expressed for example by monocytes, macrophages, natural killer (NK) cells, B and T cells, astrocytes and microglial cells (Vilček 2003). Interleukin-1ß (IL-1ß) is a member of interleukin-1 family (Vilček 2003), and it works as a pro-inflammatory agent (Henry et al. 2009; Afonina et al. 2015; Cohen 2016). Expression of IL-1ß is triggered by Toll-like receptor 4, interleukin-6 or by itself (Rosenzweig et al. 2014). IL-1ß is one of the key pro-inflammatory molecules driving systemic inflammation in for example type 2 diabetes mellitus (Knudsen & Pedersen 2015), and blocking its expression may improve the status of diabetes patients (Hensen et al. 2013). In research, IL-1ß is used as an indicator of inflammatory response in the tissue of interest (Lopez-Castejon & Brough 2011).

2.3.3. Phagocytes

Phagocytes are white blood cells (leukocytes) that are capable of ingesting and digesting bacteria and microorganisms (Gleeson et al. 2013). The major phagocytic cells of the immune system are neutrophils, monocytes, macrophages and dendritic cells (figure 3). Neutrophils are already mature cells when they enter the tissues, where they can approach a particle and phagocytize it (Ransohoff & Cardona 2010). Neutrophils are the first inflammatory cells rec-

ruited in response to inflammation or infection (Castle 2000). Monocytes are mononuclear phagocytes circulating in the blood, and macrophages are cells matured from the monocytes, now residing in the tissues (Ransohoff & Cardona 2010; Gleeson & Bosch 2013). Macrophages also take part in the regulation of immune function by producing cytokines (Castle 2000).

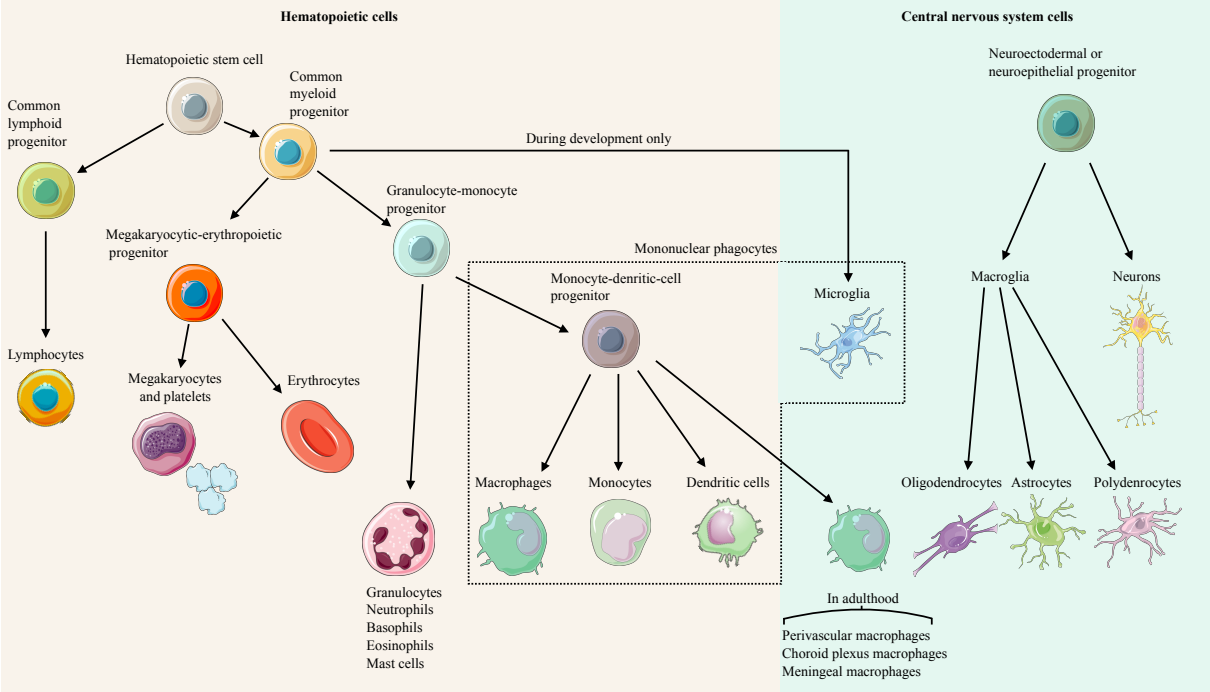

FIGURE 3. Origin and differentiation of blood cells and glial cells. Cells of the hematopoietic system and central nervous system (CNS) are depicted with arrows indicating lineage relatedness. Microglia are the only hematopoietic cells found in the CNS. (Perivascular, choroid plexus and meningeal macrophages are of unknown origin, and theirs progenitor is speculative.) (Adapted from Ransohoff & Cardona 2010; images from SmartServier s.a..)

Dendritic cells ingest pathogens and antigens in the tissues, and migrate to local secondary lymphoid organs via lymphatic vessels (Mueller et al. 2013). There dendritic cells present antigens to T cells thus marking foreign material to be removed and activate T cells (Guyton & Hall 2006, 439–450; Ransohoff & Brown 2012; Gleeson & Bosch 2013; Mueller et al. 2013). This makes dendritic cells important for initiating adaptive immune response (Ransohoff & Cardona 2010).

2.3.4. Lymphocytes

The acquired immune system is based on lymphocytes, which can be divided into T cells, B cells, and NK cells. All lymphocytes are initially similar, when formed from the pluripotent hematopoietic stem cells. After formation, they are released into circulation in immature form. Portion of these immature lymphocytes ends up in the thymus, where they mature to T cells. In the maturation phase the T lymphocytes divide rapidly, and each cell ”specializes” for recognizing just one particular antigen (figure 1). These maturing T lymphocytes also undergo a strict selection process, in which only the T cells that are not reacting with body’s own cells, but are still sensitive to invaders, will survive. (Guyton & Hall 2006, 429–450; Yatim & Lakkis 2015.) A great number of different mature T cells provide diversity for reacting against different antigens. Matured T cells exit the thymus and spread to the body via circulation. Mature T cells are responsible for forming ”cell-mediated” immunity, meaning that T cells are important at killing viruses and other pathogens that are already entered their body host cells. Different types of T cells have different roles in the defense system. T memory cells are responsible for initiating secondary response against an invader. T helper cells assist in regulating the immune response by for example secreting various interleukins, and cytotoxic T cells are direct-attack cells capable of killing micro-organisms. (Guyton & Hall 2006, 439–450; Gleeson & Bosch 2013.) T cells proliferate upon exposure to the pathogen, but when the invader has been eradicated, most of the T-cells die, leaving only memory cells alive (Mueller et al. 2013).

While T lymphocytes mature in the thymus, B lymphocytes mature in the bone marrow, and are then transferred to the lymphoid tissue, where they remain dormant until entry of a foreign antigen. In lymphoid tissue, macrophages phagocytize the antigen and present it to adjacent B lymphocytes and T cells. T helper cells contribute to activate those B lymphocytes which are specific to the given antigen. Activated B lymphocytes then enlarge, differentiate to form plasmablasts, further evolve by proliferating endoplasmic reticulum and finally rapidly divide forming plasma cells. These mature plasma cells then secrete large numbers of invader-specific antibodies, reactive protein molecules capable of destroying the antigenic substance. Antibodies can tag an invading antigen to be captured and ingested by phagocytes. Other antibody

functions are neutralization of antigen by covering its active sites, lysing the membranes of antigen, agglutination large particles into surface of antigen and amplifying effects of the complement system mentioned before. (Guyton & Hall 2006, 429–450; Gleeson & Bosch 2013.)

In contrary to the always similar pathogen-response of innate immune system, there is a difference in repeated exposure to pathogens in acquired immune system. This is because of the ”memory cells,” clones of lymphocytes that are primed to respond faster on exposure to a previously encountered antigen (Gleeson et al. 2013). In other words, B memory cells are lymphocytes that have not matured all the way to plasma cells, but have stayed in the B lymphocyte phase. Memory T cells are mature, long-living T cells which have entered ”resting state” after last encounter with their specific pathogen. B and T memory cells are numerally and functionally superior to antigen-inexperienced cells present prior the infection (Mueller et al. 2013). In the second encounter with the same pathogen, these numerous memory cells can thus launch more potent antibody response than original B lymphocytes of the specific clone. (Guyton & Hall 2006, 429–450; Gleeson & Bosch 2013.)

NK cells are subgroup of lymphocytes that are considered to be part of innate immunity system. NK cells can recognize and destroy foreign cells, tumor cells and some infected cells. The receptors of NK cells are encoded into germ line and so they are naturally capable of recognizing structures of high-molecular-weight-glycoproteins expressed on virus-infected cells. NK can kill invaders by releasing cytolysin and perforin, which cause cell membrane to break and target cell to lyse or break. (Guyton & Hall 2006, 439–450; Gleeson & Bosch 2013.) The cellular activity of an individual NK cell decreases upon aging, but it is compensated by increase of the total amount of NK cells (Castle 2000).

Finally, co-operating with all the other aspects of the immune system, different kinds of circulating proteins form a complement system. Complement system is activated by acute phase proteins. Upon activation, normally in inactive form circulating proteins break up into their active forms. These active fragments further promote phagocytosis, release of leukocytes, in-

duce an inflammatory response and also kill bacteria by attaching and making them burst (Guyton & Hall 2006, 439–450; Gleeson & Bosch 2013).

2.4. Immune system of the brain

2.4.1. Special properties of the immune system of the brain

The brain has traditionally thought to be immune-privileged area because of its special properties, specifically because of blood-brain barrier (BBB) (Erickson & Banks 2018). BBB is a layer of endothelial cells covering brain microvessels (Wolburg et al. 2009; Abbott 2013; Ericson & Banks 2018). These endothelial cells form tight junctions and are surrounded by basal lamina (Wolburg et al. 2009; Sofroniew & Vinters 2010). BBB has many functions, including control of molecular traffic, keeping out toxins, and allowing immune surveillance and response with minimal inflammation and cell damage (Abbott 2013; Kohman & Rhodes 2013; Erickson & Banks 2018). BBB is critical for preventing direct exposure of cerebrospinal fluid and neural cells from potentially harmful molecules of the blood (García-Cáceres et al. 2019). BBB can also regulate its permeability, enhancing the extent of monocyte and macrophage infiltration to the brain during a central nervous system (CNS) inflammation (Flügel et al. 2001; Erickson & Banks 2018).

In addition to BBB, another speciality of the brain immune system is the lack of DCs. As a result, for example non-traumatic microinjection into the brain area with viruses or bacterial lysates will not initiate immune response, whereas the same immunogen causes brisk immune response in peripheral exposure alarming also CNS depot of antigen. This specialty may be for the protection of the neural cells from the detrimental inflammatory responses. Pathogens should also be already recognized and immune system alarmed in draining lymph nodes or spleen, before entering the CNS. (Ransohoff & Brown 2012. Different subtypes of macrophages are also parts of the defense system of CNS (Ransohoff & Cardona 2010), forming the second line of defense, if a pathogen gets past BBB (Wolburg et al. 2009).

2.4.2. Glial cells

Glial cells are subdivided into astrocytes, oligodendrocytes and microglia (Ogata & Kosaka 2002) and together they account more than half of the cells in mammalian nervous system (Zuchero & Barres 2015). Glial cells were at first thought to be just a support cells of neurons (Zuchero & Barres 2015), but now their role as a regulators and immune cells of the nervous system has become clearer (Fields et al. 2015).

In the brain, microglia are the resident macrophages and important immune cells (Aimone et al. 2014) functioning as inflammatory cells of the CNS (Imai & Kohsaka 2002; Ransohoff & Cardona 2010). Thus, some CNS disorders can be characterized by microglial-cell activation (Ransohoff & Cardona 2010). Microglial cells are of mesodermal origin, as they are developed from bone marrow-derived monocytes (Brockhaus et al. 1993; Chan et al. 2006). Microglial cells immigrate into the brain in early postnatal development in ameba-like form (Brockhaus et al. 1993). In healthy brains, mature microglia are in their ”surveillant” form, with branched processes and small somas, and they are continually palpating the environment (Chan et al. 2006; Ransohoff & Cardona 2010; Kettenmann et al. 2011). When microglial cell recognizes an invader or receives an activation signal, it transforms into its reactive form by enlarging the soma, and retracting the processes. This transformation makes it possible for microglia to move more freely towards the invader, and more ”amoeboid” form makes them capable of phagocytosis (Chan et al. 2006; Ransohoff & Cardona 2010). IL-1ß has a role in the activation of microglia (Hoogland et al. 2015), as it mediates the inflammation reaction from circulation to the CNS via MyD88-pathway without passing BBB (Krasnow et al. 2017). In addition, reactive microglia also express pro- and anti-inflammatory cytokines, growth factors, chemokines and neurotrophins (figure 4; Ransohoff & Cardona 2010; Kettenmann et al. 2011). For example, IL-1ß is produced and released by microglia (Monje et al. 2003; Lynch 2014).

In scientific purposes, microglia are most commonly activated with a dose or doses lipopolysaccharide (LPS) (Ransohoff & Cardona 2010; Aimone et al. 2014; Fernández-Calle et al. 2017). LPS are used to simulate microglial immune response, since LPS is an endotoxin that

gram-negative bacteria contains in their cell walls (Ransohoff & Cardona 2010; Aimone et al. 2014). Moderate activation of microglia after single dose of LPS takes 3 hours, and they return to the normal resting state after 7 days (Ransohoff & Cardona 2010). Reactive microglia is commonly identified using antibody against ionized calcium-binding adaptor molecule 1 (IBA1) (Imai & Kohsaka 2002; Olah et al. 2009; Hoogland et al. 2015; Fernandéz-Calle et al. 2017). IBA1 is a small protein expressed specifically in microglia (Ito et al. 1998; Imai & Kohsaka 2002). IBA1 protein expression is greatly enhanced after neural damage as a consequence of microglial activation (Ito et al. 1998; Imai & Kohsaka 2002) or after LPS treatment (Fernandéz-Calle et al. 2017).

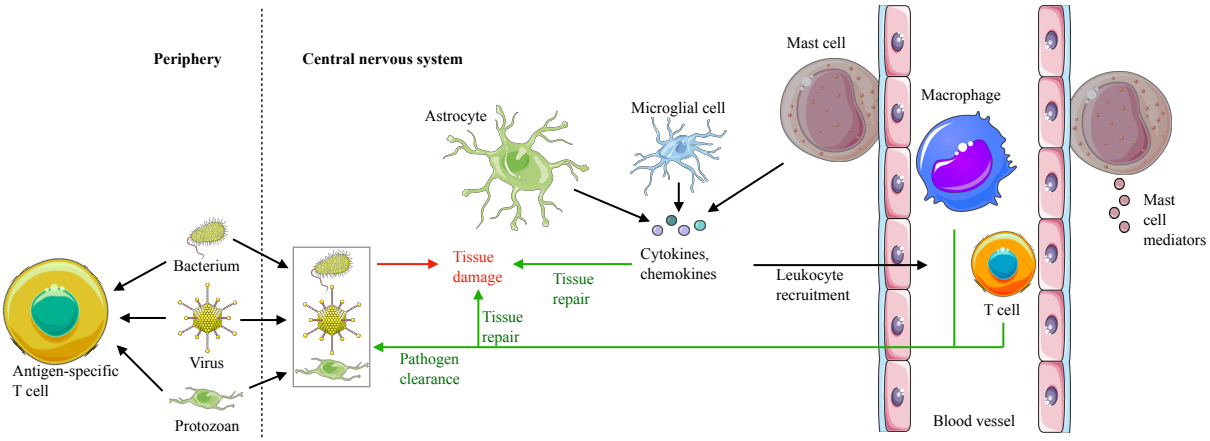

FIGURE 4. Innate immunity in the central nervous system. Pathogen enters the central nervous system and causes tissue damage. Resident microglia and astrocytes notice infection or injury. Cytokines and chemokines are expressed to promote the recruitment of circulating lymphocytes and myeloid cells from the periphery to assist in pathogen clearance. (Adapted from Ransohoff & Brown 2012; images from SmartServier s.a..)

2.4.3. Astrocytes and glial fibrillary acidic protein

Astrocytes are one of the major components of the CNS, being the most abundant cell type (Ogata & Kosaka 2002; Zuchero & Barres 2015). Each astrocyte has a very branched structure connecting to other astrocytes via gap junctions and covering many neurons (Ogata & Kosaka 2002; Halassa et al. 2007; Sofroniew & Vinters 2010) and thus astrocytes cover the entire CNS in very structured way (Sofroniew & Vinters 2010). Astrocytes have been traditional-

ly considered as supportive glial cell components of the neural tissue but their role in variety of complex functions in the CNS are gradually becoming clearer (Sofroniew & Vinters 2010; Zuchero & Barres 2015). One essential function of astrocytes is a regulation of proliferation, survival and differentiation of neuronal cells (Wilhelmsson et al. 2012; Zuchero & Barres 2015). They also control and regulate synaptic transmission (Oliet et al. 2001; Chung et al. 2015; Zuchero & Barres 2015). Finally, astrocytes contribute to immune defense of CNS by carrying different PRR's, such as TLRs, and thus alert the CNS of danger signals (Farina et al. 2007).

Glial fibrillary acidic protein (GFAP) is the main protein of the astrocytes cytoskeletons' intermediate filament network (Middeldorp & Hol 2011). GFAP, as other intermediate filament proteins, provides mechanical support for the plasma membrane of the astrocyte cells (Middeldorp & Hol 2011). GFAP functions also in cell motility and migration, proliferation, has a role in vesicle trafficking and synaptic plasticity, contributes to myelination and plays a role in protection and response to injuries (Ogata & Kosaka 2002; Middeldorp & Hol 2011; Parpura et al. 2012; Brenner 2014). GFAP, along with another intermediate filament protein vimentin, has been also proposed to mediate cell-cell signaling between astrocytes and neuronal cells, thus regulating the neural differentiation process (Wilhelmsson et al. 2012). GFAP has been used as a marker for astrocytes, since it is known to be induced and highly regulated upon brain damage (Sofroniew & Vinters 2010; Middeldorp & Hol 2011; Hol & Pekny 2015) and after LPS treatment (Fernández-Calle et al. 2017). Upregulation of GFAP may inhibit neurite growth and replenishment of neurons in some situations (Brenner 2014). However, GFAP is not an absolute marker for all astrocytes, since it is often not detectable in immunohistochemical stainings of healthy CNS tissue (Ogata & Kosaka 2002; Sofroniew & Vinters 2010).

3. SYNAPTIC PLASTICITY AND HIPPOCAMPAL NEUROGENESIS

3.1. Synaptic plasticity

Memory functions can be divided into three stages: formation, storage and recall of the memory. The recall stage is usually launched as a response to some environmental cue, and the situation in which the memory is recalled leads to adjustments for the memory, thus making learning and adaptation processes possible. (Koehl & Abrous 2011.) Even though the specific mechanisms of the memory functions are not yet solved, it is clear that the memories are stored in the brain and modified due to the plasticity of the synapses (Singh & Abraham 2017). Synaptic plasticity in simplified form means all the changes in the synapse that affect the efficiency of the synaptic function (Levenson et al. 2009).

Synapses are cell-cell adhesions between axon of one neuron and dendrite of other neuron, and synapses are key structures in controlled signal transfer in complex networks of the brain (Petzoldt & Sigrist 2014; Baldwin & Eroglu 2017). Synaptogenesis means synapse formation and maintenance processes (Petzoldt & Sigrist 2014). Astrocytes can regulate synaptogenesis and synaptic transmission by secreting factors that enhance forming of synapses (Chung et al. 2015; Singh & Abraham 2017). Regulation of the strength of excitatory and inhibitory synaptic inputs in response to neuronal stimuli through various pre- and postsynaptic mechanisms is a form of synaptic plasticity (Chung et al. 2015; Tatti et al. 2017). Proinflammatory IL-1ß impairs synaptic plasticity when released in large amounts, but it also seems to be necessary for the regulation of synaptic function in low concentrations (Lynch 2014). Abnormalities in synaptic transmission can lead to many neurodevelopmental and neuropsychiatric disorders (Petzoldt & Sigrist 2014; Tatti et al. 2017).

The synapsins are a family of phosphoproteins (Menegon et al. 2006) that regulate the kinetics of neurotransmitter release in synapses (Li et al. 1995; Hilfiker et al. 1999; Cheng et al. 2018). Synapsin-1 (SYN-1) is a protein found in a synaptic vesicles in the nerve endings (Huttner et al. 1983), and it functions in recycling the pool of synaptic vesicles (Menegon et al. 2006; Easley-Neal et al. 2013). SYN-1 connects synaptic vesicles to the nerve terminal

cytoskeleton in its dephosphorylated state, and its phosphorylation causes synaptic vesicles to dissociate from cytoskeleton making them available for releasing (Jovanovic et al. 1996; Menegon et al. 2006). Thus lowering the amount of available SYN-1 reduces ability of neuron terminals to provide synaptic vesicles for neuronal signaling in long trains of action potentials (Menegon et al. 2006). Synapsin deficient mice are more prone to epilepsy-like seizures than wild type mice (Li et al. 1995; Cesca et al. 2010). A mutation in the synapsin-expressing gene has been connected to epilepsy also in humans (Garcia et al. 2004). On the other hand, aerobic exercise increases the amount of SYN-1 compared to sedentary counterparts (Vaynman et al. 2006). SYN-1 is used as pre-synaptic marker protein (Menegon et al. 2006; Jackson et al. 2016).

Synaptophysin (SYP) is a protein of synaptic vesicles (Thiele et al. 2000; Vaynman et al. 2006). SYP is present in areas where also SYN-1 is expressed (Fletcher et al. 1991), and high expression of SYP correlates with large amounts of expressed SYN-1 (Vaynman et al. 2006). Endocytosis itself does not require SYP, but SYP is required for kinetically efficient synaptic vesicle retrieval after sustained stimulation and during prolonged neuronal activity (Daly et al. 2000; Kwon & Chapman 2011). The role of SYP is to maintain endocytic capacity in synapses (Kwon & Chapman 2011). Exclusive localization to synaptic vesicles makes SYP reliable and widely used marker for pre-synaptic terminals in research (Sarnat & Born 1999; Kwon & Chapman 2011). SYN-1 and SYP are both found in the hippocampal area, and the expression of these pre-synaptic proteins can be promoted by physical exercise (Gutierres et al. 2018a) and their levels are naturally elevated during development of the cells of hippocampal cells (Fletcher et al. 1991).

3.2. Hippocampal neurogenesis and influence of behaviour on it

The medial temporal lobe is essential for declarative memory (Squire & Zola 1996), the knowledge of events, people, things and other facts (Squire & Zola 1996; Koehl & Abrous 2011). Especially hippocampal area is crucial for memory formation, and damage in the area may cause anterograde amnesia, decreased ability to learn new things (Squire & Zola 1996). Increased volume of hippocampi has been connected to increased use of spatial memory such

as food-hiding behaviour of kangaroo rats (Jacobs & Spencer 1994) and increased gray matter volume of posterior hippocampi of London taxi drivers (Maguire et al. 2000; Maguire et al. 2006).

Adult hippocampal neurogenesis has been known to exist in mammals from at least 1960's, when the differentiation of neural cells in hippocampal area in postnatal rats was showed (Altman & Das 1965). Hippocampus has since been the area of great interest (Hoogland et al. 2015), and to the present day it has been proven that most of the neurogenesis happens in the prenatal phase and at the time of birth, but it does continue at slowed rate throughout the postnatal phase (Amrein et al. 2011). Adult hippocampal neurogenesis is, according to current knowledge, limited to two regions: subgranular zone of dentate gyrus of hippocampus, and lateral ventricles (Parpura et al. 2012; Aimone et al. 2014). Many of the new hippocampal cells die before they mature (Dayer et al. 2003), but surviving new cells that make connections with existing neural networks become functional units in the brain (van Praag et al. 2002; Dayer et al. 2003; Koehl & Abrous 2011). Hippocampal neurogenesis normally reduces with increasing age (Encinas et al. 2011). The rate of neurogenesis is affected by several behavioural and environmental aspects (figure 5; Aimone et al. 2014).

Inflammation in the brain inhibits the continuous formation of the new neurons in the "normal" undamaged brain, but on the other hand inflammation status can also promote neurogenesis after a brain lesion or radiation therapy (Ekdahl et al. 2003; Monje et al. 2003). Activation of microglia decreases survival of the new hippocampal neurons (Ekdahl et al. 2003; Butovsky et al. 2006). On the other hand, microglia can express anti-inflammatory cytokine that is able to promote neural stem cell differentiation and thus neurogenesis (Battista et al. 2006). Microglia also induce neural cell renewal in the adult brain (Butovsky et al. 2006). Overall, microglia can either promote or inhibit hippocampal neurogenesis, depending on the degree of microglial activation and cytokine expression (Butovsky et al. 2006). Astrocytes take part in controlling the integration and survival of newly formed neurons (Parpura et al. 2012). They regulate hippocampal neurogenesis by releasing ephrin-B2, which activates ß-catenin signaling in neural stem cells, increasing the expression of proneural transcription factors and guiding neuronal differentiation (Song et al. 2002; Lie et al. 2005; Ashton et al. 2012).

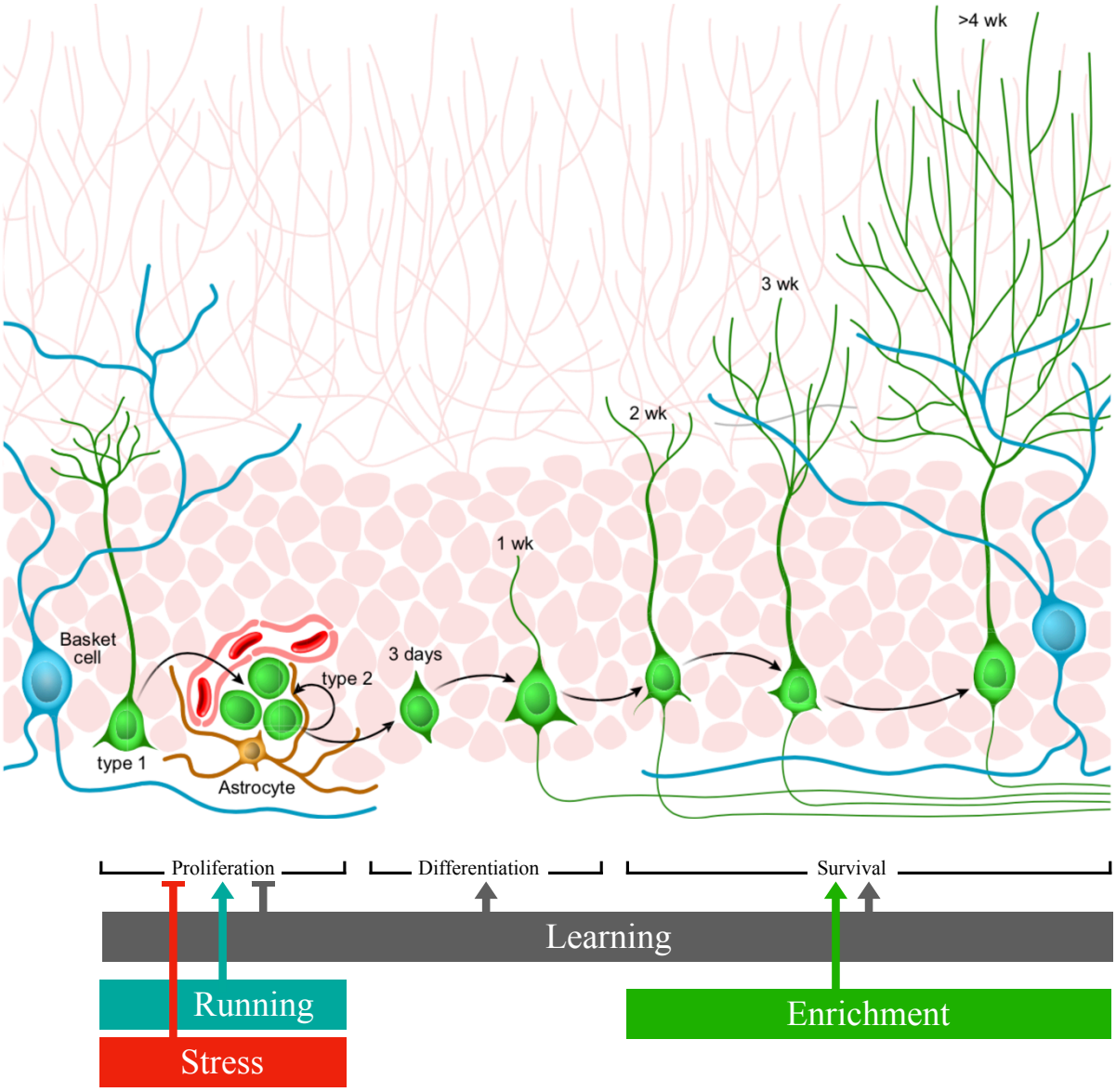

FIGURE 5. Neurogenesis and its regulation by behavior. Granule cells of dentate gyrus develop from stem cells to functional neurons in a timescale of about four weeks, but the growing and maturation phase continues also after that. Running promotes proliferation of the neural progenitor cells, while stress is detrimental to the process. Enriched environment increases survival of the neurons in their maturation phase. Learning may suppress the proliferation phase, but promotes later the differentiation and survival of neural cells. (Adapted from Aimone et al. 2014.)

Voluntary aerobic exercise in a running wheel promotes neurogenesis in rats (Trejo et al. 2001; Nokia et al. 2016) and mice (van Praag et al. 1999; Fabel et al. 2009; Olah et al. 2009; Bolz et al. 2015). Exercising elevates the expression levels of insulin-like growth hormone

and vascular endothelial growth factor, which increase hippocampal neurogenesis of rats (Åberg et al. 2000; Trejo et al. 2001; Fabel et al. 2003). Along with the voluntary exercise, also different forced motor tasks can increase synaptic density in hippocampal area (Moser 1999). Motor skill training in acrobatic course improves acrobatic performance (Black et al. 1990; Kleim et al. 1996; Lee et al. 2007; Garcia et al. 2012) and also facilitates restoration of functional capacity after brain lesion (Jones et al. 1999) in rats. Acrobatic skill training increases synaptic density in motor cortex (Kleim et al. 1996; Jones et al. 1999) and spine density and length of cerebellar Purkinje neurons (Black et al. 1990; Lee et al. 2007).

Survival of the newborn hippocampal neurons can also be promoted by training spatial skills for example in Morris water maze (Trouche et al. 2009). Morris water maze was introduced in early 1980s for testing spatial memory of rats (Morris 1981), and has since been popular tool for testing spatial learning and memory in rodents (D'Hooge & Deyn 2001; Cao et al. 2004; Barnhart et al. 2015). Spatial skill training promotes the integration of newborn neural cells to the existing neural networks that contribute to the spatial memory function (Trouche et al. 2009). Correspondingly, decrease in the adult hippocampal neurogenesis in mice led to reduced performance in Morris water maze (Goodman et al. 2010).

In addition to training, enriched environment provides sensory, social and motor stimulation (Aimone et al. 2014) and it alone has been proven to induce neurogenesis (Kempermann et al. 1997; Cao et al. 2004; Fabel et al. 2009; Neidl et al. 2016) and synaptogenesis (Moser 1999). The neurogenesis-promoting effect is even greater, if enriched environment is combined with voluntary physical activity such as wheel running (Fabel et al. 2009).

4. WESTERN BLOT AS A TOOL FOR IMMUNOLOGICAL STUDY

4.1. Western blot

Western blot is a technique developed in the 1970s (Towbin et al. 1979). It has since become a common method in biomedical research, and is used worldwide in daily basis (Mahmood & Yang 2012; Ghosh et al. 2014; Gilda et al. 2015). By western blotting one can identify a single protein of interest in a complex mixture even if expressed in low quantities (Ghosh et al. 2014; Danbolt et al. 2016), and western blotting can also be used to simultaneously resolute multiple immunogenic antigens (Kurien & Scofield 2006). These traits along with reasonable cost of time and money have resulted western blotting to be one of the most popular methods in the world's cellular and molecular laboratories (Ghosh et al. 2014). Western blot is used, for example, to study the regulation of neurogenesis (Wilhelmsson et al. 2012), immunological signaling (Park et al. 2006; Piao et al. 2013), differences in the strains and diets of rats (Novak et al. 2010; Morris et al. 2017) and the effects of different training regimens for different rat groups (Haram et al. 2009; Garcia et al. 2012; Park et al. 2017; Zidon et al. 2018).

Western blot method in general consists of a number of steps, and nowadays it can be executed in a couple of hours (Silva & McMahon 2014). Before the western blotting itself, the samples must be prepared and protein concentration measured (Taylor et al. 2013; Gilda et al. 2015). Then proteins are separated by electrophoresis, commonly using sodium dodecyl sulfate — polyacrylamide-gel electrophoresis (SDS-PAGE) (Alberts et al. 2008, 517–518; Nelson & Cox 2008, 88–90). After electrophoresis proteins are transferred to nitrocellulose membrane in the actual blotting phase (Mahmood & Yang 2012). Then membranes are exposed to antibodies, that is "labeling" of the proteins of interest in the sample, and the membranes can be visualized for example by a film or a charge-coupled device (CCD) (Degasperi et al. 2014; Ghosh et al. 2014). Finally, obtained images can be quantified using appropriate software to get numeral, comparable results of the amounts of the proteins of interest (Gilda et al. 2015).

Research has been done to validate western blotting and suggestions have been given to fine-tune the method to be more authentic (Wu et al. 2014; Gilda et al. 2015; Gorr & Vogel 2015). Still, these validations focus mainly on quality of antibodies (Gilda et al. 2015) or overall methodology (Wu et al. 2014; Gorr & Vogel 2015; Bass et al. 2017). Moreover, the validation studies have touched the differences between different devices (Gassman et al. 2009) or between the persons executing the quantification procedure (Gallo-Oller et al. 2018).

4.2. Devices and software for western blot imaging and quantification

Traditional method for image acquisition requires X-ray film to which the blots are exposed in dark for a period of time (Towbin et al. 1979; Degasperi et al. 2014; Ghosh et al. 2014). For shorter overall duration of the western blotting and for the ability to easier digital processing of the results, office scanners and CCD imager systems are used for image acquisition (Gassmann et al. 2009; Silva & McMahon 2014; Ghosh et al. 2014). However, normal office scanners have not been recommended, because they provide poor linear dynamic range, meaning that devices are capable of sensing only few samples in dilution series of increasing amounts of proteins before the results become saturated (Gassmann et al. 2009; Degasperi et al. 2014). CCDs have thus proven to be more suitable for image acquisition in western blotting compared to X-ray films or office scanners (Gassmann et al. 2009; Degasperi et al. 2014; Ghosh et al. 2014).

ChemiDoc™ and ChemiDoc™ MP imaging systems have been introduced by Bio-Rad laboratories as a practical and reliable CCDs for western blotting (Posch et al. 2013; Taylor et al. 2013). Since that, they have also been used in research (Tan et al. 2014; Lerner et al. 2016; Hunt et al. 2018; Yermakov et al. 2018) and also in some validation studies (Taylor et al. 2013; Ghosh et al. 2014; Gilda et al. 2015). Odyssey CLx (LI-COR) imaging system has also been used in research as a CCD for the visualization of western blotting results in recent years (Collins et al. 2015; Pekkala et al. 2015; Song et al. 2017; Koch et al. 2018).

Quantification of western blot results begun soon after the western blotting method was invented. At first, quantification was mostly of confirming the correlation between blot density

and loaded antigen (Lin et al. 1985). Now, decades later, method is usually called ”semi-quantitative” allowing simultaneous analysis of different protein on the same membrane. Thus, the method provides comparable, numerical values as results but is still being somewhat dependent on the exact methodology, devices and software used (Gassmann et al. 2009; Heidebrecht et al. 2009; Gorr & Vogel 2015). Despite semi-quantitative nature, western blots can still be executed with good reproducibility at present (Gallo-Oller et al. 2018). Accurate densitometric analysis is possible with Image Lab -software (Taylor et al. 2013) and it has been used in research (Posch et al. 2013; Taylor et al. 2013; Osier et al. 2018; Yermakov et al. 2018). Also Image Studio™ is commonly used software for quantification (Collins et al. 2015; Pekkala et al. 2015; Song et al. 2017; Koch et al. 2018).

In the research papers, the information of used software, the principles of software use for quantification, and the settings used are generally poorly presented (Gassmann et al. 2009), that compromises the credibility of the whole study (Gilda et al. 2015). In addition, validation and reliability studies for devices used in image acquisition and quantification are rare, if they exist at all. The same stands for intra-user reliability, which has seldom been tested (Gallo-Oller et al. 2018).

5. RUNNING CAPACITY OF RATS

Running capacity is dependent of biomechanical, anthropometrical and physiological factors, such as running efficiency, body weight and maximal oxygen uptake (Joyner 1991; Lundby et al. 2017; Thompson 2017). Genetics (Barbato et al. 1998; Koch & Britton 2001; Almeida et al. 2014; Nokia et al. 2016) and training (Rossiter et al. 2005; Almeida et al. 2014; Nokia et al. 2016) play big parts in defining the level of individual's running capacity. Also behavioral aspects are to be taken into account, as for example tenth generation of mice artificially selected for their voluntary wheel-running behavior show marked increase in voluntary wheel-running tendency when compared to randomly bred mice (Swallow et al. 1998a).

There are differences in innate aerobic running capacities between different inbred lines of rats (Barbato et al. 1998; Koch et al. 2005; Almeida et al. 2013). For studying the effects of these differences for health, Koch and Britton (2001) have produced two strains of rats artificially selected for their running capacity. These lineages started from heterogenous N:NIH stock rats, whose innate endurance running capacity were tested. After testing, rats achieving the best results were used for breeding for high capacity running strain, and correspondingly those rats who ran the smallest distance, were selected for the founders of low capacity lineage. In subsequent generations, the "best" or correspondingly "worst" male and female runner in each rat family has been selected for breeding to increase the running capacity difference between high-capacity runners (HCR) and low-capacity runners (LCR) strains. The mating couples have been rotated in each generation to minimize inbreeding. (Britton & Koch 2001; Koch & Britton 2001.) After over 30 generations of artificial selecting, the difference in intrinsic running capacity is more than 10-fold between the two strains (Vieira-Potter et al. 2015; Cooper et al. 2017) when tested in ramping treadmill protocol (Koch & Britton 2001).

HCR rats have greater aerobic capacity and thus they are capable of achieving greater working rates in running compared to the LCR rats (Gonzalez et al. 2006). LCR rats have higher body mass than age-matched HCR rats (Henderson et al. 2002; Beighley 2013; Pekkala et al. 2017), but HCR rats have greater heart mass and maximal cardiac output in relative to the body mass (Gonzalez et al. 2006). Also, HCR rats have higher tissue-level capacity for oxy-

gen transport (Henderson et al. 2002; Gonzalez et al. 2006), greater capillary density, better perfusion capacity in microvessels (Beighley et al. 2013) and increased oxidative enzyme activity muscles (Howlett et al. 2003; Wisløff et al. 2005) compared to LCR rats. Architecture of the microvasculature has still stayed unchanged between high and low capacity runner rats (Beighley et al. 2013). Overall maximal oxygen uptake ($VO_{2\ max}$) is higher (Henderson et al. 2002; Gonzalez et al. 2006), and oxygen expenditure during walking is lower (Novak et al. 2010) in HCR rats compared to LCRs. HCR rats are also more active than LCR rats when measured in voluntary wheel running (Karvinen et al. 2016b; Park et al. 2016). As an overall effect of high running capacity and its beneficial aspects, HCR rats live even 45 % longer than do LCR rats with similar sedentary lifestyle (Koch et al. 2011).

An individual's aerobic capacity is affected by genetics, but also habits such as exercising play a big role (Koch et al. 2005). For example treadmill training in the maximal lactate steady state speed induces training adaptations and increases aerobic capacity within weeks of training (Almeida et al. 2014). Also high-intensity interval training (Wisløff et al. 2001; Wisløff et al. 2005; Nokia et al. 2016), submaximal running protocols (Koch et al. 2005; Haram et al. 2009) and voluntary wheel-running promote aerobic capacity in rats (Nokia et al. 2016) and mice (Swallow et al. 1998b). Improvement in aerobic capacity is mainly due to the improvement in VO_{2max} relative to the body weight (Swallow et al. 1998b; Wisløff et al. 2001; Wisløff et al. 2005). Besides the effects to aerobic capacity, exercising can also protect against inflammation in adipose tissue (Peppler et al. 2017).

In addition to forced exercising with treadmill or voluntary wheel-running, spontaneous physical activity of rats is commonly studied. Spontaneous physical activity is usually measured by video-analyses (Brodkin et al. 2014), infra-red beams (Smyers et al. 2014) or force-plate systems (Karvinen et al. 2016b). HCR rats are more active compared to LCR rats when measured in spontaneous physical activity (Novak et al. 2009; Novak et al. 2010; Smyers et al. 2014; Karvinen et al. 2016b) and also expend more energy relative to the body weight (Novak et al. 2009; Novak et al. 2010). Higher energy expenditure may be due to higher body temperature of HCR rats compared to LCR rats (Karvinen et al. 2016a).

6. RESEARCH QUESTIONS AND HYPOTHESES

1. **Does greater aerobic capacity of HCR rats decrease the expression of inflammation markers (TLR3, TLR5, IL-1ß, IBA1, GFAP) in hippocampus compared to LCR rats?**

Yes. LCR rats have impaired regulation and resolving mechanisms of inflammation reactions which suggests higher inflammatory status of LCR rats compared to HCR rats (Su et al. 2013; Feng et al. 2015). Low aerobic capacity is also connected to poor overall health and increased inflammation status (Bowden-Davies et al. 2015; Cooper et al. 2017), while exercising has anti-inflammatory effects (Pedersen & Febbraio 2005 & Petersen & Pedersen 2005). Passive lifestyle human subjects have elevated inflammation levels and pronounced immune responses compared to active counterparts when tested with LPS stimulation (McFarlin et al. 2006), suggesting a correlation poor aerobic capacity and chronic inflammation status.

2. **Do acrobatic training rats have lower expression of inflammation markers (TLR3, TLR5, IL-1ß, IBA1, GFAP) in hippocampus compared to control rats?**

No. According to the PubMed searches (acrobatic training; rats / acrobatic training; mice) executed 4.5.2019, total of 26 studies were found, one of which was review of effects of acrobatic exercise on brain plasticity (Gutierrez et al. 2018b) and none considered inflammation markers. Acrobatic training may enhance recovery of function after brain insult, but the mechanisms proposed were linked to the synaptogenesis, not to the reduction of inflammatory agents (Jones et al. 1999). It is likely that low metabolic activation due to the acrobatic training is not comparable with high metabolic activation of aerobic exercise (Black et al. 1990), and thus does not induce reaction in inflammation markers.

3. **Does greater aerobic capacity of HCR rats increase the expression of markers for synaptic plasticity (SYN-1, SYP) in hippocampus compared to LCR rats?**

Yes. Aerobic training increases expression of SYN-1 in motor cortex, cerebellum and striatum and also SYP in motor cortex, pre-frontal cortex and striatum of rats (Garcia et al. 2012; Gu-

tierrez et al. 2018a). Also Vaynman et al. (2006) showed that voluntary running increased expression of SYN-1 and SYP. Voluntary running promotes learning in pattern recognition task (Bolz et al. 2015). Aerobic training and higher aerobic capacity can be hypothesized to result similar outcomes, and the widely distributed changes in the brain should also reach the hippocampal area.

4. **Do acrobatic training rats have higher expression of markers for synaptic plasticity (SYN-1, SYP) compared to control rats?**

Yes. Acrobatic training has been shown to increase the expression of SYN-1 in cerebellum and in striatum, and also SYP in motor cortex, and in striatum of the rats (Garcia et al. 2012). Also Jones et al. (1999) found the increase in synaptic density of motor cortex of rats following acrobatic exercise.

5. **Do the results differ between two users of Bio-Rad quantification software?**

No. Gallo-Oller et al. (2018) found inter-observer Pearson's correlation coefficient to be over 0.97 ($p < 0.0001$) in two western blot analyses made with the same densitometer but with different scientists.

6. **Do the results differ between Odyssey and Bio-Rad imaging devices and quantification softwares?**

No. Gallo-Oller et al. (2018) compared Bio-Rad densitometer with office scanner and ImageJ-software (not designed for western blot quantification) and resulted Pearson's correlation coefficients around 0.6 ($p < 0.005$) in two different resolutions. As Odyssey and Bio-Rad imaging devices used in this thesis are utilizing similar hardware and software, difference should be smaller.

7. METHODS

The present thesis consists data from two different animal studies, one of which used high and low running capacity rats and the other Sprague-Dawley rats trained for acrobatic skills. Overview of the study design is presented in the figure 6.

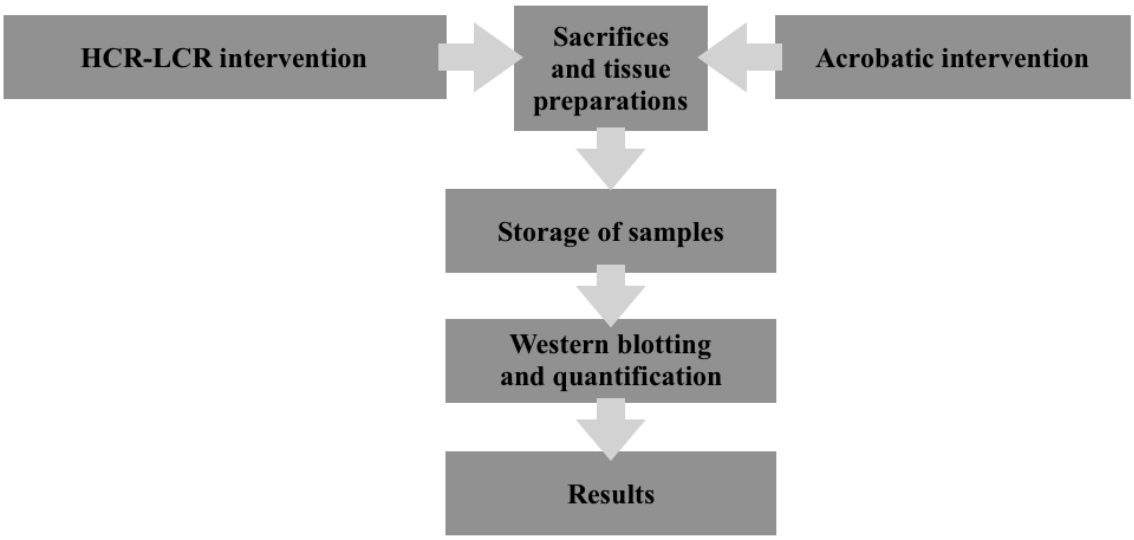

FIGURE 6. Overview of the study design.

7.1. Animals and sample preparation

High- and low running capacity rats. 19 HCR and 20 LCR male rats were divided to the groups of young (HCR: n = 8; LCR: n=10) and adult animals (HCR: n = 11; LCR: n = 10). Their parents were of the 35th generation of artificial selection for high and low aerobic capacity (Koch & Britton 2001). Animals lived sedentary life. Young group was euthanized at the age of 8 weeks. Adult group was euthanized at the age of 40 weeks, when rats were middle-aged. The group is called ”old” for differentiation from the young group. General characteristics of experimental animals is presented in table 1. For more detailed description, see Pekkala et al. (2017).

Acrobatic training rats. Total of 24 Sprague-Dawley male rats were divided to acrobatic training (Acro; n = 12) and control (Ctrl; n = 12) groups. Rats of Acro-group were trained for 8

weeks (3 times per week, 8 trials per session) in acrobatic training course with obstacles, and rats of Ctrl-group were handled correspondingly at the same track without obstacles. Rats lived sedentary life apart from training. Rats were euthanized at the age of approximately 22 weeks. General characteristics of experimental animals is presented in table 1. For more detailed description, see Honkanen (2017).

TABLE 1. General characteristics of the experimental animals at the time of sacrifice.

Group	n	Age (wk)*	Mean body mass (g)
Young HCR	8	8	178 ± 25
Young LCR	10	8	183 ± 23
Old HCR	11	40	377 ± 48
Old LCR	10	40	477 ± 43
Acro	12	22	451 ± 57
Ctrl	12	22	444 ± 68

* age is an approximation.

Tissue preparation. HCR and LCR rats were killed by cardiac puncture after stunning with rising CO_2 concentration. Animal brains were immediately extracted and left hippocampus was cut out, frozen with liquid nitrogen and stored in -80 °C. The tissue was later homogenized and hippocampal proteins extracted. For procedure, see Kontro (2016). Identical method was used for acrobatic training rats. The concentrations of hippocampal protein homogenates were determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, USA) according to manufactures instructions for microplate procedure (ThermoFisher Scientific 2013). Protein homogenates were stored at the temperature of -80 °C until they were used for western blotting.

7.2. Western blotting

Electrophoresis. 30 µg of total protein was diluted to milli-Q-H_2O to equalize protein concentration between samples. 6x sample buffer (30 % glycerol (Sigma-Aldrich G 5516,

Saint Louis, MO, USA), 15 % ß-mercaptoethanol (Sigma-Aldrich M3148), 20 % (w/v) SDS (sodium dodecyl sulfate) (Sigma-Aldrich 71725), 18.8 % (1 M, pH 6,8) Trizma base (Sigma-Aldrich T6066), 12 % (w/v) bromophenolblue, (Sigma-Aldrich B0126) was added. Samples were heated for 10 minutes at 95 °C, and shortly centrifuged (Biofuge Pico, Heraeus Instruments, Osterode, Germany) at 8000 rpm before loading to the gel (4–20 % Criterion™ TGX Stain-Free™ Precast Gels, Bio-Rad, Hercules, CA, USA). Samples from each group were placed to each gel, total of 12 - 15 samples per gel. The molecular weight standard (Precision Plus Protein™ All Blue Standards, Bio-Rad) was loaded to one well at the both ends of the gel comb. Electrophoresis chamber was filled with running buffer (0.3 % (w/v) Trizma base, 1.4 % (w/v) glycine (Sigma-Aldrich 50046), 0.1 % (w/v) SDS). Gel electrophoresis was executed by conducting 250 V voltage to the gel for approximately 50 minutes in cold room (+4 °C).

Blotting. After electrophoresis the gel was activated with ChemiDoc™ MP Imaging System (Bio-Rad) using default set up for stain free gel ultraviolet activation. Then the gel was balanced in blotting buffer (0.3 % (w/v) Trizma base, 1.4 % (v/w) glycine, 10 % methanol (Sigma-Aldrich 322415). Blotting sandwich was assembled as follows: (1) a sheet of scouring pad, (2) a layer of whatman paper, (3) the gel, (4) the membrane (Nitrocellulose Blotting Membrane, Amersham™ Protran™, GE Healthcare Life Science, Chicago, USA), (5) layer of whatman paper and (6) sheet of scouring pad. Air bubbles between the gel and membrane were removed using plastic roll when assembling layers. Blotting sandwich was placed to a plastic rack, and it was filled with blotting buffer. Proteins were transferred approximately 2.5 hours at 300 mA in the cold room (+4 °C) on ice and buffer solution was constantly stirred with magnetic stirrer.

Total protein and Ponceau S staining. After blotting, picture of the membrane was obtained using default set up for stain free blots with ChemiDoc™ MP Imaging System. Membrane was then stained with Ponceau S dye (0.2 % (w/v) Ponceau S (Sigma-Aldrich 141194), 5 % acetic acid (Sigma-Aldrich A6283) for couple of minutes until protein bands became visible. Ponceau S was washed away with milli-Q-H_2O.

Membrane cutting and primary antibody incubation. Membrane was cut either to four strips for TLR3, GFAP, IL-1ß and IBA1 or to three strips for TLR5, SYN-1 and SYP (table 2). The cut membranes were blocked for 1 hour at room temperature (RT) with Odyssey^{R} Blocking buffer (LI-COR 927-40000, Lincoln, NE, USA) with gentle rocking. Membranes were washed for 2 minutes using Tris-buffered saline (TBS; 0.1 % (w/v) Trizma base; 0.9 % (w/v) sodium chloride (NaCl, Sigma-Aldrich 746398). Primary antibodies were mixed with 50 % Odyssey^{R} Blocking buffer and 50 % TBS solution in 1:500, 1:700 or 1:1000 dilution according to table X. Primary antibodies were incubated with the membranes overnight in cold room (at +4 °C) with gentle rocking.

TABLE 2. Primary antibodies and margins for membrane strips.

Antibody	Size (kDa)	Dilution	Upper limit (kDa)	Lower limit (kDa)	Product number and manufacturer
TLR3	130	1:500	250	100	#SAB2900405-50UG, Sigma-Aldrich, Saint Louis, MO, USA
GFAP	55	1:5000	100	~45	#ab7260, Abcam, Cambridge, UK
IL-1ß	30	1:500	~45	~22	#AB1832P, Merck, Darmstad, Germany
IBA1	17	1:700	~22	10	#PA5-27436, ThermoFisher Scientific, Rockford, IL, USA
TLR5	100	1:500	250	75	#SAB3500374, Sigma-Aldrich, Saint Louis, MO, USA
SYN-1	60	1:1000	75	50	#ANR-014, Alomone labs, Jerusalem, Israel
SYP	40	1:1000	50	15	#ANR-013, Alomone labs, Jerusalem, Israel

Upper limit and lower limit columns indicate the margin used for cutting the membrane.

Secondary antibody and scanning. In the next morning membranes were washed 4 times 5 minutes with TBS-Tween (0.12 % (w/v) Trizma base, 0.9 % (w/v) NaCl, 0.1 % Tween 20 (Sigma-Aldrich P9416). The membranes were then incubated with IRDye® 800CW (LI-COR)

diluted 1:20 000 in 50 % Odyssey^{R} Blocking buffer and 50 % TBS for 1 hour in RT, protected from light and with gentle rocking. Afterwards the membranes were washed 4 times 5 minutes with TBS-Tween. Pictures from the membranes were obtained using ChemiDoc™ MP Imaging System with 60-second exposure time and Odyssey CLx (LI-COR) imaging system with default set up.

7.3. Quantification of western blots

Total protein quantification. Total amount of protein in each lane of membrane was determined from the stain-free image obtained right after the blotting phase. Quantification was made with Image Lab –software (version 6.0, Bio-Rad, Hercules, CA, USA). Total protein image was cropped so that the area for analysis covered proteins sized from 10 kDa to 250 kDa, which is the range of the molecular weight standard. Total lane protein volume was defined as total lane signal reduced with automatically detected background noise. The amount of total protein per lane was used as a correction factor for variances in total protein in wells.

Individual band quantification. Pictures of western blots obtained with ChemiDoc™ MP and Odyssey CLx (LI-COR Lincoln, NE, USA) imaging systems were quantified using manufacturers' own softwares: Image Lab 6.0 for ChemiDoc™ MP and Image Studio™ (version 5.2) for Odyssey CLx. The area and optical density of each individual blot was measured using the parameters of each software. The data was exported to and further analyzed in Excel 2016 (Microsoft, Redmond, WA, USA). The resulting signal number was divided with each lane's amount of total protein to normalize the results to the total protein loaded.

Normalization for final score. Average score for each group in each membrane was calculated. Then those average scores were divided with the average score of the first membrane for their group in order to get inter-membrane normalization factor. Finally, the score for each individual sample (the original number divided by total protein) was divided with this normalization factor for each group and membrane, resulting a final number that is corrected from the variances in protein amounts in wells and also with possible differences between the gels.

Inter-user variation. The whole quantification process from total protein quantification to normalization for final scores was executed independently by two persons. The same images obtained by ChemiDoc™ MP Imaging System and the same Image Lab software were used for quantification. Both persons followed the routine described above, and the comparison of the results was made between final scores. Variations in the results of SYN-1, IBA1 and SYP were studied. For comparison, the results were scaled from 0 to 1 using equation:

$$x' = \frac{x - \min(x)}{\max(x) - x(\min)}$$

where x' is normalized value, x is original value and min(x) and max(x) are lowest and highest values of the group.

7.4. Statistical analyses

Possible outliers from each group were identified using IBM SPSS Statistics. Any number exceeding 1.5 interquartile lengths from 75 percentile or respectively undercut 25 percentile by 1.5 interquartile lengths was defined as outlier and deleted from further analyses. In IBA1 results, one case in Acro group and two cases in Ctrl group were missing, and one case in Ctrl group was deleted as outlier. In IL-1ß results, one old HCR case was missing, and two cases from young LCR group and one case from old LCR group was deleted as outliers. In TLR3 results, two cases from young LCR group and one case in Ctrl group were deleted as outliers. In SYP results, one case from young LCR group and one case in Acro group were deleted as outliers. Final numbers of cases for each antibody is presented in table 3. Correlation graphs and Bland Altman -plots between the two devices and the two persons were made for methodological comparison. Two missing values were deleted pairwise from the analysis.

The normality of the data was tested with IBM SPSS Statistics 24 for Windows (Chicago, IL, USA) using Shapiro-Wilk normality test. Due to that the data were not entirely normally distributed, non-parametric tests were chosen. The group comparison were studied using non-parametric Mann-Whitney U test of independent samples. The p-value of 0.05 was chosen as the level of significance. Mean protein expression levels were compared between young HCR

(Yng-HCR) and young LCR (Yng-LCR) rats, Yng-HCR and old HCR (Old-HCR) rats, Yng-LCR and old LCR (Old-LCR) and between Old-HCR and Old-LCR. Acrobatic-trained rats were compared to their control group rats. Numerical values of HCR-LCR -study values and values of acrobatic intervention are not comparable, because results are not normalized. For methodology comparison, Spearman's rank correlation coefficient was used.

TABLE 3. Final number of cases (n) in each group for each antibody.

Antibody	Yng-HCR	Yng-LCR	Old-HCR	Old-LCR	Acro	Ctrl
SYN-1	8	10	11	10	12	12
TLR5	8	10	11	10	12	12
IBA1	8	10	11	10	11	9
TLR3	8	8	11	10	12	11
GFAP	8	10	11	10	12	12
IL-1ß	8	8	10	9	12	12
SYP	8	9	11	10	12	12

8. RESULTS

8.1. Inflammatory markers

The expression of TLR3 protein in hippocampal area of the young HCR rats (Yng-HCR) were lower than TLR3 expression of the old HCR rats (Old-HCR; $p = 0.036$). The young LCR rats (Yng-LCR) rats had lower TLR3 expression than the old LCR rats (Old-LCR; $p < 0.001$). The Old-HCR rats had lower TLR3 expression than the Old-LCR rats ($p = 0.036$). Also the acrobatic-trained (Acro) rats had lower TLR3 expression than their control group (Ctrl) rats ($p = 0.004$). Results of TLR3 expression are presented in figure 7.

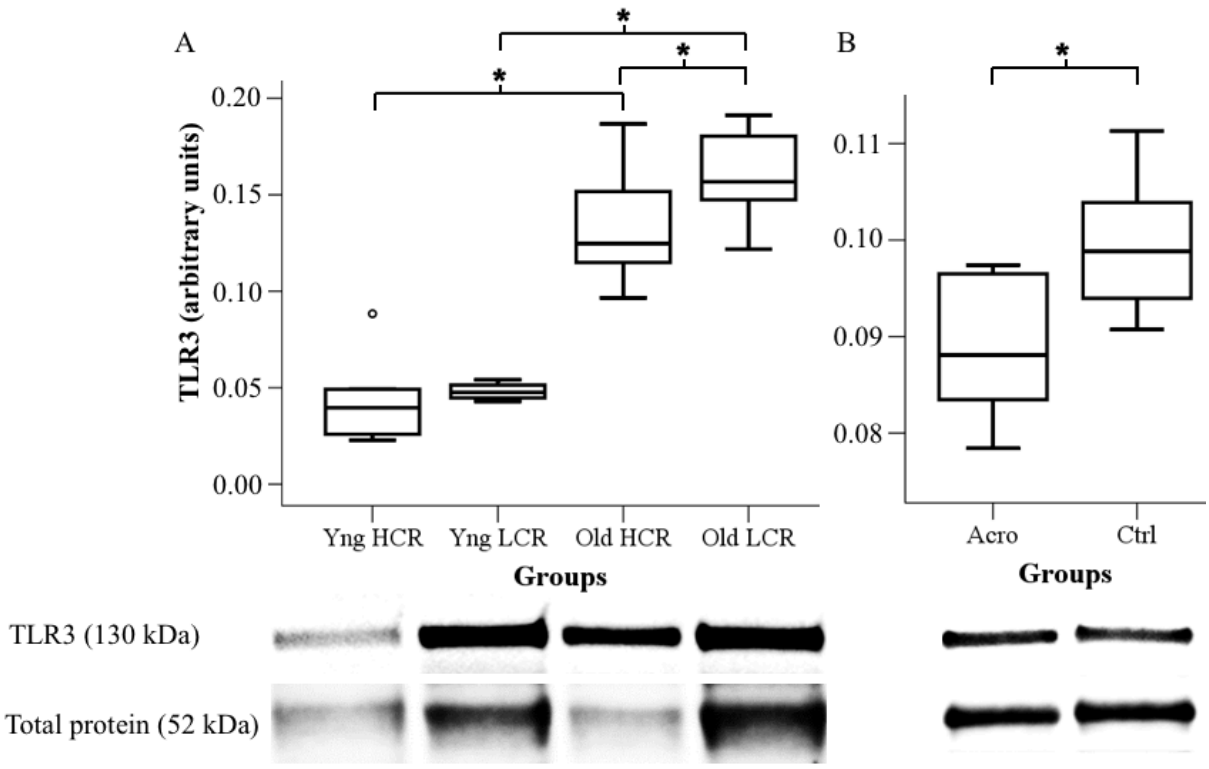

FIGURE 7. Toll-like receptor 3 expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. TLR3, Toll-like receptor 3; * $p < 0.05$; ° data point exceeding 1.5 interquartile lengths from 75 percentile. Values of A and B are not comparable.

The expression of TLR5 in Yng-HCR was lower compared to Yng-LCR (p = 0.21) and higher compared to Old-HCR (p = 0.33; figure 8). The Yng-LCR rats expressed more TLR5 than the Old-LCR rats (p = 0.009). There were no differences in TLR5 levels between the Acro and Ctrl groups.

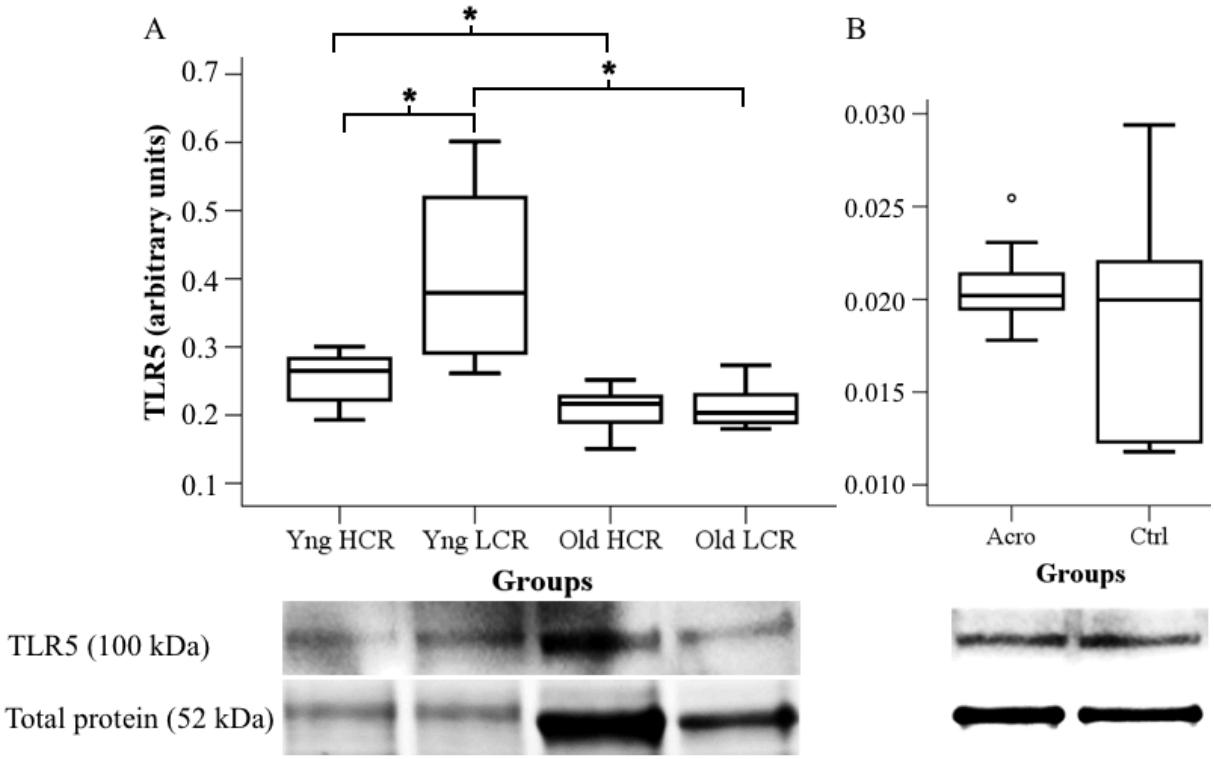

FIGURE 8. Toll-like receptor 5 expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. TLR5, Toll-like receptor 5; * p < 0.05; ° data point exceeding 1.5 interquartile lengths from 75 percentile. Values of A and B are not comparable.

The Yng-HCR rats had lower IL-1ß expression levels than the Yng-LCR (p < 0.001) or Old-HCR rats (p < 0.001). The Old-LCR rats had higher IL-1ß expression levels than the Yng-LCR (p = 0.002) or Old-HCR rats (p = 0.010). There were no differences in IL-1ß levels between the Acro and Ctrl rats. The results are presented in the figure 9.

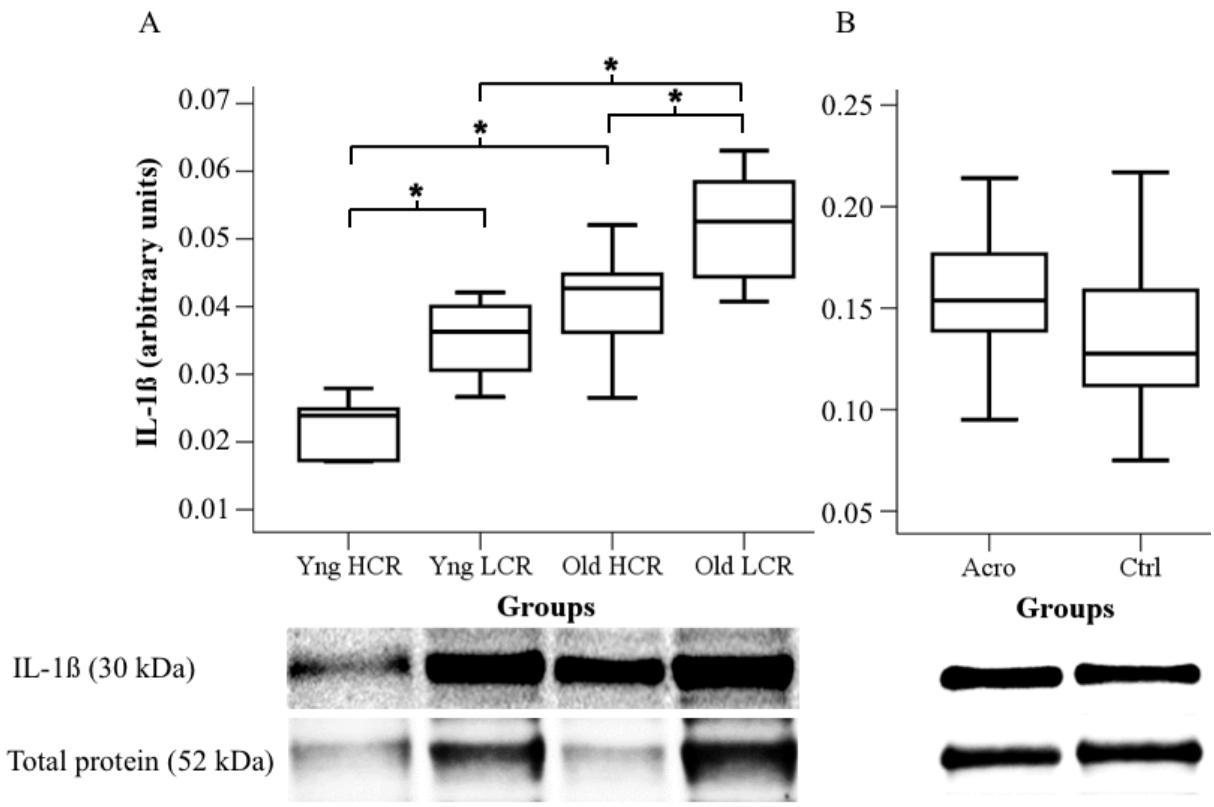

FIGURE 9. Interleukin-1ß expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. IL-1ß, interleukin-1ß; * $p < 0.05$. Values of A and B are not comparable.

IBA1 expression level of the Old-HCR group was lower than that of the Yng-HCR group ($p = 0.005$) or the Old-LCR group ($p = 0.010$). There were no differences in IBA1 levels between the Acro and Ctrl rats. The results are expressed in the figure 10.

The expression GFAP was higher in Yng-HCR than in the Old-HCR rats ($p < 0.001$). GFAP levels of Old-LCR were lower than in Yng-LCR ($p = 0.009$) but higher than in the Old-HCR rats $p = 0.006$). There were no differences in GFAP levels between the Acro and Ctrl rats. The results are expressed in the figure 11.

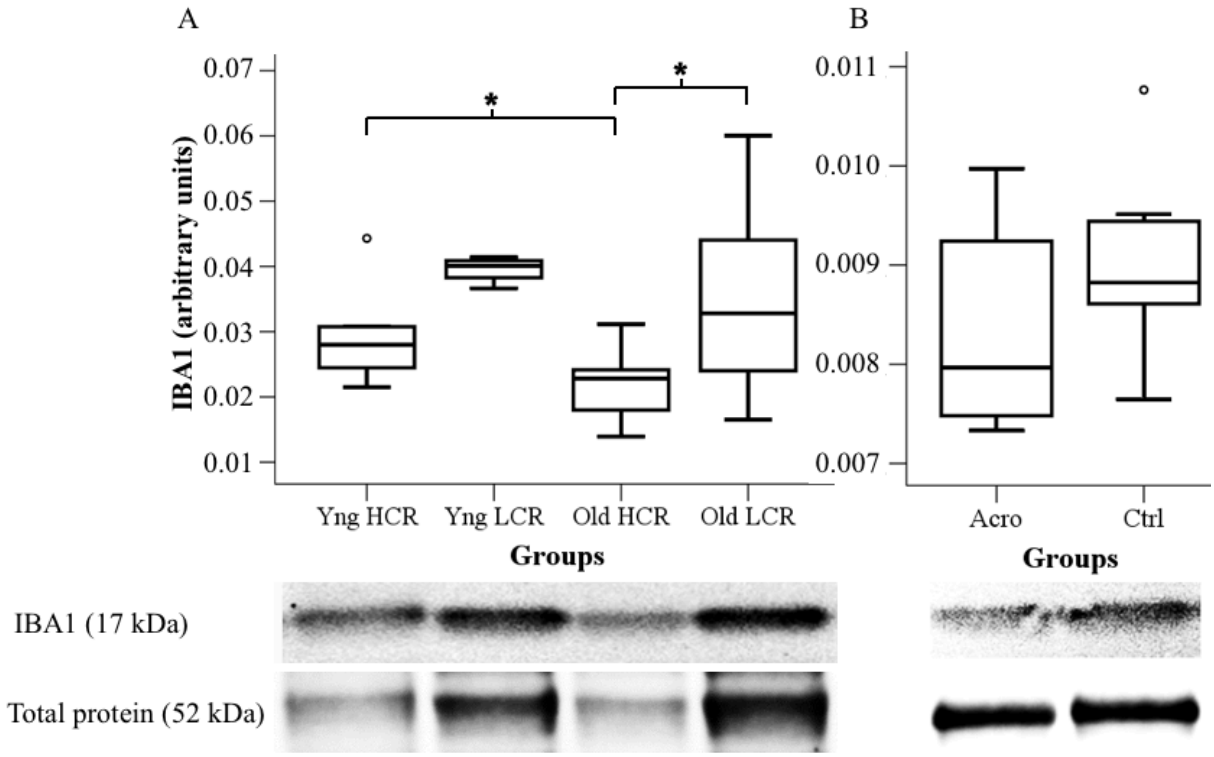

FIGURE 10. Ionized calcium-binding adaptor molecule 1 expression of HCR-LCR rats. * $p < 0.05$; ° data point exceeding 1.5 interquartile lengths from 75 percentile. Values of A and B are not comparable.

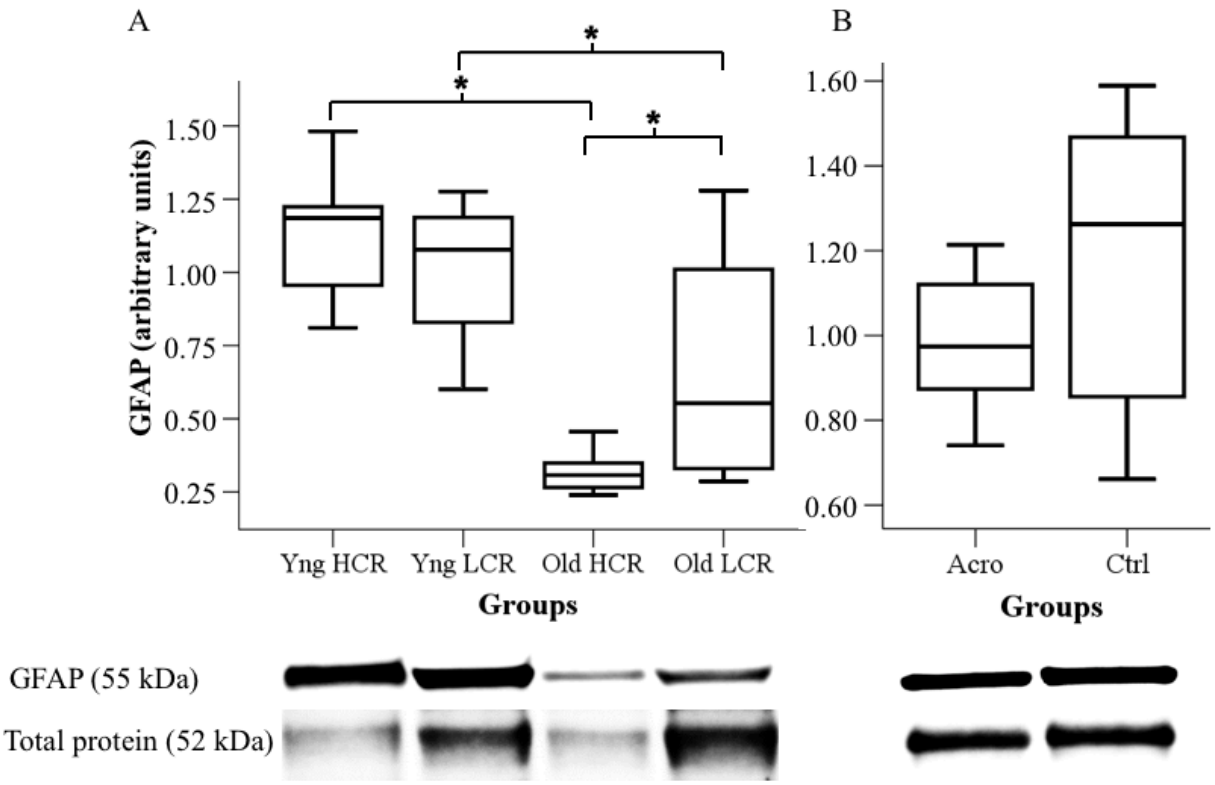

FIGURE 11. Glial fibrillary acidic protein expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. GFAP, glial fibrillary acidic protein; * $p < 0.05$. Values of A and B are not comparable.

8.2. Synaptic plasticity

The expression of SYN-1 was higher in the Yng-LCR group than in the Yng-HCR ($p < 0.021$) or Old-LCR rats ($p < 0.001$). The results are presented in the figure 12. SYP expression (figure 13) of the Yng-HCR rats was higher than in the Yng-LCR ($p = 0.011$) or Old-HCR rats ($p = 0.003$). The Old-LCR rats had lower SYP expression compared to the Yng-LCR ($p < 0.001$) or Old-HCR rats ($p < 0.001$). The results are presented in the figure 13. There were no differences in SYN-1 or SYP levels between the Acro and Ctrl rats.

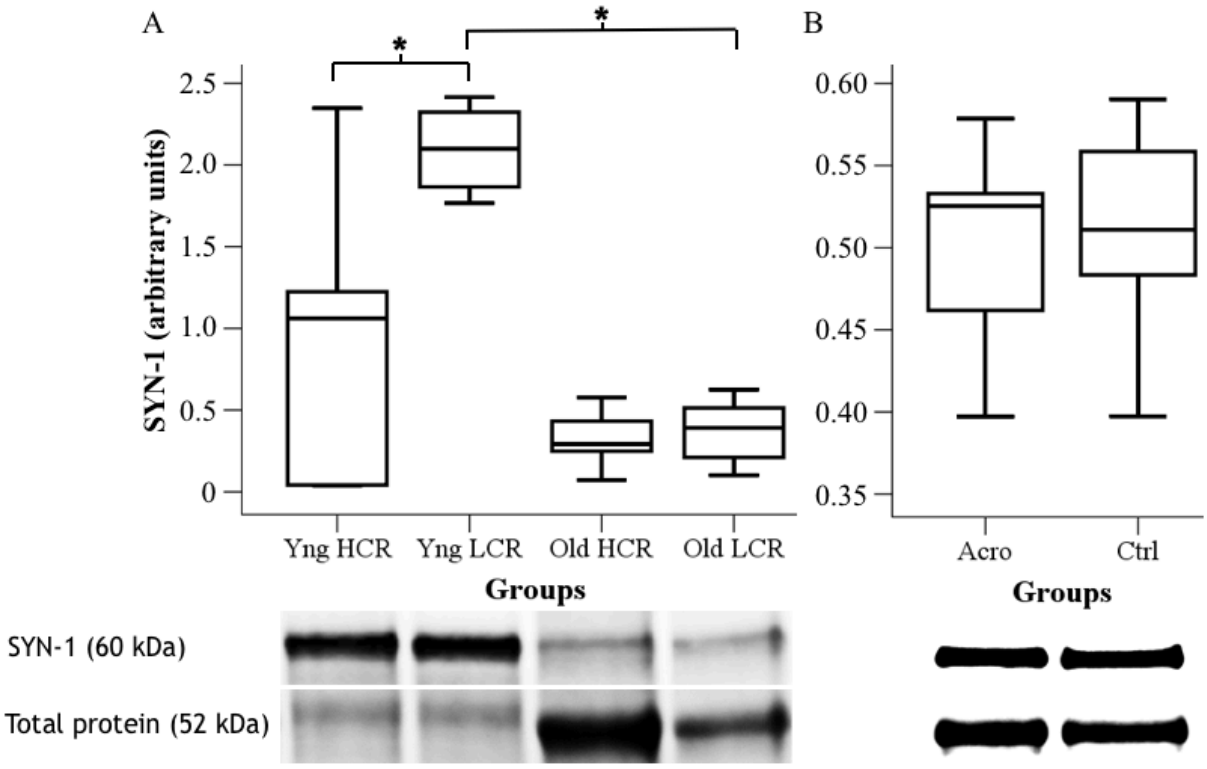

FIGURE 12. Synapsin-1 -expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. SYN-1, synapsin-1; * $p < 0.05$. Values of A and B are not comparable.

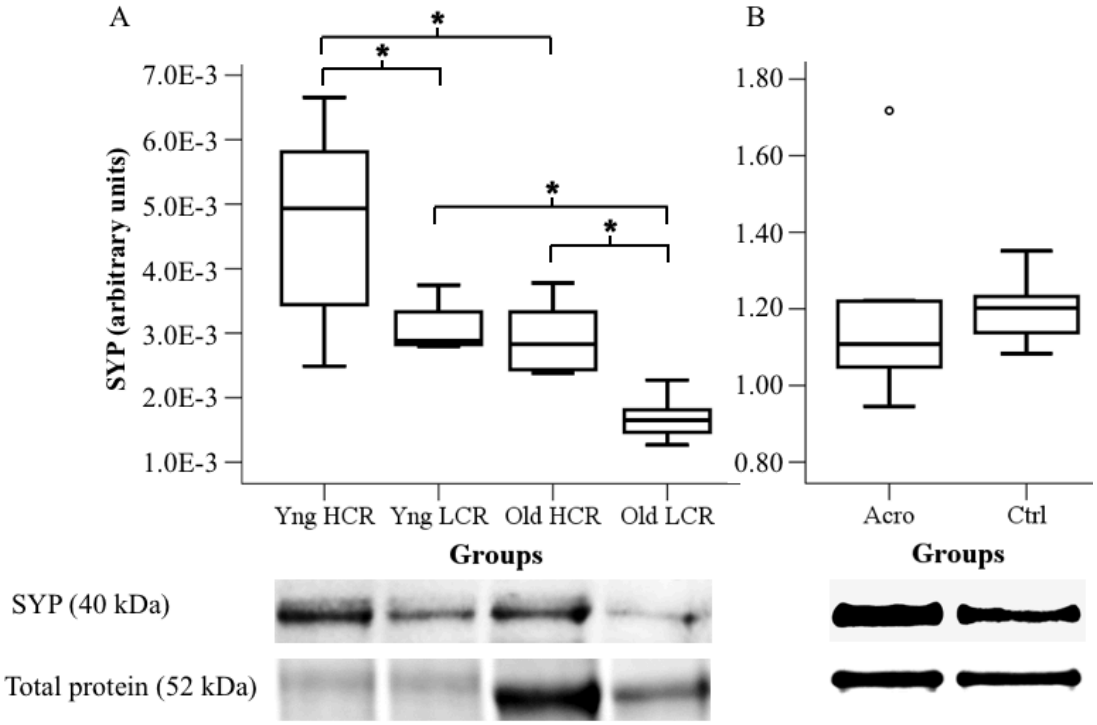

FIGURE 13. Synaptophysin expression of A: HCR-LCR -rats and B: Acro-Ctrl -rats. SYP, synaptophysin; * $p < 0.05$; ° data point exceeding 1.5 interquartile lengths from 75 percentile. Values of A and B are not comparable.

8.3. Inter-user and inter-device comparison

The correlations and Bland Altman plots for user-to-user-variation comparison between Bio-Rad 1 (results from the analyst 1) and Bio-Rad 2 (analyst 2) are presented in figure 14 for SYN-1, figure 17 for IBA1 and figure 20 for SYP. Respectively, comparisons of Bio-Rad 1 and Odyssey device and software are presented in figures 15 (SYN-1), 18 (IBA1) and 21 (SYP). Comparison between Bio-Rad 2 and Odyssey is presented in figures 16 (SYN-1), 19 (IBA1) and 22 (SYP). All the correlations had p-values below 0.001.

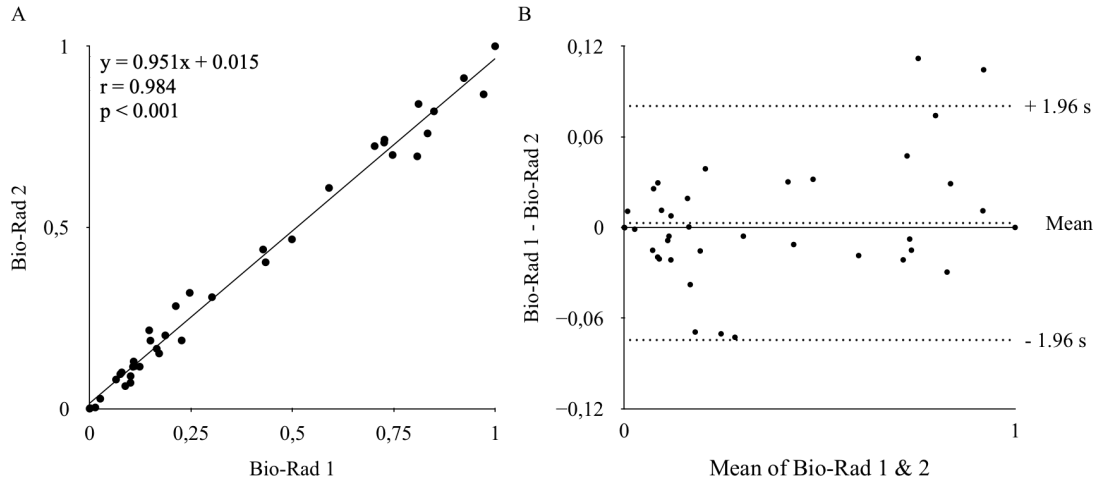

FIGURE 14. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Bio-Rad 2 in SYN-1. y, linear equation; r, correlation coefficient; s, standard deviation.

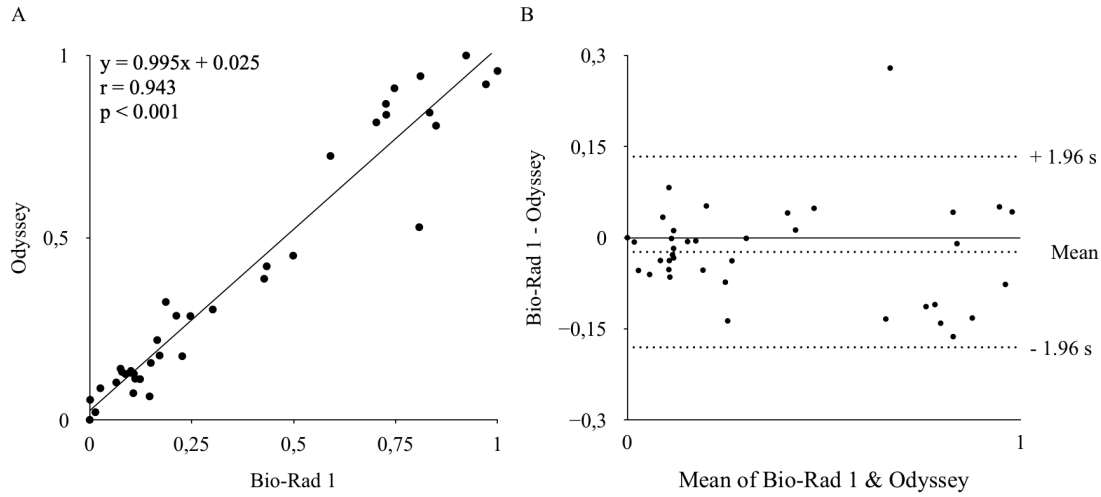

FIGURE 15. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Odyssey in SYN-1. y, linear equation; r, correlation coefficient; s, standard deviation.

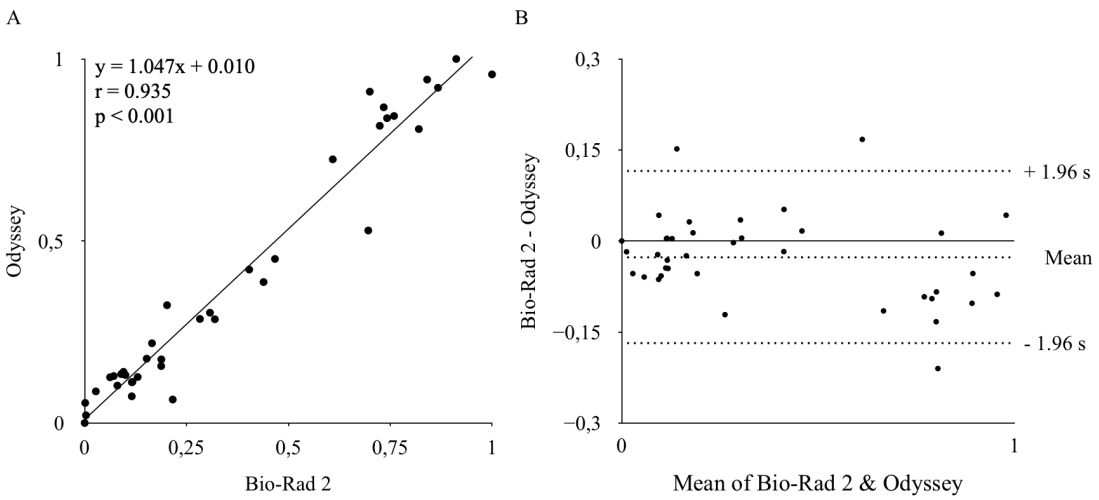

FIGURE 16. A, Correlation and B, Bland Altman plot of Bio-Rad 2 and Odyssey in SYN-1. y, linear equation; r, correlation coefficient; s, standard deviation.

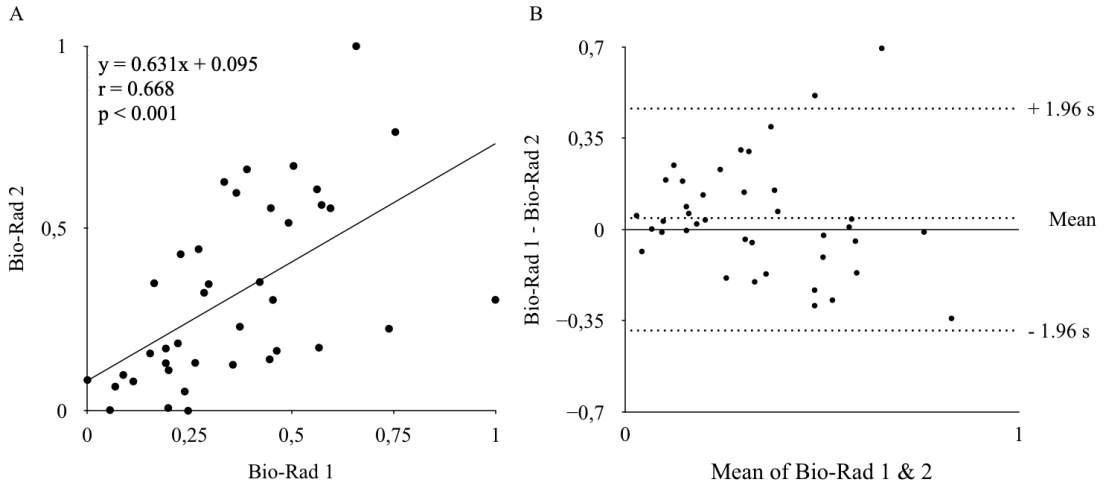

FIGURE 17. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Bio-Rad 2 in IBA1. y, linear equation; r, correlation coefficient; s, standard deviation.

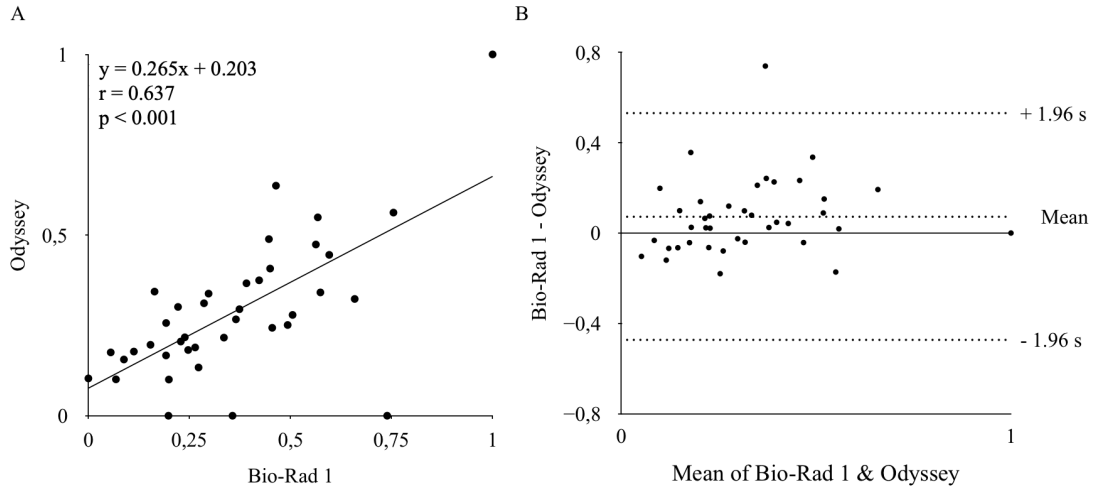

FIGURE 18. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Odyssey in IBA1. y, linear equation; r, correlation coefficient; s, standard deviation.

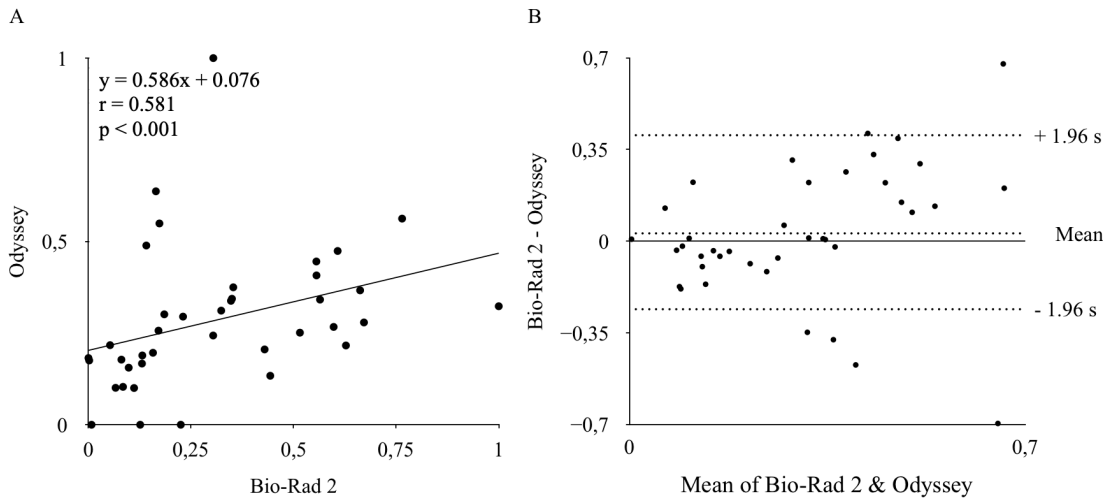

FIGURE 19. A, Correlation and B, Bland Altman plot of Bio-Rad 2 and Odyssey in IBA1. y, linear equation; r, correlation coefficient; s, standard deviation.

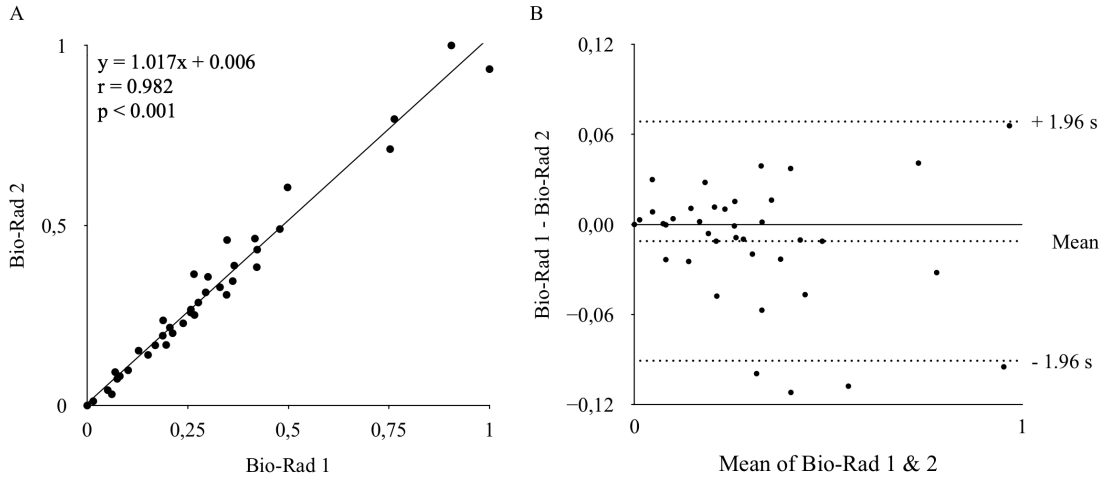

FIGURE 20. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Bio-Rad 2 in SYP. y, linear equation; r, correlation coefficient; s, standard deviation.

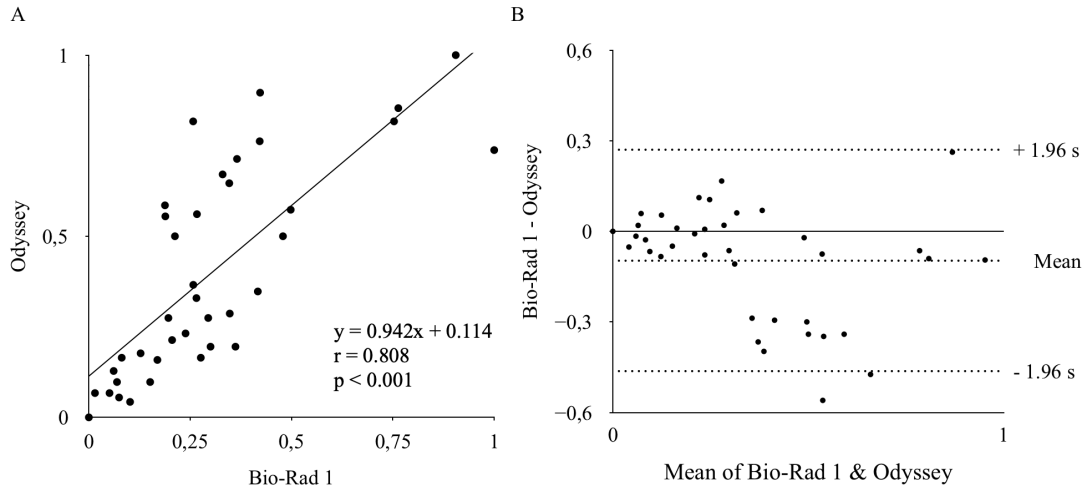

FIGURE 21. A, Correlation and B, Bland Altman plot of Bio-Rad 1 and Odyssey in SYP. y, linear equation; r, correlation coefficient; s, standard deviation.

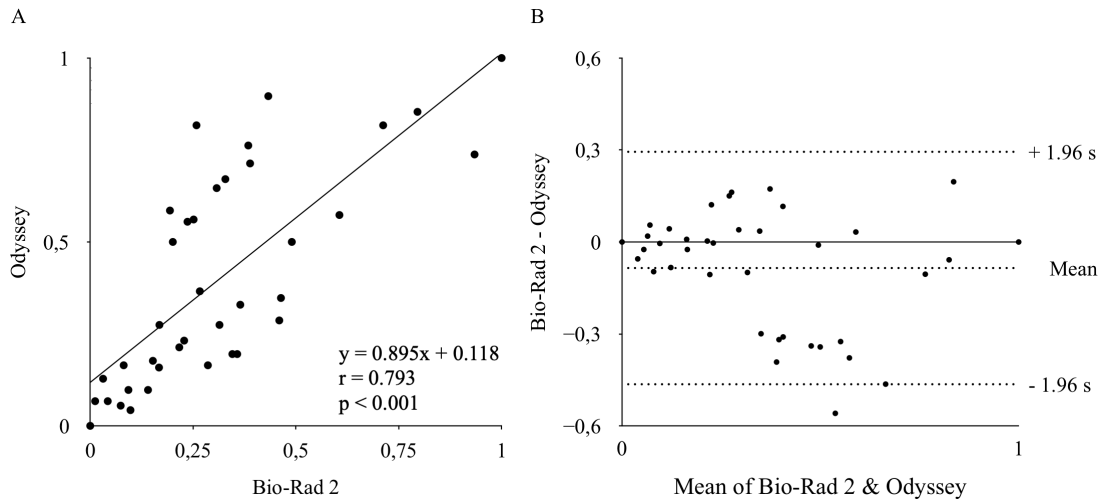

FIGURE 22. A, Correlation and B, Bland Altman plot of Bio-Rad 2 and Odyssey in SYP. y, linear equation; r, correlation coefficient; s, standard deviation.

9. DISCUSSION

The purpose of this study was to test if the better aerobic capacity of the HCR rats reduces the expression of inflammation markers in hippocampal proteins compared to the LCR rats and if the possible difference is already present in young animals or if it only appears upon aging. Also, the effect on acrobatic training on the brain inflammation markers was studied. Finally, the reliability of western blotting was tested by using two different devices and softwares or two experimenters for the same images to see if the results differ. The main finding of the present study was the increased level of inflammation markers in the LCR rats compared to the HCR rats as a summary of the results. However, the results were not absolute, as many of the variables did not represent differences. The analyst and device may cause variation to the results depending on the antibody used.

In the present thesis, the HCR and LCR rats were used for studying the differences of intrinsic high and low aerobic capacity to the inflammation in the hippocampal area. In most of the references cited, the set up has been to study the effects of exercising compared to the sedentary lifestyle. This difference between these assemblies makes the comparison between studies problematic but interesting. The biggest divergences of these two approaches are of course the intrinsic aerobic capacity versus the exercising factor. Training can induce over 30% increase in running capacity in standardized 8-week running protocol in rats bred for high response for training (Koch et al. 2013), but the training effect is small compared to over ten-fold difference in the intrinsic aerobic capacity of the HCR and LCR rats in the same test (Vieira-Potter et al. 2015; Cooper et al. 2017). The end result is still the difference between aerobic capacity of the HCR and LCR rats, or between running and sedentary groups, but mechanisms do differ from one another. Exercising causes increases in expression of growth factors such as brain-derived neurotrophic factor (BDNF), while the non-trained HCR and LCR rats do not differ on BDNF messenger RNA expression levels (Groves-Chapman et al. 2011). Increased BDNF expression has been connected also to improvement in cognitive performance after brain-injury (Griesbach et al. 2009).

9.1. Inflammatory markers in the brain

In the present study, TLR3 expression of the Old-LCR rats was higher compared to the Old-HCR rats, and TLR5 expression of Yng-LCR was higher compared to Yng-HCRs (figures 7A and 8A). IL-1ß was higher in the LCR rats compared to the HCR rats regardless their age (figure 9A). The Old-LCR rats expressed also more IBA1 and GFAP than Old-HCR (figure 10A & 11A). Thus, all of the inflammatory markers used in the present study supported the hypothesis: Higher aerobic capacity reduces the expression of inflammatory markers and can be concluded to offer some protection against inflammation in brains. Exercising prior to traumatic brain injury (Chio et al. 2017) or weeks after brain lesion (Piao et al. 2013) has been shown to reduce inflammation in the brain area, suggesting preventive role of the high aerobic capacity for neuroinflammation. Physically active lifestyle has anti-inflammatory and health-promoting effects (Smith et al. 1999; McFarlin et al. 2006), while chronic and systemic low-grade inflammation status has been connected to poor overall fitness (Karstoft & Pedersen 2016) and obesity (Lumeng & Saltiel 2011). As the end result of the differences, the HCR rats live longer than the LCR rats (Koch et al. 2011). Inflammatory reactions in the brain also impair cognitive function even in tasks learned before (Sparkman et al. 2006), and the HCR rats have shown to outperform the LCR rats in cognitive tasks requiring adaptation to the changed rule (Wikgren et al. 2012).

Results were inconsistent between different age groups. Both of the older groups expressed more TLR3 and IL-1ß than the younger groups. In TLR5 and GFAP, results were opposite. IBA1 levels was lower in Yng-HCR compared to Old-HCR, but there were no differences in the LCR rats. Aging should increase the expression of inflammation markers in the brain (Norden & Godbout 2013; Yin et al. 2016). The rats from HCR and LCR lines are normally tested for their aerobic capacity at the age of 10 weeks, when the rats have already established the difference in their running capacity (Koch & Britton 2001). Sedentary LCR also rats develop several risk factors for metabolic syndrome under the age of six months (Wisløff et al. 2005). Also in this study, differences were found between the groups even with the young animals, but the results were inconsistent. These factors indicate the genotype to be indeed the determinant factor of the aerobic capacity in these rats. However, older rats used in this study

were only middle-aged, and may not have had yet developed an increased inflammation status shown in elderly LCR rats (Koch et al. 2011). GFAP expression was decreased in older animals compared to younger in both LCR and HCR groups. Also, GFAP is lower in Old-HCR compared to Old-LCR. Even though elevated GFAP expression is a stress response indicating the activation of astrocytes (Zhang et al. 2017), also treadmill running has been seen to increase GFAP expression (Li et al. 2005; Ferreira et al. 2011). This is probably due to the increased metabolic activity (Black et al. 1990; Li et al. 2005), and increase in the vascular density leads to increase in the astrocytic cover on the vessels (Wolburg et al. 2009). Thus increased GFAP expression may also indicate higher activity level and increased vascularization in the brain of young animals compared to older counterparts, and represent inflammation status of Old-LCR compared to Old-HCR.

Acrobatic training resulted significant difference in TLR3, which was lower in the Acro group compared to the Ctrl group. No other significant differences in inflammatory markers were noted between the groups, as was hypothesized. TLR3 deficiency has been connected to increased neurogenesis and enhanced working memory in mice (Okun et al. 2010). However, Okun et al. (2010) studied TLR3 knockout mice, which is not a comparable situation with the effects of acrobatic training. The acrobatic training intervention resulted improvement in the acrobatic performance (Honkanen 2017), but seems not to cause a decrease in inflammation marker expression in the brain. Also, methods used in present study may not have had high enough resolution to detect the changes and some markable effects may also have happened in the brain areas or variables not studied in this thesis.

9.2. Synaptic plasticity

Contrary to the hypothesis, the expression of SYN-1 was lower in Yng-HCR than in Yng-LCR (figures 12A). There was no difference in the SYN-1 levels in the older animals. SYP levels were higher in HCR animals compared to LCR rats in both young and old groups (figure 13A). The SYP results are supporting the hypotheses that higher aerobic capacity leads to higher synaptic plasticity, while SYN-1 suggested opposite outcome. In previous studies, SYN-1 has been increased by exercise training (Vaynman et al. 2006; Ferreira et al. 2011).

Treadmill running has been shown to increase also SYP in pre-frontal cortex and motor cortex especially with aged rats (Gutierrez et al. 2018a). The amount of aerobic exercising should correlate with the expression level of SYN-1 and SYP (Vaynman et al. 2006).

Enhanced neurogenesis results better outcome in tasks of cognitive function, and it can be achieved, for example, by enriched environment (Kempermann et al. 1997) or exercise training (Bolz et al. 2015). Synaptic plasticity links to the neurogenesis by the notion that newborn neural cells are more potent to create synaptic connections (Wang et al. 2000). SYN-1 is a protein contributing to synaptic plasticity (Fornasiero et al. 2010) and SYP is an integral protein of the synapses itself (Thiele et al. 2000) resulting SYN-1 and SYP to be common markers for synaptic plasticity. As SYN-1 was higher in Yng-LCR compared to Yng-HCR, and there was no difference between older animals, further conclusions cannot be made in the regard of this study. In SYP, the HCR rats had also higher synaptic plasticity, but the conflict between SYN-1 and SYP still diminishes the value of this finding. Overall, young animals of higher aerobic capacity did have elevated rate of neurogenesis compared to the LCR rats, and the difference increased with age (Lensu et al. 2016). This suggests also elevated synaptogenesis in the HCR rats compared to the LCR, even with suppressed levels of SYN-1 in the Yng-HCR group.

There were no difference between in SYN-1 or SYP between the Acro and Ctrl groups (figures 12B & 13B). The present results were contrary to the hypothesis that acrobatic training increases the expression of SYN-1 and SYP. Acrobatic training has been shown to increase SYP in motor cortex and in dorsomedial striatum (Salame et al. 2016; Gutierrez et al. 2018a) and to induce synaptogenesis in motor cortex (Kleim et al. 1996; Gutierrez et al. 2018a) and cerebellum (Black et al. 1990; Kleim et al. 1998). Overall, acrobatic exercise should induce plastic changes in various areas of rat brain, but the most effective training regimen for enhancing synaptic plasticity remains to be determined (Gutierrez et al. 2018b).

9.3. Methodology discussion

In the present study, it seems that the antibody used has a remarkable effect on the correlation of the results between two analysts or two devices. In SYN-1, the correlations were high (r-values from 0.935 to 0.984), and there were no big variations between analysts or between Bio-Rad and Odyssey. These results were as expected. However, IBA1 resulted only moderate correlations (r-values from 0.581 to 0.668) in all three comparisons. The r-values of approximately 0.6 can be counted poor, as the only variable in the process were either the analyst or the device and software used for analysis. Especially for the same software and two analysts, the variation between each pair of results is quite big, as Bland Altman plot in figure 17B presents. Slightly lower correlations between the two methods is understandable, as there are more altering variables, but still both of the methods used CCDs and softwares developed for quantitative western blotting. Previously, r-value of 0.974 has been obtained when comparing two analysts using the same densitometer device (Gallo-Oller et al. 2018). That result was of the same order of magnitude as the present results with SYN-1 (r = 0.984), but is higher than the present IBA-1 correlation (0.668). Also comparison between two western blot quantification methods has been made between office scanner and densitometer, resulting correlations from 0.495 to 0.670 depending on the used pixel-per-inch value of the office scanner (Gallo-Oller et al. 2018). The correlations of the present study were higher than those in the Gallo-Oller et al. (2018), but that was expected since the devices and software were similar, both being CCDs and softwares designed for western blotting.

The correlation in SYP between the two Bio-Rad results is very high (r = 0.982), but when comparing each Bio-Rad result with Odyssey results, the correlation decreases (r = 0.808 or 0.793). This indicates that the quantitation process with Odyssey method resulted slightly different outcome than Bio-Rad method did. This can be seen in figures 21A and 22A as a cluster of ten dots over the regression line, and probably the same ten data points are also clustered below the mean line in the middle of the Bland Altman plot in figures 21B and 22B. This may be due to, for example, saturation of some strong bands in Bio-Rad analysis, resulting cutting-off of the high end of results and thus lower relative values compared to those of Odyssey. On the other hand, the difference might be from systematic over-indication of bands

or under-indication of background in some certain gel in Odyssey method, thus resulting group of prominent data points. Overall, with all of the present results, also intra-user variation has to be taken into account. Gallo-Oller et al. (2018) found correlation 0.950 between the two quantifications of the same images of western blots. Intra-user variation was not studied in the present study, but as both of the analysts were inexperienced, the variation from data set to data set may explain some of the differences.

Western blot protocol contains many steps, each of which is crucial for the end results (Ghosh et al. 2014; Gilda et al. 2015). In the present study, all of the steps from tissue preparation to secondary antibody staining were the same, since the same samples were used for images. Thereby all of the differences in the results are either from the differences in Bio-Rad and Odyssey imaging devices and their quantification software, or from the variance of two analysts in the same Bio-Rad method. In general, high variation in the group of cases leads to higher correlation between methods (Giavarina 2015), which can be seen in contrary with the IBA1 results. The difference between means of groups in IBA1 were lower than the differences between groups of SYN-1 and SYP, and also correlations were lower in IBA1 than in the other two. Overall, it is still impossible to say which method or which analyst was more accurate, as there is no precise and accurate result to compare with. As a conclusion, it seems that quantitative western blotting can provide reliable results regardless of the analyst or the device used, provided that the device is designed for western blotting, and all the procedures are executed in careful manner. However, due to the nature of western blotting method and the fact there is always slight inter- and also intra-user variation, one analyst should always execute all of tests in one study using the same device and software. Also, suggestive results should be confirmed, for example, with immunohistochemistry or some other suitable method to ensure the results are correct.

9.4. Strengths and weaknesses of the study

The HCR-LCR model is excellent for studying various health-related differences in the rats. Also, the present study gives a viewpoint to the differences between lines without any invasive lesions or stressful interventions interfering with the results. Negative aspect in the HCR-

LCR set up was the quality of the hippocampal protein homogenates. The amounts of expressed total proteins obtained from stain-free images varied considerably from sample to sample, even though the measured and loaded protein amounts should have been the same in every sample. The normalization of the quantitative scores tackled some of the problem, but the origin of the variances remained unknown. The Acro-Ctrl samples extracted with the same protocol resulted homologous total protein amounts. One explanation might be the longer storage time and more freeze-thaw cycles of the HCR-LCR samples compared to newer Acro-Ctrl samples (Ghosh et al. 2014). Overall execution of the laboratory work was done mostly by inexperienced person, and as western blot method is susceptible to defects from the experimenter, it may have caused variation or inaccuracy to the results.

The acrobatic intervention set up was carefully organized, as the control group received an equal amount of handling and motor movement, the obstacles being the only difference between groups. However, acrobatic intervention might not be effective enough to induce noticeable changes that could be detected in the semi-quantitative western blotting method, as only difference was noticed in TLR3. The variation in the behaviour and stress reactions of each rat may surpass the effects of the training response itself. Overall, the dose-response relationship is hard to evaluate when using a novel approach in animal model in behavior study. In addition to training response, other thing affecting to the results is the accuracy of the western blot method. Garcia et al. (2012) suggested that western blot may lack the spatial resolution for significant changes from hippocampal area.

Methodological comparison was successful as it produced both nearly identical results and also analyst-or device-dependent variation to the results. This variation highlights the aspect that the results obtained from western blotting may not be perfect, and that careful description of the process could increase the value of research (Gilda et al. 2015). Comparison between results of more experienced analyst and amateur could have resulted an interesting outcome in this thesis. Also intra-user variation should have been included to the comparison, as Gallo-Oller et al. (2018) found even greater variation within two analyses of the same experimenter than between two analysts.

9.5. Conclusions and future perspectives

Inflammation in the brains has previously shown to impair neurogenesis (Ekdahl et al. 2003; Monje et al. 2003). It was hypothesized that high aerobic capacity associates with inflammation and enhances synaptic plasticity. Overall results were indicative of more inflammation in the LCR rats compared to the HCR rats, but the effect on age resulted contradictory results. In addition, synaptic plasticity markers were not conclusive, as SYN-1 was higher in the Yng-LCR rats compared to the Yng-HCR group even if SYP and previous work of Lensu et al. (2006) suggests elevated synaptogenesis. The knowledge of the relation between intrinsic aerobic capacity and aerobic capacity obtained due to training is still not completed. Study set up containing both HCR and LCR with sedentary and exercising groups would answer the questions if detrimental health effects of poor physical fitness can be prevented by exercising, and if the physical fitness itself or the effects on training is more beneficial to the healthy state of brain. Also the roles of BDNF and other growth factors in the synaptic plasticity and learning should be taken into account in future studies.

Acrobatic training intervention did not result in significant outcomes in present thesis. The reason may lie in insufficient resolution of western blotting in the present thesis or in the intervention inadequate to results significant changes. However, acrobatic training has already previously been shown to induce synaptogenesis (Black et al. 1990; Kleim et al. 1998), but the most effective way to use acrobatic exercising as a tool for skill learning or rehabilitation is yet to be found (Gutierrez et al. 2018b).

The methodological comparison in this thesis was a good opening for the careful and critical walkthrough of the whole western blot process and analysis of its results. There were differences between the analysts and devices used, depending on the current antibody. In SYN-1, all the comparisons resulted excellent correlations, while correlations with IBA1 where moderate at best. SYP outcomes are indicative of some fault in the image obtaining or quantification process, resulting a difference between Bio-Rad and Odyssey results. To get insight of the actual reasons behind the variation between the analysts and devices, next step would be to see in which kind of situation variation grows and when it does not matter who does the

analyses. Also more profound analysis should be made with larger pool of results to get reliable results. Western blot analyses have been developing fast, and from time to time steps back should be taken to see if results of the analyses are true or not.

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APPENDICES

APPENDIX 1. Protocol for extraction of proteins from rat hippocampus.

Homogenizing buffer:

HEPES pH 7.4	20 mM	(Sigma H 0887, Saint Louis, MO, USA)
EDTA (Ethylenediaminetetraacetic acid) (Sigma-Aldrich 60-00-4, Saint Louis, MO, USA)	1 mM	
EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) (Sigma H E3889, Saint Louis, MO, USA)	5 mM	
sodium deoxycholate	0,2 %	(Sigma-Aldrich D6750, Saint Louis, MO, USA)
$MgCl_2$	10 Mm	(Sigma M 8266, Saint Louis, MO, USA)
DTT (dithiothreitol)	2 mM	(Sigma-Aldrich D0632, Saint Louis, MO, USA)
NP-40	1 %	(Sigma-Aldrich NP40S, Saint Louis, MO, USA)
Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific 78444, Waltham, USA)	1 %	
Na_3VO_4 (Sodium orthovanadate)	1 mM	(Sigma-Aldrich 450243, Saint Louis, MO, USA)
ß-glyserophosphate	100 mM	(Sigma-Aldrich G9422, Saint Louis, MO, USA)
milli-Q-H_2O		

Homogenizing buffer was prepared as prescribed above. Pulverized tissue were kept in liquid nitrogen prior mixing with the buffer in a tube. Steal beads were added to tubes. Samples were lysed with TissueLyzer 2 x 2 min at 20 Hz. Then samples were were incubated in cold room (+4 °C) for 30 minutes and centrifuged at 10000 g for 10 minutes. Then protein homogenate was extracted with pipette and stored in -80 °C until used.

APPENDIX 2. Compositions of used solutions.

Blotting buffer, 10x stock solution:

3.03 % (w/v) Trizma base (Sigma T6066, Saint Louis, MO, USA)

14.41 % (v/w) glycine (Sigma-Aldrich 50046, Saint Louis, MO, USA)

1 l milli-Q-H_2O

Blotting buffer, 1x use solution:

0.3 % (w/v) Trizma base

1.4 % (v/w) glycine

10 % methanol (Sigma-Aldrich 322415, Saint Louis, MO, USA)

10x stock solution of blotting buffer was diluted prior usage to 1x with methanol and milli-Q-H_2O.

Ponceau S

0,2 % (w/v) Ponceau S (Sigma-Aldrich 141194, Saint Louis, MO, USA)

5 % acetic acid (Sigma-Aldrich A6283, Saint Louis, MO, USA)

Running buffer, 10x stock solution:

3.03 % (w/v) Trizma base (Sigma T6066, Saint Louis, MO, USA)

14.41 % (v/w) glycine (Sigma-Aldrich 50046, Saint Louis, MO, USA

1.00 % (w/v) SDS (Sigma-Aldrich 71725, Saint Louis, MO, USA)

1 l milli-Q-H_2O

10x stock solution of running buffer was diluted prior usage to 1x with milli-Q-H_2O.

6x Sample Buffer:

30 % glycerol (Sigma G 5516, Saint Louis, MO, USA)

15 % ß-mercaptoethanol (Sigma M3148, Saint Louis, MO, USA)

20 % (w/v) SDS (Sigma-Aldrich 71725, Saint Louis, MO, USA)

18.8 % (1 M, pH 6,8) Trizma base (Sigma T6066, Saint Louis, MO, USA)

12 % (w/v) bromophenolblue (Merck 108122, Darmstadt, Germany)

TBS (Tris-buffered saline), 10x stock solution:

1.21 % (w/v) Trizma base (Sigma T6066, Saint Louis, MO, USA)

9.00 % (w/v) NaCl (Sigma 746398, Saint Louis, MO, USA)

10x stock solution of TBS was diluted prior usage to 1x with milli-Q-H_2O.

TBS-Tween, use solution:

0.121 % (w/v) Trizma base (Sigma T6066, Saint Louis, MO, USA)

0.900 % (w/v) NaCl (Sigma 746398, Saint Louis, MO, USA)

0.1 % Tween 20 (Merck Cas: 9005-64-5, Hohenbrunn, Germany)

The use solution of TBS-Tween were diluted from 10x TBS to 1x with Tween 20 and milli-Q-H_2O added.

APPENDIX 3. Antibodies used:

Primary antibodies:

TLR3, Toll-like receptor 3 (130 kDa) (#SAB2900405-50UG, Sigma-Aldrich, Saint Louis, MO, USA)

TLR5, Toll-like receptor 3 (100 kDa) (#SAB3500374, Sigma-Aldrich, Saint Louis, MO, USA)

IL-1ß, Interleukin-1ß (30 kDa) (#AB1832P, Merck, Darmstad, Germany)

GFAP, Glial fibrillary acidic protein (55 kDa) (#ab7260, Abcam, Cambridge, UK)

IBA1, Ionized calcium-binding adaptor molecule 1 (17 kDa) (#PA5-27436, ThermoFisher Scientific, Rockford, IL, USA)

SYN-1, Synapsin-1 (60 kDa) (#ANR-014, Alomone labs, Jerusalem, Israel)

SYP, Synaptophysin (40 kDa) (#ANR-013, Alomone labs, Jerusalem, Israel)

Secondary antibody:

IRDye® 800CW Infrared Dye (LI-COR, Lincoln, NE, USA)