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Exercise-induced neuroprotection in the hippocampus of EAE mice

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Abbreviations

Ab	Antibody
ACTN4	Alpha-Actinin-4
AD	Alzheimer’s disease
AHN	Adult hippocampal neurogenesis
bacTRAP	Bacterial artificial chromosome-translating ribosome affinity purification
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
CAPN1	Calpain-1
CFA	Complete Freund’s adjuvant
CNS	Central nervous system
DAB	3,3’-diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco’s phosphate buffered saline
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
FNDC5	Fibronectin type III domain-containing protein 5
GM	Grey matter
HBSS	Hanks' Balanced Salt Solution
HC	Hippocampus
HLA	Human leucocyte antigen
IGF-1	Insulin-like growth-factor-1
IHC	Immunohistochemistry
LTP	Long-term potentiation
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte protein
MRI	Magnetic resonance imaging

MS	Multiple sclerosis
N2A	Neuro-2A
NAWM	Normal appearing white matter
NDS	Normal Donkey Serum
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
p.m.	Postmortem
PNGM	Primary neuron growth medium
PPMS	Primary progressive multiple sclerosis
RNS	Reactive nitrogen species
ROI	Regions of interest
ROS	Reactive oxygen species
RRMS	Relapsing remitting multiple sclerosis
SEM	Standard error of the mean
SGZ	Subgranular zone
SPMS	Secondary progressive multiple sclerosis
SVZ	Subventricular zone
Tbp	TATA-binding protein
TRIM59	Tripartite Motif-Containing Protein 59
UVB	Ultraviolet B
VEGF	Vascular endothelial growth factor
WM	White matter

1 Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS). MS mainly affects young women, with a varying clinical course (Reich et al., 2018). The most common relapsing-remitting MS (RRMS) is characterized by relapses of neurological symptoms primarily affecting the motor, sensory, visual, and autonomic systems, with clinical recovery in between (Compston and Coles, 2008). Many patients with RRMS develop a secondary progressive phase (SPMS) after 10-20 years, with progressive worsening of neurological symptoms and disorders, often leading to severe disability. 15% of MS patients present with progredient symptoms without prior relapses, the primary progressive MS (PPMS) (Reich et al., 2018). Important pathophysiological factors include the invasion of autoreactive lymphocytes across the blood-brain barrier (BBB) into the CNS with activation of innate immune cells such as microglia alongside early and progressive neurodegeneration. In RRMS, CNS inflammation leads to demyelination and promotes neurodegenerative processes such as neuronal and axonal damage in mainly circumscribed regions, so-called lesions (Friese et al., 2014, Compston and Coles, 2008). The localization of white matter (WM) and grey matter (GM) lesions determines the nature of temporary clinical symptoms. The progressive phase is characterized by global and gradual neurodegeneration of the GM and WM (Dendrou et al., 2015). However, a single trigger causing MS has not been detected so far and it is assumed that a complex combination of genetic predisposition, environmental factors and infectious events contributes to MS pathogenesis (Compston and Coles, 2008). Despite great progress in the development of disease modifying drugs for especially RRMS and the inflammatory component, there is an urgent need for therapeutic strategies that address the ongoing neurodegeneration (Hauser and Cree, 2020).

1.1.1 Epidemiology and Etiology

MS affects more than 2.5 million people worldwide, with women at least twice as likely to develop the disease. The average age of diagnosis is about 30 years (Walton et al., 2020). Prevalence is highest in Northern Europe countries and North America and lowest in regions around the equator, increasing with higher latitudes while decreasing from north to south (Wallin et al., 2019). A genetic predisposition has been supposed for more than a century due to characteristics in the epidemiology. Women have an increased risk of developing MS compared to men. This suggests both a genetic predisposition and an autoimmune component as women show a higher prevalence of autoimmune diseases (Reich et al., 2018).

Furthermore, first-degree relatives are about 30 times more likely to develop MS (3%) than the general population (0.1%) (Patsopoulos, 2018). And finally, twin studies revealed that monozygotic twins have significantly higher concordance (25-30%) than dizygotic twins (3-7%). Altogether, a meta-estimation revealed that approximately 50% of MS susceptibility is caused by hereditary factors (Fagnani et al., 2015). The heredity pattern does not indicate that MS is caused by a single gene and inherited according to the Mendelian rules. Certain human leukocyte antigens (HLA) class I and II molecule variants, especially the HLA-DRB1*15:01 haplotype, are associated with increased or decreased susceptibility for MS (Hollenbach and Oksenberg, 2015). Until the early 2000s, it was not possible to identify further risk genes outside the HLA locus that contribute significantly to MS genesis (Patsopoulos, 2018). A breakthrough was reached with genome wide association studies revealing more than 200 gene variants and polymorphisms that are more prevalent in patients with MS compared to the general population. These genes are predominantly associated with immunological processes in mainly regulatory gene regions that, among other functions, modify splicing (Patsopoulos, 2018).

Besides the genetical predisposition, several environmental and behavioral factors are crucial in MS pathogenesis. Migration studies reveal a strong influence of environmental factors, as migration from a high-risk region to a low-risk region during childhood significantly reduces the risk of developing MS (Compston and Coles, 2008). Differences in prevalence depending on latitude can be explained by different ultraviolet B (UVB) exposure besides genetic factors. Many studies have been conducted and most of them describe an inverse correlation between sun exposure and MS prevalence (Marrie, 2004). In part, this could be attributed to enhanced vitamin D levels with higher sun exposure. Low levels of vitamin D were shown to enhance both the risk for MS and an increased disease activity (Sintzel et al., 2018). Furthermore, smoking, traumatic injury and several comorbid diseases contribute to disease risk (Belbasis et al., 2015). Obesity, notably during adolescence, is associated with a higher risk of developing MS later in life (Olsson et al., 2017). This is consistent with findings that intense physical activity in the same period of life is associated with a reduced likelihood of MS (Wesnes et al., 2017).

Infections have long been studied in the context of MS etiology, with a focus on the Epstein-Barr virus (EBV). Recent findings indicate an up to 32-fold increased risk of developing MS in people infected with EBV (Bjornevik et al., 2022). Conversely, EBV antibody (ab) titers and reactivity were increased in patients with MS compared to healthy EBV-seropositive people (Olsson et al., 2017, Sundström et al., 2009). It is assumed that an EBV infection in genetically predisposed people can lead to an aberrant immune response causing MS (Bar-Or et al., 2020). Here, molecular mimicry plays a decisive role. Such a cross-reaction was recently identified between EBNA1, an EBV protein, and GlialCAM, a glial adhesion protein expressed

in the CNS suggesting a causal link between MS and EBV infection (Lanz et al., 2022). Overall, according to current knowledge, only a complex combination of genetic predisposition and additional environmental and lifestyle factors can explain the genesis of MS.

1.1.2 Pathophysiology with focus on neurodegeneration

The term "multiple sclerosis" refers to the characteristic sclerotic plaques as the main feature of the disease. These plaques can be visualized via radiological imaging and examination of postmortem (p.m.) brain tissue and are the result of predominantly circumscribed inflammation, demyelination and neurodegeneration (Compston and Coles, 2008). Sequence and cause of these pathological processes remain elusive and are subject of current research. The *outside-in* model of MS pathophysiology assumes peripheral activation of T lymphocytes which gain ability to cross the BBB and attack brain tissue in an autoreactive manner (Stys et al., 2012, Thompson et al., 2018). Together with macrophages and B lymphocytes, they infiltrate distinct areas in the WM but also the GM of the CNS. This proinflammatory environment results in activation of resident immune cells, especially microglia, and other glial cells such as astrocytes, leading to demyelination and axonal damage (Compston and Coles, 2008). These locally and temporally disseminated lesions are described as the hallmark of RRMS whereas the progressive phase according to the *outside-in* model is thought to be a consequence of CNS inflammation and mainly neurodegenerative. Characteristic pathological hallmarks have also been identified for progressive MS. These include demyelination of the GM and diffuse neurodegenerative processes such as axonal damage and neuron loss predominantly in the normal appearing white matter (NAWM) and deep GM including the hippocampal area (Filippi et al., 2012). In addition, cortical lesions play an important role, whereby a correlation between the degree of cortical demyelination and localized B and T cell follicles, especially in the meninges, was observed, although the overall global inflammatory activity is not as high as in RRMS (Machado-Santos et al., 2018, Magliozzi et al., 2018). At the edge of some chronically demyelinated and slowly expanding lesions, macrophages and microglia with iron accumulation could be detected (Dal-Bianco et al., 2017). These rim lesions presented to be most evident in progressive MS potentially driving ongoing neurodegeneration and causing poorer neurocognitive performance (Dal-Bianco et al., 2021). Altogether, these processes lead to progredient atrophy of the brain and irreversible disability of affected patients (Eshaghi et al., 2018, Dendrou et al., 2015).

On the other hand, the *inside-out* model assumes pathological processes primarily taking place in the CNS and secondarily activating the immune system by systemically released antigens (Stys et al., 2012). This theory is accompanied by the assumption that neurodegeneration is not only the consequence of inflammation, but an early phenomenon

that occurs independent from the amount of relapses and immune cell infiltration (Friese et al., 2014). It could be shown that especially in PPMS, but also in SPMS, the entire CNS is diffusely affected by pathological processes. This is mainly reflected in a global activation of innate immune cells, cortical lesions and pathological changes in the NAWM. These processes develop independently of the WM lesions that characterize RRMS, calling into question the *outside-in* model (Kutzelnigg et al., 2005). The MS disease course could therefore be explained as neuroprotective and neurodegenerative processes initially compensating for early neurodegeneration and then exhausting themselves in the progressive phase resulting in irreversible disability (Friese et al., 2014). Studies have demonstrated brain atrophy resulting from especially GM loss at very early stages of MS (Calabrese et al., 2007, Chard et al., 2002b). Besides measuring of brain atrophy, a decrease of N-acetylaspartate, a marker only expressed in neurons and whose decrease correlates with neuronal cell loss or dysfunction, could be detected in the GM of patients with early MS (Chard et al., 2002a, Kapeller et al., 2001). Interestingly, the degree of disability that correlates best with neurodegeneration does not depend on the number of MS relapses (Confavreux et al., 2000). This observation is in line with the observation that although anti-inflammatory drugs reduce the number of relapses, they can only improve or delay the progression of disability to a limited extent (Hauser and Cree, 2020).

Neurodegeneration

Although many aspects of the immunopathological and neuropathological processes, especially the question of cause and consequence, remain to be elucidated, neurodegeneration certainly plays a crucial role in MS. It is known that irreversible, long-term disability of MS patients correlates best with the degree of neurodegeneration which progresses continuously especially in the progressive phase of MS (Trapp et al., 1999). A substantial loss of neurons, glial cells and synapses as well as severe damage and reduction of axons could be shown in the CNS of MS patients (Wegner et al., 2006, Popescu and Lucchinetti, 2012). Cortical and subcortical atrophy is already evident at early stages of the disease but the hallmark of the progressive phase of MS (Eshaghi et al., 2018). This indicates both inflammation-independent and -dependent neurodegeneration. There is still no clarity about the exact underlying neurodegenerative processes, but some important pathways have been identified. Chronic CNS inflammation by innate immune cells, more precisely microglia and macrophages, leads to the release of reactive oxygen species (ROS), reactive nitrogen species (RNS), cytokines and glutamate, all promoting neuroaxonal damage (Friese et al., 2014). ROS and RNS evoke oxidative stress causing damage of cellular structures in neuronal cells (Lassmann and van Horssen, 2011). Ion channels are crucial for maintaining the electrical properties of neurons and their dysfunction appear to have a central influence on progressive neurodegeneration. The excitotoxicity caused by glutamate, which is released in

excess both by damaged cells and by inflammatory cells (Piani et al., 1991), is important in this context. Glutamate induces a harmful calcium influx into the cell by activating N-methyl-D-aspartate (NMDA) receptors (Friese et al., 2014, Hardingham and Bading, 2010). Mitochondria produce the energy necessary for cell survival and functioning and are severely impaired in MS (Witte et al., 2010). This leads to energy deficiency and therefore hypoxia in neurons, which is a central pathological hallmark of neurodegeneration (Halder and Milner, 2020). Their dysfunction was shown to be cause and consequence of several other neurodegenerative processes such as ion channel dysfunction and oxidative stress (Dutta et al., 2006, Friese et al., 2014). Furthermore, the disruption of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and their neuroprotective pathways during MS can hinder the repair and regeneration of neurons (Bernardes et al., 2013, Aharoni et al., 2005).

1.1.3 Experimental Autoimmune Encephalomyelitis

CNS imaging and p.m. tissue analysis of MS patients are essential for exploring disease mechanisms of MS, but not sufficient to understand underlying processes of neurodegeneration. This requires *in vivo* models that provide an approximation of the still unknown pathomechanism of MS. A widely used model is the experimental autoimmune encephalomyelitis (EAE) most frequently induced in mice. Much of what is known today about the pathophysiology of MS and many of the drugs being used to treat MS derived from studies in EAE (Constantinescu et al., 2011). Just as the clinical course of MS is very heterogeneous, there are also different methods to induce EAE, providing models for monophasic acute or chronic MS as well as RRMS. This is due to differences in induction as well as the use of different mouse strains (Constantinescu et al., 2011). What EAE protocols have in common, however, is that the disease is triggered by peripheral immunization with antigens inducing an inflammatory response that secondarily spreads to the CNS. This most closely reflects the *outside-in* model of MS, so some bias in research results must be assumed when assumptions are applied from EAE to MS (Sriram and Steiner, 2005). To actively induce EAE, mice are injected with specific CNS antigens together with complete Freund´s adjuvant (CFA). The immune response is additionally enhanced by application of pertussis toxin (Racke, 2001). To induce a monophasic EAE course, myelin oligodendrocyte protein (MOG) is applied as CNS antigen in C57BL/6 mice. $CD4^+$ T cells that specifically recognize the injected antigen, cross the BBB, get reactivated in the CNS, where, together with resident immune cells, they trigger focal inflammation, demyelination and neurodegeneration (Stromnes and Goverman, 2006). The average time between immunization and clinical symptoms is 10-15 days. Mice show characteristic symptoms that are mainly due to damage to the spinal cord and thus affect the musculoskeletal system. An acute phase of symptom deterioration is followed by a chronic phase in which some of the animals partially recover, but others do not experience any

improvement and suffer from progressive disability, similar to SPMS (Constantinescu et al., 2011, Bittner et al., 2014). This EAE model is particularly suitable to study neurodegenerative processes because it reflects the chronic progressive rather than relapsing remitting components of MS (Voskuhl and MacKenzie-Graham, 2022). Interestingly, the animals exhibit impaired spatial orientation even before disturbances in motor function, indicating early damage to, among other CNS regions, the hippocampus (HC) (Mancini et al., 2017).

1.1.4 Cognitive impairment

MS presents with a variety of symptoms that, in case of RRMS, mostly result from the localization of the lesions. To name a few examples, lesions on the optic nerve cause visual impairment, if they occur in the cerebellum, tremor and dysarthria may result. Lesions of the cerebrum or spinal cord could lead to sensory and motoric impairment (Compston and Coles, 2008). An important symptom complex that cannot be exclusively attributed to a specific brain region and whose underlying mechanisms remain largely unknown is cognitive impairment. Overall, about 40-70% of MS patients suffer from cognitive impairment, which is a major socioeconomic burden for the community, given that MS affects mainly young people of working age (Kavaliunas et al., 2022). Until today, there is no effective treatment addressing this symptom complex of the disease. Cognitive impairment subsumes a range of possible dysfunctions severely affecting the ability to manage everyday life and ability of social integration (Chiaravalloti and DeLuca, 2008). To evaluate these impairments, there are several test batteries. The minimal neuropsychological assessment of MS (MACFIMS) is often used and covers the domains language, spatial processing, new learning and memory, executive functions, processing speed and working memory (Benedict et al., 2006). Other frequently used testing instruments are the Multiple Sclerosis Functional Composite (MSFC) score and the Paced Auditory Serial Addition Task (PASAT) (Motl and Pilutti, 2012). It has become apparent that particularly processing speed and the episodic memory are impaired in MS (Chiaravalloti and DeLuca, 2008, Sumowski et al., 2018).

It has been recognized for several decades that cognitive impairment is a central symptom in MS, but there is still disagreement about the pathological processes underlying this disability. At first, lesion load could be shown to correlate with the severity of cognitive decline (Rao et al., 1989). This was consistently demonstrated in subsequent studies (Lazeron et al., 2005, Sperling et al., 2001). Over time, brain atrophy in MS became more of a focus and magnetic resonance imaging (MRI) studies of human brains revealed an even stronger correlation of brain atrophy than lesion load with cognitive decline (Benedict et al., 2004). Furthermore, progressive and early GM degeneration is central in MS and has been shown to correlate closely with cognitive impairment (Rocca et al., 2015). Since brain atrophy as a sign of

neurodegeneration can be detected at very early stages of MS, it fits well with the results of a prospective study which revealed that cognitive decline often occurs years prior to other typical MS symptoms such as visual or sensory impairment (Cortese et al., 2016). Especially subcortical atrophy seems to be related to poorer cognitive performance (Bermel et al., 2002). Two central subcortical regions whose impairment has been linked to cognitive disability are the thalamus and the HC. The volume of the thalamus was not only found to be reduced in MS patients compared to healthy controls but also significantly smaller in patients with lower cognitive performance (Houtchens et al., 2007). The HC is central to the formation and retrieval of memories, an important cognitive function. In the following, the multiple functions of the HC as well as its structural damage in patients with MS will be discussed in more detail in order to establish a connection between the damage to the HC and the cognitive impairment of MS patients.

1.2 Hippocampus

1.2.1 Structure and functions

The HC is a unique and complex structure. As part of the archicortex, it belongs to the phylogenetically oldest part of the brain. In humans, the HC is located in the medial temporal lobe, adjacent to the inferior horn of the lateral ventricle. The HC in the narrow sense consists of the C-shaped dentate gyrus (DG) and cornu ammonis (CA), which in turn is organized in several subareas (CA1-CA4). However, these areas are not connected in series, but the CA winds in and around the DG, enabling a complex interaction. In rodents, the HC occupies a larger part of the brain and, due to the lack of lobation, is located in the superior-medial part, which is adjacent to the third ventricle in both hemispheres. Unlike the neocortex, the HC is characterized by its three instead of six cortical layers. Principal cells in the DG are the granule cells, which are closely packed in the granule cell layer. There, dendrites branch in the molecular layer forming the outer layer of the C-shaped DG. The third and inner layer is called polymorphic layer, forms the hilum and contains small polymorphic cells and the axons of the granule cells (Schultz and Engelhardt, 2014). The CA, as part of the archicortex, also has a three-layer structure. Here, pyramidal cells are the principal cells forming the middle layer. The stratum oriens mainly contains basal dendrites of the pyramidal cells. Above the pyramidal cells is the molecular layer, which is once again divided into the stratum lucidum and radiatum, where axons of the DG granule cells project on dendrites of the CA pyramidal cells (Schultz and Engelhardt, 2014).

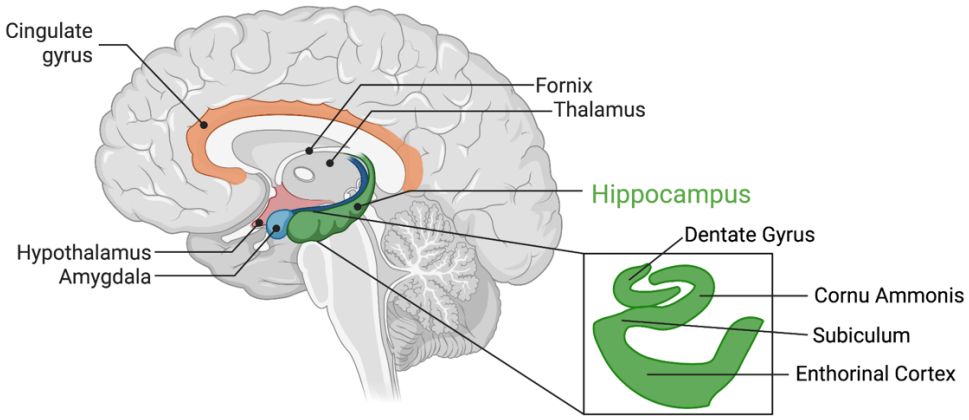


Figure 1.1 Limbic System and Hippocampus

Illustration of important areas of the limbic system with magnified representation of the components of the hippocampal formation (Created with BioRender.com).

The HC is a central part of the limbic system, which comprises several subcortical and cortical structures, the most important of which are labeled in **Figure 1.1**, surrounding the thalamus and basal nuclei. The areas are anatomically distributed in the CNS but closely interconnected by specific fiber tracts. First described by Broca as the limbic lobe, it was initially assigned to the olfactory system (Broca, 1878). However, with the help of animal experiments, other central functions of the limbic system were discovered. These include its critical role in learning processes and memory formation, emotions, motivation and autonomic and behavioral regulation (Mega et al., 1997). Over time it became apparent that defining one big limbic system is too simple and that the classification into anatomical and, subsequently, functional subsystems is more appropriate. Most attempts separate one subsystem that is crucial for emotions from another for memory formation (Rolls, 2015, Catani et al., 2013). The latter subsumes structures that are directly connected to the HC as the central structure. The best known neuronal circuit that must be mentioned in this context is the Papez circuit, which subsumes the following structures: HC, fimbria, fornix, mamillary body, anterior nucleus of the thalamus and cingulate gyrus, and was initially described as “the anatomic basis of emotions” (Papez, 1937). Later, however, the circuit was attributed primarily to memory formation, or more precisely the transfer of contents of the short-term into the long-term memory (Rolls, 2015). After bilateral resection of temporal lobe structures including the HC in order to cure his epilepsy, the patient H.M. developed anterograde amnesia. His working memory remained intact, but no new content could be transferred to long-term memory and the retrieval of contents of the long-term memory was partly impaired (Corkin, 2002). More specifically, the HC is primarily responsible for the episodic memory, meaning memories of autobiographical events with temporal and spatial classification.

Within the HC, synaptic circuitry can be described as a trisynaptic loop. Simplified, afferents from sensory cortices enter the DG via the perforant path. Granule cells of the DG send their axons, called mossy fibers, to the CA3 pyramidal cells, from where Schaffer collaterals project to CA1 pyramidal cells. The efferents exit the HC via the subiculum. To amplify or attenuate synaptic signaling, the circuits are modulated by associational and commissural fibers as well as inhibitory neurons (Mancini et al., 2017). Closely related to semantic memory is spatial mapping. The HC exerts this function, in part, through networks of so-called place cells that facilitate the learning of orientation in space creating a cognitive map. Place cells show a location-specific firing pattern and were first described in rodents (O'Keefe and Nadel, 1979) and later in the human HC (Ekstrom et al., 2003). Both the functionality of place cells and the consolidation and recall of memories require a high degree of plasticity. A key mechanism first described in the HC is long-term potentiation (LTP). LTP describes the following phenomenon: when two neurons are activated in a time-coupled manner, their synaptic connection is strengthened generating cell assemblies encoding memories and a cognitive map (Hebb, 1949, Neves et al., 2008). In addition to this synaptic plasticity, another unique form of plasticity occurs in the HC, namely adult hippocampal neurogenesis (AHN).

1.2.2 Adult hippocampal neurogenesis

Santiago Ramon y Cajal postulated in 1913 „In the adult centers, the nerve paths are something fixed and immutable. Everything may die, nothing may be regenerated". This notion persisted primarily because mature neurons exhibit postmitotic characteristics. Regeneration of neurons was therefore long considered impossible and the potential of neurogenesis was thought to end at birth (Surget and Belzung, 2022, Altman and Das, 1965). Today it is known that neuronal plasticity, the constant adaption of brain structures and functions, is a basic requirement for learning and memory and one fascinating phenomenon of plasticity is adult neurogenesis. Besides the subventricular zone (SVZ), the HC, and more specifically the subgranular zone (SGZ) of the DG, is one of two regions of the adult human brain, where neurogenesis, the generation of neuronal precursors, has been detected (Kumar et al., 2019). The SGZ provides the necessary conditions for the proliferation and differentiation of neuronal stem cells, such as mild hypoxia (Mohyeldin et al., 2010) and production of BDNF (Kempermann et al., 2015).

The HC is crucial for memory formation, emotions and motivation. All these tasks require a high cognitive flexibility, which, among other mechanisms, is maintained by AHN. Newborn neurons from the SGZ undergo maturation **(Figure 1.2)** until they are finally integrated into the granule cell layer of the HC with electrophysiological and morphological properties of mature granule cells (van Praag et al., 2002) and even higher LTP potential and decreased threshold

for activation (Ge et al., 2007). AHN is not a static phenomenon. Environmental factors such as physical activity (van Praag et al., 1999), enriched environment (Kempermann et al., 1997) and dietary restrictions (Lee et al., 2000) as well as in conditions after events that damage the brain were shown to enhance AHN. These include ischemia (Liu et al., 1998), seizures (Parent et al., 1997) and trauma (Shapiro, 2017), suggesting a regenerative potential of AHN. The idea of using neurogenesis therapeutically in the context of these diseases, and particularly in the context of neurodegenerative diseases, has been discussed for some time (Sailor et al., 2006). As it is proving difficult to translate animal studies on the transplantation of exogenously generated neurons into the CNS to human studies, the use of environmental conditions known to stimulate neurogenesis, such as physical activity, is an interesting therapeutic option.

AHN in rodents and other species is a recognized and well-studied phenomenon (von Bohlen und Halbach, 2011, Lledo et al., 2006), but studies in humans still reach conflicting conclusions about whether AHN exists at all and, if so, up to what age (Boldrini et al., 2018, Sorrells et al., 2018). In humans, it was first described by Eriksson et al. (1998) via Bromodeoxyuridine (BrdU) immunohistochemistry (IHC). BrdU is a synthetic thymidine analogue that, after *in vivo* injection, incorporates in the deoxyribonucleic acid (DNA) of dividing cells (Kuhn et al., 2016). However, this marker can only be quantified in p.m. brain tissue, which significantly limits its application in human studies. It remains a critical problem that there are no valid methods to detect neurogenesis *in vivo.* In p.m. brain tissue, various markers could be defined that indicate the maturation status of newborn neurons (von Bohlen und Halbach, 2007). **Figure 1.2** illustrates the maturation starting from radial glial cells which are considered as precursors of neurons and glial cells (Moreno-Jimenez et al., 2019). Doublecortin (DCX), a microtubule-associated protein, could be validated as a reliable and specific marker of newborn neurons (Couillard-Despres et al., 2005). It is exclusively expressed in certain maturation stages of neurons **(Figure 1.2)** and restricted to newborn neurons in the SVZ and SGZ (Brown et al., 2003).

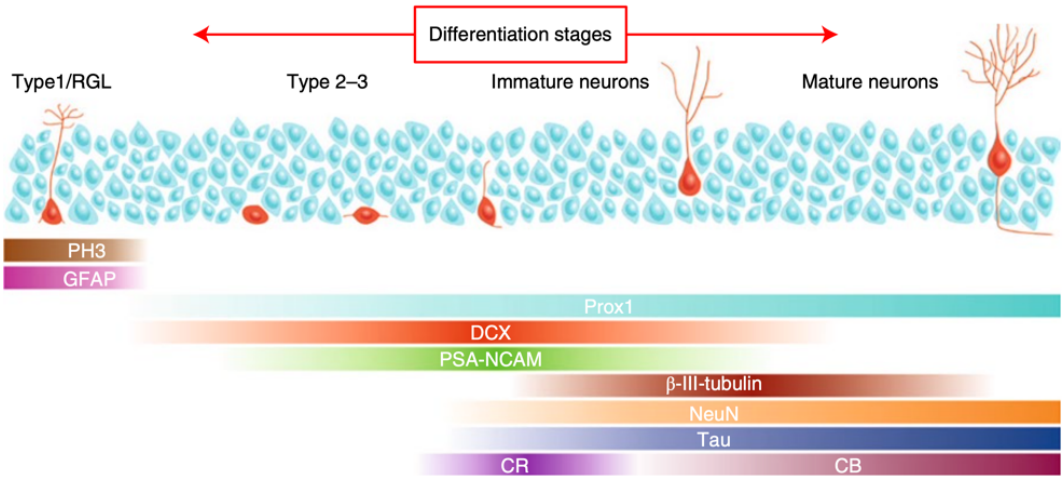


Figure 1.2 Differentiation stages in neurogenesis

Illustration of the most important differentiation stages during hippocampal neurogenesis together with representation of certain cell markers specifically expressed at different stages of maturation. DCX is expressed during variable maturation stages and therefore suitable for investigation of immature neurons in AHN (Moreno-Jimenez et al., 2019).

1.2.3 Hippocampal alterations in MS and EAE

Cognitive impairment is a symptom of MS that is at least in part attributed to damage to the HC. The following summarizes these impairments and their functional implications in MS and EAE. Lesion load of the WM quantified by imaging or p.m. tissue analysis is often used to assess how severely a brain region is affected in MS. In recent years, lesions and alterations of the GM, including the HC, have become an increasing focus of interest and evidence of hippocampal lesions was found in p.m. brain tissue from MS patients (Papadopoulos et al., 2009, Geurts et al., 2007). Shortly thereafter, new MRI methods were developed that allowed *in vivo* detection of hippocampal lesions in both RRMS and SPMS (Geurts et al., 2012, Roosendaal et al., 2008). Furthermore, these imaging methods showed early-onset alteration of GM not affected by lesions, supporting the previously established hypothesis of early-onset neurodegeneration (Inglese et al., 2004, Oreja-Guevara et al., 2005). At the functional level, higher lesion burden in the HC has also been shown to correlate with greater cognitive impairment (Geurts et al., 2007). In addition, the HC seems to be already affected by neurodegenerative processes visualized by diffusion tensor imaging in patients with CIS and these changes were seen to correlate negatively with cognitive performance (Planche et al., 2017). Measurable hippocampal atrophy has been described in MS patients as a sign of progressive neurodegeneration compared to healthy controls. Furthermore, atrophy is significantly more pronounced in MS patients with cognitive impairment compared to patients with preserved cognition (Damjanovic et al., 2017). Analyzing the subregions of the HC in more detail, the CA1 region seems to be most affected by atrophy, which starts early and is

most pronounced in the progressive phase of MS associated with impaired cognitive performance (Sicotte et al., 2008). Similarly, hippocampal atrophy and demyelination, particularly of the CA1 region and studied by MRI and p.m. tissue examination, is also evident in EAE most pronounced in the chronic phase (Ziehn et al., 2010, Ziehn et al., 2012, Hamilton et al., 2019). Interestingly, during EAE, limitations in spatial orientation occurred even before motor deficits that mainly define the course of the disease indicating early onset neurodegeneration in EAE (Dutra et al., 2013, Kim et al., 2012).

Histological and morphological alterations

Histological studies revealed CNS inflammation as an important component in MS pathogenesis and microglial activation in the HC could be detected both during the acute and chronic course of EAE (Ziehn et al., 2010, Di Filippo et al., 2016, Di Filippo et al., 2013). However, there are no consistent results on whether infiltration with lymphocytes occurs. Some studies describe that no lymphocytes are detectable in the HC of EAE mice or primarily in adjacent meninges (Ziehn et al., 2010, Kyran et al., 2018). In other studies, in turn, the detection of lymphocytes in parenchyma of the HC was successful (Mori et al., 2014). Supplementary, it must be said that EAE induction protocols vary between studies and thus explain differences in results to some extent. In MS patients, microglial activation in the HC is also evident and has been linked to functional deficits (Colasanti et al., 2016).

Consistent with the results of new MRI methods, profound demyelination in and around the HC was identified in histological studies of MS patients (Geurts et al., 2007). Regarding a significant loss of neurons, there is more disagreement in the literature. While one study describes a severe reduction of neurons (Papadopoulos et al., 2009), another study saw only minimal neuronal cell loss in the HC of MS patients. Instead, a severe loss of synapses was reported (Dutta et al., 2011). Even though EAE is known to primarily affect the spinal cord, atrophy of especially the CA1 hippocampal region is reported together with demyelination but no significant axonal loss (Ziehn et al., 2010). A 40% loss of pyramidal cells in CA1 and CA4 was reported at day 30 postimmunization (Kurkowska-Jastrzębska et al., 2013). More intensively than synapse density, the functional level such as changes in synaptic plasticity during MS and EAE has been studied (Mancini et al., 2017). Both induction (Nisticò et al., 2013) and impairment of LTP in hippocampal CA1 neurons (Di Filippo et al., 2016) are described in the literature. Differences were also found with respect to the timing of the analysis, with LTP being affected exclusively in the chronic but not in the acute phase of EAE consistent with the presence of spatial memory impairment (Novkovic et al., 2015). Equally controversial are studies examining the influence of MS and EAE on AHN. Giannakopoulou et al. (2017) reported an induction of AHN with an increased number of DCX^+ neurons during chronic EAE but no differences in acute EAE (Giannakopoulou et al., 2013). Other studies report enhanced neuronal proliferation in acute and chronic EAE (Huehnchen et al., 2011) or,

in contrast, a decline in chronic EAE (Aharoni et al., 2005, Guo et al., 2010) partly after an initial enhancement shortly after EAE induction (Aharoni et al., 2005). Although induced AHN has been observed in some studies, the extent to which the newborn neurons can mature and replace injured neurons remains to be explored. In summary, analyses of hippocampal changes, especially during EAE, yield contradictory results.

1.3 Exercise

Regular moderate aerobic exercise has numerous positive effects on body functions and systems. These include the cardiovascular system, the immune system, the metabolism and the musculoskeletal system preventing, to name a few, cardiovascular disease, diabetes, obesity and cancer (van Praag et al., 2014, Hillman et al., 2008). Focused on the brain, endurance exercise is used, for example, as a non-drug migraine prophylaxis and in the treatment of depression (Dimeo et al., 2001, Lemmens et al., 2019). Especially hippocampal functions appear to be positively influenced by exercise. Physical activity during childhood was shown to be correlated with better cognitive performance in school (Sibley and Etnier, 2003). A systematic review revealed a strong influence of acute and chronic exercise on short- and long-term memory (Loprinzi et al., 2018). In rodents, exercise improves the cognitive performance, as tested, for example, by spatial memory in the Morris water maze (Vaynman et al., 2004). A large meta-analysis revealed that physical activity has a significant positive effect on multiple domains such as improved quality of life, mood, and cognitive performance in patients with chronic brain diseases such as Alzheimer’s disease (AD), Huntington's disease and MS (Dauwan et al., 2019). Exercise is a broad term and encompasses both acute and chronic physical activity including endurance and strength training. In the following, potential neuroprotective properties of aerobic endurance exercise will be discussed, which could be at least partly responsible for the observed positive effects.

1.3.1 Neuroprotective properties with focus on the hippocampus

The neuroprotective influences of aerobic exercise on the CNS are numerous, and the HC is particularly susceptible to these effects. Exercise has anti-inflammatory properties and can therefore reduce neurodegeneration accelerated by inflammation and oxidative stress (Małkiewicz et al., 2019). These include downregulation of proinflammatory and upregulation of anti-inflammatory cytokines, as well as enhancement of antioxidant signaling pathways in the CNS (Liu et al., 2019, de Sousa et al., 2017). Furthermore, the CNS is largely protected from the intrusion of toxins, pathogens and the immune system by the BBB. Exercise seems to have an effect on the integrity and function of the BBB, Souza et al. (2017) described an

restoration of tight-junction proteins to normal levels in EAE mice after an exercise intervention. The major energy generation of the CNS occurs within the mitochondria through oxidative phosphorylation. Mitochondrial dysfunction and consecutive oxidative stress are key elements of neurodegenerative diseases and also described in MS and EAE (Burtscher et al., 2021, Witte et al., 2010, Rosenkranz et al., 2021). Exercise can increase mitochondrial biogenesis, enhance their function and consequently reduce oxidative stress while promoting neuronal resilience to stress and detrimental influences (reviewed in Burtscher et al., 2021).

Neurotransmitters, as chemical messengers of the brain, are essential for the communication between neurons and thus the functionality of neuronal circuits. They have the potential to induce the expression of genes important for neurogenesis, neuronal survival and plasticity and thus central functions of the HC. Regular exercise can lead to changes in neurotransmitter levels and activity and therefore counteract age- or disease-related alterations of brain functions such as cognition (Vecchio et al., 2018). Closely related to the altered expression of neurotransmitters is the increased production of neurotrophins. Well studied in this context are BDNF, nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and Insulin-like growth-factor-1 (IGF-1). IGF-1 for example is a growth hormone essential for cell division and normal length growth in children and adults. IGF-1 is produced mainly in the liver with the ability to cross the BBB but also in the CNS. In the CNS, it has been shown to induce neurogenesis, synaptic plasticity, and promote neuronal survival (Bianchi et al., 2017). Elevated peripheral and central levels of IGF-1 following exercise are described (Ding et al., 2006). Another important neuroprotective pathway starts with the secretion of the myokine Irisin, the soluble form of Fibronectin type III domain-containing protein 5 (FNDC5). After the exercise-induced release of Irisin from the muscle, it increases the expression of BDNF, which is important for nerve cell survival, induction of neurogenesis, differentiation of neurons and plasticity (Wrann et al., 2013) **(Figure 1.3)**. BDNF is expressed in the brain especially in the hippocampal area while stimulated by neuronal activity. It is furthermore peripherally produced with the potential to cross the BBB (Leal et al., 2017). Increased levels of BDNF in the HC were detected due to exercise (Neeper et al., 1995, Wrann et al., 2013).

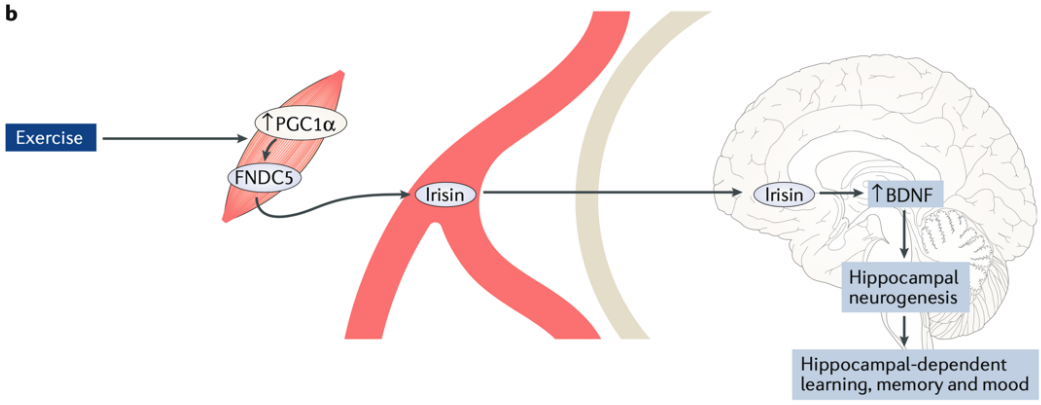


Figure 1.3 FNDC5/BDNF pathway

Exercise induces the PGC1α-dependent formation and release of Irisin, the soluble form of FNDC5, from the muscle. After crossing the BBB, Irisin enhances levels of BDNF in the HC with the potential to induce hippocampal neurogenesis and therefore learning, memory and mood (Pedersen, 2019).

All these pathways induce visual and measurable changes in the HC. It could be demonstrated that exercise can increase hippocampal volume in humans (Erickson et al., 2011). On a cellular level, the induction of AHN by physical exercise is widely recognized. An enhanced number of $BrdU^+$ cells could be detected in the mouse HC following a voluntary exercise intervention (van Praag et al., 1999). Not only altered signaling of neurotransmitters and neurotrophins, but also an enhanced blood and energy supply supports the AHN (Ma et al., 2017). Whether these newborn neurons have the potential to integrate into pre-existing hippocampal circuits is largely unknown. Exercise seems to induce LTP (Loprinzi, 2019) and therefore promotes plasticity. Closely linked to this, it was demonstrated that exercise increases the density of dendritic spines and could therefore potentially expand the number of synaptic contacts (Eadie et al., 2005). The investigation of changes in neuroinflammation, especially microglial activation, led to differing results. Soch et al. (2016) described an increase in number and activation of microglia in the HC after voluntary exercise. Furthermore, no changes in hippocampal cell count could be detected. Other studies emphasize anti-inflammatory properties of exercise with inhibition of microglial activation and proliferation (Mee-Inta et al., 2019).

1.3.2 Exercise in MS and EAE

EAE, as a widely used model of MS, offers the opportunity to investigate the influence of exercise on disease progression and hippocampal changes. Since EAE is externally induced, the acute phase of the disease can be correlated with immune cell infiltration in the CNS, although there is evidence of early neurodegenerative processes. In the chronic phase, however, neurodegeneration predominates (Voskuhl and MacKenzie-Graham, 2022). Studies

investigating the effect of an exercise intervention prior or at timepoint of EAE induction report some positive impacts. Previous Treadmill-running was shown to delay time of disease onset and attenuate disease severity in acute and chronic EAE (Souza et al., 2017, Einstein et al., 2018). Comparable results were obtained in a study that used previous forced swimming as an endurance exercise model (Bernardes et al., 2016). Both exercise models potentially induce stress in the animals, which in turn could bias the results, as discussed in detail later in this work **(see Chapter 5.2)**. Other investigators chose voluntary exercise models with simultaneous exercise intervention and EAE induction. Interestingly, two studies (Rossi et al., 2009, Rizzo et al., 2021) reported that time of disease onset was comparable between exercise and sedentary mice while a third study (Pryor et al., 2015) showed a delayed disease onset in the exercise group. A reduced disease severity in acute and chronic exercise EAE was consistent in all three studies.

Few studies have investigated cognitive and hippocampal changes associated with exercise intervention in EAE mice. Cognitive impairment appears early in EAE disease course and could be related to hippocampal neurodegeneration (Dutra et al., 2013, Kim et al., 2012). Voluntary exercise has been shown to counteract cognitive decline in EAE mice (Kim and Sung, 2017, Rizzo et al., 2021). Even before the onset of motor symptoms, exercise mice showed better performance in different cognitive tests than sedentary mice (Rizzo et al., 2021). Investigations of the HC have shown that exercise counteracts the impaired synaptic plasticity during EAE. Furthermore, microglial activation was significantly milder in exercise mice compared to sedentary mice with EAE (Rizzo et al., 2021). In chronic EAE, less demyelination, less cell apoptosis and an enhanced neurogenesis together with higher BDNF levels could be detected in the HC of exercise mice compared to sedentary mice (Kim and Sung, 2017).

A direct transfer of the animal experiments to MS patients is difficult. On the one hand, many models of the experiments, e.g. exercise intervention before disease onset, are not possible. On the other hand, there are very limited techniques to study the HC in living patients. Sandroff et al. (2016) reviewed existing studies on the impact of exercise on cognition in MS patients. In conclusion, there seem to be indications of a beneficial effects, but studies show to be inconsistent in design and cognition is often not the primary endpoint of existing studies. Furthermore, there were major differences in the methods used to measure cognition (Sandroff et al., 2016). Various studies have attempted to design standardized exercise models for trials in MS patients, but so far, no uniform method has become established (Dalgas et al., 2009, Petajan and White, 1999, Rosenkranz et al., 2023). In general, exercise training was found to have a positive effect on various symptoms of MS, such as fatigue, pain and cognition (Dalgas et al., 2019). The relapse rate in MS patients seems to be reduced due to exercise (Pilutti et al., 2014). But no study has yet been shown a positive effect on the progressive disability correlating best with ongoing neurodegeneration (Heesen and

Rosenkranz, 2022, Dalgas et al., 2022, Motl and Sandroff, 2022). One reason for the lack of evidence that exercise has disease-modifying properties in MS patients could be the design of previous studies (Heesen and Rosenkranz, 2022). To date, there are no biomarkers that reliably reflect neurodegenerative processes such as those underlying cognitive impairment in MS patients, even though the requirements for future biomarkers are already the subject of current considerations (Rosenkranz et al., 2023). MRI studies are suitable for investigating brain functions and brain volume in living humans. An MRI imaging study has shown that cardiorespiratory fitness correlates positively with the volume of certain GM regions. At the same time, performance in cognitive tasks was improved (Prakash et al., 2010). Specific measurements of hippocampal volume showed a positive correlation between volume and cardiorespiratory fitness in MS patients (Motl et al., 2015). Similarly, an aerobic exercise intervention in cognitively impaired MS patients led to an increase in hippocampal volume and significantly improved memory function (Leavitt et al., 2014). Treadmill walking was shown to improve hippocampal neuroplasticity measured by an MRI technique investigating mechanical properties of hippocampal tissue in line with improved learning and memory (Sandroff et al., 2017). There is evidence that regular physical activity can delay the onset of the first symptoms of MS, such as cognitive impairment indicating preventive properties (Cortese et al., 2018, Wesnes et al., 2018). Underlying mechanisms of exercise-induced neuroprotective properties in MS are widely unknown. Increased peripheral blood levels of the neurotrophic factor BDNF were measured in MS patient following an exercise intervention (Schulz et al., 2004, Castellano and White, 2008, Briken et al., 2016), potentially contributing to the observed effects of exercise.

1.4 Aims

Cognitive impairment is a cardinal symptom of MS and at least partly attributed to neurodegenerative processes in the HC. The underlying mechanisms are largely unknown and resulting histological alterations in the HC differentially described in the literature. The first step is therefore to define the histological changes in the HC in a mouse model of MS, the EAE. Exercise has a range of neuroprotective properties. A non-stress-related exercise intervention will be used to evaluate the impact on the EAE disease course and hippocampal structures to confirm the effectiveness of the exercise model. Subsequently, exercise-induced changes in gene expression in exclusively hippocampal neurons will be analyzed, offering the potential to develop therapeutic approaches to improve cognition in patients with MS.

2 Material

2.1 Reagents and chemicals

Table 1: Reagents and chemicals for animal experiments

Name	Company
CO2/O2 gas mixture (80%/20%)	SOL
D(+)-Saccharose ≥39,5%	Carl Roth
DietGel® Recovery	Clear H2O
Dulbecco's phosphate buffered saline (DPBS)	PAN-Biotech
Freund's adjuvant, incomplete	Becton, Dickinson and Company
Isofluran	Baxter
Ketanest® S 25mg/ml (Ketamine)	Pfizer Pharma
Mouse/rat MOG35–55 peptide, MEVGWYRSPFSRVVHLYRNGK	Peptides & elephants
Mycobacterium tuberculosis H37	Becton, Dickinson and Company
Nekrolyt® Salbe	CP-Pharma
Paraformaldehyde (PFA) reinst	Carl Roth
Pertussis toxin (Bordetella pertussis)	Millipore
Rompun® 2% (Xylazine)	Bayer
Tissue-Tek® O.C.T.TM Compound	Sakura

Table 2: Reagents and chemicals for genotyping

Name	Company
dNTP Mix (10mM)	Thermo Fisher Scientific
DreamTaq™ Hot Start Green DNA Polymerase, 5 U/µl	Thermo Fisher Scientific
DreamTaq™ Hot Start Green polymerase chain reaction (PCR) Master Mix (10x)	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA), 0.5 mM	Sigma-Aldrich
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
LiChrosolv® Water for chromatography	Merck
Primer	Biomers
QuickExtract™ DNA Extraction Solution	Lucigen
Roti®-Safe GelStain	Carl Roth
Tris ultrapure	Applichem
UltraPure™ Agarose	Invitrogen

Table 3: Reagents and chemicals for cell culture

Name	Company
Cytosine β-D-arabinofuranoside	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM), high glucose GlutamMAX™ Supplement	Thermo Fisher Scientific
DPBS sterile filtered (1x)	Pan-biotech
Fetal Calf Serum (FCS)	Roth
Gibco™ DMEM/F-12	Thermo Fisher Scientific
Gibco™ GlutaMAX™ Supplement	Thermo Fisher Scientific
Gibco™ HBSS, no calcium, no magnesium	Thermo Fisher Scientific
Gibco™ Opti-MEM™	Thermo Fisher Scientific
Gibco™ TrypLE™ Express Enzyme (1x)	Thermo Fisher Scientific
Gibco™ Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific
Penicillin-Streptomycin (10.000 U ml^{-1})	Invitrogen
PNGM™ Primary NeuronGrowth Medium BulletKit™	Thermo Fisher Scientific
Poly-D-Lysine hydrobromide	Sigma–Aldrich
Trypan Blue solution 0.4%	Sigma-Aldrich

Table 4: Reagents and chemicals for IHC on cryosections and microscopy

Name	Company
AffiniPure Fab Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch
Immersol™ 518 F	Zeiss
Normal Donkey Serum (NDS)	Merck
Pap Pen, Ready Probes™ Hydrophobic Barrier	Thermo Fisher Scientific
Paraformaldehyde reinst	Carl Roth
Roti®-Mount FluorCare 4',6-diamidino-2-phenylindole (DAPI)	Carl Roth
Triton-X® 100 reinst	Carl Roth

Table 5: Reagents and chemicals for IHC on paraffin sections

Name	Company
Acetic acid puriss.	Merck
Antibody Diluent	ThermocFisher Scientific
Bluing Reagent	Ventana Roche
Cell Conditioning Solution 1	Ventana Roche

Cell Conditioning Solution 2	Ventana Roche
Certistain®, Eosin Y (yellowish)	Merck
Ethanol 96%, 100%	Apotheke UKE
EZ Prep Concentrate (10x)	Ventana Roche
Hematoxylin	Ventana Roche
Hematoxylin solution acc. To Harris	Roth
Histofine® Simple Stain™ MAX PO (Rat, Mouse and Rabbit)	Nichirei Biosciences
Hydrochloric acid 25%	Merck
LCS (Predilute)	Ventana Roche
Lithium carbonate	Merck
Luxolechtblau MBS	Chroma-Gesellschaft Schmidt GmbH
Mayer´s hemalum solution	Merck
Normal goat serum	Cell Signaling Technology
OSTEOSOFT®	Merck
Phloxine B	Merck
Reaction buffer Concentrate (10x)	Ventana Roche
Tissue-Tek® Glas™ mounting medium	Sakura
Tissue-Tek® Paraffin Wax TEK III	Sakura
UltraView Universal 3,3'-diaminobenzidine (DAB) Detection Kit	Ventana Roche
Xylol, min 99.0%	TH. Geyer

Table 6: Reagents and chemicals for qPCR

Name	Company
2-Mercapthoethanol	Sigma-Aldrich
AllPrep® DNA/RNA Mini Kit	Qiagen
Ethanol ROTIPURAN® ≥99,8%	Carl Roth
RevertAid H Minus First Strand, cDNA Synthesis Kit	Thermo Scientific
RNAse-Free DNAse Set	Qiagen
RNase-Free Water	Qiagen
RNeasy® Mini Kit	Qiagen
TaqManTM Gene Expression Master Mix	Applied Biosystems

2.2 Antibodies

Table 7: Primary antibodies for IHC on cryosections

Name	Host	Clonality	Dilution	Company
Anti-alpha Actinin 4, HPA001873	rabbit	polyclonal	1:500	Merck
Anti-Calpain 1, ab155677	rabbit	polyclonal	1:500	Abcam
Anti-Doublecortin, ab18723	rabbit	polyclonal	1:500	Abcam
Anti-GFP, ab13970	chicken	polyclonal	1:1000	Abcam
Anti-Homer 1/2/3, 160 103	rabbit	polyclonal	1:500	Synaptic Systems
Anti-Iba1, 019-19741	rabbit	polyclonal	1:500	Wako
Anti-NeuN, ABN91	chicken	polyclonal	1:500	Millipore
Anti-NeuN, MAB377	mouse	monoclonal	1:200	Millipore/Chemicon
Anti-Synapsin 1/2, 106 004	guinea pig	polyclonal	1:500	Synaptic Systems
Anti-TRIM59, ab69639	rabbit	polyclonal	1:100	Abcam

Table 8: Primary antibodies for IHC on paraffin sections

Name	Host	Clonality	Dilution	Company
Anti-CD3, ab16669	rabbit	monoclonal	1:50	Abcam
Anti-Iba1, 019-19741	rabbit	polyclonal	1:1000	Wako

Table 9: Secondary Antibodies for IHC

Name	Clonality	Dilution	Company
Alexa Fluor® 488 Donkey Anti-chicken, 703-545-155	polyclonal	1:600	Jackson ImmunoResearch
Alexa Fluor® 488 Donkey Anti-guinea pig, 706-545-148	polyclonal	1:600	Jackson ImmunoResearch
Alexa Fluor® 488 Donkey Anti-mouse, ab150105	polyclonal	1:600	Abcam
Alexa Fluor® 647 Donkey Anti-rabbit, ab181347	polyclonal	1:600	Abcam
Cy™3 donkey Anti-chicken, 703-165-155	polyclonal	1:600	Jackson ImmunoResearch
Cy™3 Donkey Anti-guinea pig, 706-165-148	polyclonal	1:600	Jackson ImmunoResearch
Cy™3 donkey Anti-mouse, 715-166-150	polyclonal	1:600	Jackson ImmunoResearch

2.3 Primers

Table 10: Primers for qPCR

Name	Assay ID	Company
Actb	Mm00607939_s1	Taqman™ by Applied Biosystems
Actn4	Mm00502489_m1	Taqman™ by Applied Biosystems
Btk	Mm00442712_m1	Taqman™ by Applied Biosystems
Capn1	Mm00482964_m1	Taqman™ by Applied Biosystems
CD93	Mm00440239_g1	Taqman™ by Applied Biosystems
Fzd9	Mm01206511_s1	Taqman™ by Applied Biosystems
Gstk1	Mm00504022_m1	Taqman™ by Applied Biosystems
Kif26a	Mm01339746_m1	Taqman™ by Applied Biosystems
Tbp	Mm01277042_m1	Taqman™ by Applied Biosystems
Tex9	Mm00493619_m1	Taqman™ by Applied Biosystems
Trim59	Mm02527285_s1	Taqman™ by Applied Biosystems

2.4 Buffers, solutions and media

Table 11: Buffers, solutions and media

Name	Ingredients
4% PFA	Dissolve 4 g PFA in 100 ml 80°C Phosphate Buffer 0.1M cool to 4°C
Blocking solution	0.1% Triton X-100 10% NDS in 1 x Phosphate buffered saline (PBS)
Neuro-2A (N2A) medium	500 ml DMEM, high glucose GlutaMAX™ Supplement 10% FCS 100 U ml-1 Penicillin-Streptomycin 0,05% Amphotericin B
PBS, 10x, 7.4 pH	80g Natriumchlorid 2g Kaliumchlorid 17,8g Natriumhydrogenphosphat-Dihydrat

	2,7g Kaliumhydrogenphosphat 2,7g Kaliumhydrogenphosphat
Tris-acetate-EDTA (TAE), 50X	2 M Tris 0.05 M EDTA 5.7% Acetic Acid in ddH2O

2.5 Consumables

Table 12: Consumables

Name	Company
384 Well PCR Plate	Biozym Scientific
96 Well PCR-Plate	Sarstedt
Biosphere® Filter Tips	Sarstedt
Biosphere® SafeSeal Tubes	Sarstedt
C-Chip, Neubauer Improved Disposable Counting Chamber	NanoEnTek
Cell Culture Dishes	Thermo Fisher Scientific
Cell scrapers, handle length 16 cm	Sarstedt
CELLSTAR® Cell Culture Multiwell plates	Greiner Bio-One
CELLSTAR® Polypropylene Tubes, 15 ml and 50 ml	Greiner Bio-One
Cover Slips, Ø 12 mm	Thermo Scientific
Deckgläser Stärke 1, 24x50 mm	Carl Roth
Filter tips	Sarstedt
Microscope slides, Superfrost® Plus	Thermo Scientific
Pasteur pipette glass, 230 mm	Heinz Herenz Medizinbedarf
PCR plate sealing tape	Sarstedt
Pipette tips	Sarstedt
QIAshredder™ columns	Qiagen
RNAse Zap™	Invitrogen
SafeSeal Micro tubes	Sarstedt
Serological pipettes (sterile), 2ml, 5ml, 10ml and 25ml	Sarstedt
Shandon™ Einweg-Ausgießform	Thermo Scientific
StarGuard® Comfort gloves	Starlab
Superfrost Plus Microscope Slides	VWR
Syringes, needles, cannulas	BD and Braun
Tissue culture flask T-157, T-25	Sarstedt

2.6 Software

Table 13: Software

Name	Company
Adobe Illustrater	Adobe
ImageJ (Fiji)	NIH, Bethesda
Manual Whole Slide Imaging (ManualWSI)	Microvisioneer
Mendeley Reference Manager	Elsevier
Microsoft Excel	Microsoft
NanoDrop 1000 V3.8.1	Thermo Fisher Scientific
Prism 8	GraphPad Software
QuantStudio™ Real-Time PCR	Applied Biosystem
QuPath (0.2.0-m8)	GitHub
RQ Manager 1.2.1	Applied Biosystem
SDS 2.4	Applied Biosystem
Tbase Client 4Dv12sql	Tbase Server
ZEN black	Zeiss
ZEN blue	Zeiss

2.7 Equipment

Table 14: Equipment

Name	Company
7900HT Fast Real-Time PCR System	Applied biosystems
ASP300S Fully Enclosed Tissue Processor	Leica Biosystems
Biological Microscope CX43	Olympus
Biometra® Low Voltage Power Supply	Analytic Jena
Centrifuge 5417R	Eppendorf
Comfort Freezer (-20°C)	Liebherr
EG1160 Embedding Center, Dispenser + hot Plate	Leica Biosystems
Electrophoretic system	Peqlab
FlexCycler^2	Analytik Jena
Incubator (cell lines)	Memmert
Incubator (primary neurons)	Thermo Fisher Scientific
Intas Gel-documentation	Intas Science Imaging

Kimble®Kontes Disposable Pellet pestle	Merck
Kontes^{2} Pellet Pestle^{Tm}	DWK Life Sciences
LSM600 confocal laser scanning microscope	Zeiss
Microme HM 560 Cryostat	Thermo Fisher Scientific
Microscope CX21	Olympus
MR 3002 magnetic stirring hotplate	Heidolph
Multifuge® 3 S-R	Heraeus
Multifuge® 3L Centrifuge	Heraeus
NanoDrop® 1000 Spectrophotometer	Thermo Fisher Scientific
Perfusion System	Ismatec
Picus® NxT Electronical Pipettes	Sartorius
PIPETBOY acu 2	Integra Biosciences
Pipettes	Sartorius, Ep
QuantStudio^{TM} 6 Flex Real-Time PCR System	Applied biosystems
REAX 2000 Vortexer	Heidolph
Refrigerator (4°C)	Liebherr
RM25125 RTS The Essential Microtome	Leica Biosystems
Safe 2020 Biological Safety Cabinets	Thermo Fisher Scientific
Scale PM4000	Mettler Toledo
Sprout^{TM} Microcentrifuge	Biozym
Staining jars	Marienfeld
StainTray slide staining system	Merck
Surgical instruments	FST Fine Scientific Tools
Thermomixer 5436	Eppendorf
Thermomixer comfort, 1.5 ml block	Eppendorf
TSX Series ultra-low freezers	Thermo Fisher Scientific
Ultra-low Temperature Freezer (-80°C)	Sanyo
VACUBOY Handoperator	Integra Biosciences
VARIOLAB Mobilien W90 extraction tables	Waldner
VENTANA BenchMark XT	Roche
Water bath	GFL Gesellschaft für Laborbedarf
Wild M3Z Stereo Microscope	Leica Biosystems
Willy Wheel	Karlie

3 Methods

3.1 Mice

C57BL/6 wildtype mice were obtained from the Jackson Laboratory and *Lypd1-L10-eGFP* mice (background strain C57BL/6) were generated by The Rockefeller University and purchased from the Jackson Laboratory. Animals were housed and bred in the Central Animal Facility at the University Medical Center Hamburg-Eppendorf (UKE). Shortly before starting the experiments, mice were transferred to the local Animal Facility at the INIMS where the experiments were conducted. All mice were kept under specific pathogen-free conditions in individually ventilated cages with access to food and water ad libitum. All animal experiments were approved by the local ethics committee (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz in Hamburg) and approved by the Tierversuchsantrag 112-17.

3.2 Genotyping

In order to determine the genotype, biopsies were taken from the ears of mice at the age of three weeks. Biopsies were first lysed in QuickExtract™ DNA Extraction Solution for 6 minutes at 65°C and 500 rpm followed by 2 minutes at 98°C and 350 rpm. The lysate was then cooled down on ice before the extracted gDNA could be used for PCR experiments. 23 µl PCR reaction mix was prepared containing 16.03 µl deionized H_2O, 2.5 µl DreamTaq™ Hot Start Green PCR Master Mix (10x), 0.5 µl dNTP Mix (10nM), 0.9 µl of each the forward (CGG CGA GCT GCA CGC TGC CGT CCT C) and reverse (CCT ACG GCG TGC AGT GC TTC AGC) *egfp* primer and 1 µl of each the forward (AGA GGG AAA TCG TGC GTG AC) and reverse (CAA TAG TGA TGA CCT GGC CGT) *β-actin* primer and 0.17 µl DreamTaq™ Hot Start Green DNA Polymerase. Finally, 2 µl of the gDNA lysate were added to the PCR reaction mix and the PCR was performed using the FlexCycler^2. First, the mixture incubated for 2 minutes at 94°C followed by 38 cycles of the following program. 30 seconds at 94°C, 30 seconds at 64°C and 30 seconds at 72°C. Subsequently, the mixture incubated for 5 minutes at 72°C and cooled down to 4°C. PCR products were separated by gel electrophoresis using a 1.5 % agarose gel in 1 x TAE buffer. Gel und buffer were heated up until agarose was completely dissolved and Roti®-Safe GelStain was added in a 1:20000 ratio. The solution was then filled into the gel chambers and incubated for 45 minutes until polymerization of the agarose. Thereafter, gel pockets were filled with 10 µl DNA GeneRuler 1 kb DNA Ladder or 20 µl of the PCR reaction mix containing amplified gDNA, respectively. The gel was run at 140 mV for approximately 20 minutes. The PCR resulted in gel bands at 350 bp (*egfp*) and 150 bp (*β-actin*) (genotyping was performed by student assistants of the INIMS).

3.3 Experimental autoimmune encephalomyelitis

Mice for EAE experiments without prior exercise experiments were all wildtype females with an age of 8-10 weeks. They were immunized by subcutaneous injection of 200 µg MOG_{35-55} peptide in complete Freund´s adjuvant containing 4 mg/ml *Mycobacterium tuberculosis.* Additionally, 200ng pertussis toxin was injected intraperitoneally at the same day and 48 h later (EAE´s were performed by Charlotte Schubert and Nicola Rothammer). Mice for EAE experiments following eight weeks of an exercise experiment were all wildtype males. Regarding their higher age with 14 weeks, they were immunized by subcutaneous injection of 100 µg MOG_{35-55} peptide in complete Freund´s adjuvant containing 2 mg/ml *Mycobacterium tuberculosis.* Additionally, 100ng pertussis toxin was injected intraperitoneally at the same day and 48 h later. Mice were scored daily starting at day six postimmunization by the following clinical signs: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; 5, premorbid or dead. Animals with a clinical score of ≥ 4 were killed according to the Animal Welfare Act. During EAE experiments, soft food was provided ad libitum.

3.4 Exercise setting

Mice for exercise experiments were wildtype and *Lypd1-L10-eGFP* mice of both genders, which were randomly assigned to either the exercise or the sedentary cohort. Animals joining the exercise cohort were individually housed in type III cages with free access to a functional running wheel they could voluntarily use during the whole experiment. Animals in the sedentary cohort were housed in identical cages but with magnetically blocked running wheels. To measure the running distance of exercise mice, wheels were connected to a counting instrument. With knowledge of the wheels´ extent (Ø: 13 cm), the running distance in km/24h could be calculated for each exercise mouse (exercise experiments without subsequent EAE induction performed with help from Lukas Raich). The experimental set-up was built by Torsten Renz.

3.5 Perfusion

Mice were deeply anesthetized by the intraperitoneal injection (100 µl per 10 g body weight) of Ketamine/Xylazine (12 mg ml^{-1}/ 1.6 mg ml^{-1}) in 1 x PBS. Subsequently, mice were transcardially perfused with cold 1 x PBS for 3 minutes to eliminate the blood. In order to generate tissue for IHC, perfusion with 1 x PBS was followed by the perfusion with 4% PFA

for 7 minutes to fixate the tissue (perfusion was performed with help of Vanessa Vieira and Nina Kursawe).

3.6 Histological analysis

3.6.1 Tissue preparation

Tissue preparation for cryosections

For immunohistochemical experiments on cryosections, brains were manually dissected from the skull und post-fixated in 4% PFA for another 30 minutes. Brains were dehydrated and cryoprotected in 30% sucrose solution in 1 x PBS for at least one week at 4°C and thereafter embedded in TissueTek® for storage at -80°C. The whole brains were cut into 12 µm thick cryosections using a cryostat, mounted on glass slides and stored at -80°C.

Tissue preparation for paraffin sections

Brains inside the cranial bone were dissected from mice, postfixated in 4% PFA for 24 h and stored in 1 x PBS, both at 4°C. Further tissue preparation procedures and immunohistochemical stainings were performed by the UKE Mouse Pathology Facility. For decalcification, brains incubated in Osetosoft for 10 days, subsequently dehydrated overnight inside the tissue processor and embedded in paraffin by the Embedding center. Brains were cut with a microtome into 3 µm thick slices and transferred on glass slides. For paraffin removal, slices were incubated in Xylol for 20 minutes and thereafter in decreasing dilutions of ethanol three minutes each (100%, 96%, 90%, 80%, 70%). Finally, slides were rinsed for 5 minutes in ddH20.

3.6.2 Immunohistochemistry on paraffin and cryosections

Immunohistochemical DAB staining for IBA1 and CD3 on cryosections and paraffin sections was performed by the automated Ventana BenchMark XT. The system automatically deparaffinized slices, stained them with antibodies against IBA1 and CD3 and visualized the staining by the Universal DAB detection kit and associated kits and reagents following the manufacturer´s instructions. In order to counterstain DAB stained tissue, sections incubated with Bluing Reagent and Hematoxylin for 4 minutes each. Sections were manually scanned with a microscope using the ManualWSI software. For fluorescent immunohistochemical staining, cryosections were air dried, washed in 1 x PBS for 5 minutes and thereafter incubated with blocking solution (10% NDS in 0.1% Triton X-100 in 1 x PBS) for 45 minutes at room temperature. When antibodies from mouse hosts were used, a further blocking step to avoid

unspecific binding of antibodies was performed. Therefore, AffiniPure Fab Fragment Donkey Anti-Mouse IgG (H+L) was diluted 1:200 in 0.1% Triton X-100 and sections were incubated for 1 h at room temperature followed by a washing step in 1 x PBS. After this intermediate step or after incubation with blocking solution, respectively, slices were incubated with primary antibodies diluted in 3% NDS in 0.1% Triton X-100 in 1 x PBS overnight at 4°C. The next day, sections were washed three times in 1 x PBS and incubated with fluorophore-labelled secondary antibodies diluted in 3% NDS in 0.1% Triton X-100 in 1 x PBS for 2.5 h at room temperature. The stained sections were washed three times in 1 x PBS and finally mounted in a medium containing DAPI for nuclei counterstaining and covered by coverslips. Sections dried protected from light overnight at room temperature before they were stored at 4°C. All images were taken with a laser scanning microscope (LSM 600, Zeiss) and identical settings within each experiment.

3.6.3 Immunohistochemical analyses

Quantification of adult and newborn neurons

For quantification of adult and newborn neurons, cryosections of the brain were stained for NeuN as a biomarker for adult neurons and DCX to visualize neuronal precursor cells. Z-stack images covering the thickness of the whole slices and representing the areas CA1, CA3 and the DG of the left HC were taken using a x20 magnification and 0.5 Zoom. Analysis was performed with the ImageJ software. Adult and newborn neurons were manually counted within regions of interest (ROI) of same size and comparable localization between mice (two ROIs within CA1, one ROI for each CA3 and the DG). Up to two hippocampal sections were analyzed per animal. Counterstaining with DAPI was used to identify and separate neuronal cell bodies.

Quantification of synaptic density

The quantification of synaptic density was performed by staining cryosections of the brain for Synapsin 1/2 as a presynaptic and Homer 1/2/3 as a postsynaptic marker. Four images per animal at comparable localization within the stratum radiatum of the hippocampal CA (two images in the CA1 region and one image in each CA2 and CA3) were taken with x63 magnification. ROIs of same size and comparable localization were analyzed with ImageJ within these images. A synapse was defined as the overlap of pre- and postsynapse represented by colocalization of Synapsin1/2 and Homer 1/2/3 and detected by the image calculator plugin. Subsequently, stained particles of each partner as well as colocalizations were automatically quantified with the analyze particles plugin. Mean fluorescence intensity (MFI) of both Synapsin 1/2 and Homer 1/2/3 was measured in the regions of colocalization.

Quantification of T cells and microglial activation

To visualize immune cells, antibodies against CD3 for T cells and IBA1 for microglia were used on deparaffinized (CD3 and IBA1) or frozen (IBA1) brain sections. For quantification, the left HC in up to three sections per animal was analyzed with the QuPath software. The entire hippocampal parenchyma was defined with help of the Coronal Allen Mouse Brain Atlas and circled by the polygon tool in QuPath. Within this area and along the meninges, $CD3^+$ cells were manually counted. To quantify microglial activation, the IBA1 stained area was automatically quantified by the positive pixel count tool after preprocessing the image in order to estimate stain vectors.

Immunohistochemical validation of bacTRAP candidates

For the immunohistochemical validation of candidates from the hippocampal bacterial artificial chromosome-translating ribosome affinity purification (bacTRAP), cryosections of *Lypd1-L10-eGFP* mice were stained for Tripartite Motif-Containing Protein 59 (TRIM59), Alpha-Actinin-4 (ACTN4) and Calpain-1 (CAPN1). Images from up to three slices of the left HC and comparable regions in CA1 were taken with x40 magnification and 0.7 Zoom for TRIM59 validation and 0.5 Zoom for ACTN4 and CAPN1 validation, respectively. Analysis was performed with the ImageJ software. To quantify protein expression, the MFI of all neurons within the CA1 region in an image was measured using the threshold and analyze particles plugins. Subsequently, the MFI in exclusively GFP^+ neurons representing the neurons analyzed by the bacTRAP was determined. For this, the Image Calculator plugin was used to analyze colocalizing regions of GFP and candidate protein.

3.7 Cell culture

3.7.1 Primary hippocampal neurons

If not mentioned otherwise, all procedures were carried out at 4°C or on ice. In order to culture primary hippocampal neurons, pregnant mice were sacrificed by CO_2 inhalation when embryos reached embryonic day 16.5. Embryos were collected from the uterus, placed in Hank´s Balanced Salt Solution (HBSS) and decapitated. HC were dissected from brain and placed in HBSS until they settled to the bottom of the tube. For cell dissociation, HC were incubated for 6 minutes at 37°C in Trypsin/EDTA. HC were placed in DMEM containing FCS until settling to the bottom, incubated in HBSS and dissociated using a pipette followed by centrifugation for 2 min at 600g. The supernatant was aspirated and palleted cells were dissolved in PNGM. 10 µl of the cell solution were mixed with equal amounts of Trypan blue whereafter cells were counted in a Neubauer chamber. Subsequently, cells in PNGM were seeded with a density of

50.000-100.000 cells/cm^2 to Poly-D-Lysine coated wells. For RNA purification, cells were seeded in 6 well plates without coverslips. To inhibit growth of glial cells Cytosin-D-Arabinofuranoside was added to the wells 72h later. After 14 days in culture, cells were either used for qPCR experiments (experiments with primary hippocampal neurons were performed with help of Lukas Raich).

3.7.2 N2A cells

Cells from the neuroblastoma cell line N2A were purchased from ATCC Manufacturing. Cells were cultured in TC flasks with N2A medium. When 80% confluence of the flask was reached, cells were passaged as followed. The medium was discarded and cells were washed in 1x DPBS followed by an incubation step in TrypLE^{TM} at 37°C in order to detach cells from the flask surface. After 3 minutes, N2A medium was added and cell density was determined by counting in a Neubauer chamber as described previously. Suspended cells were subsequently cultured in new TC flasks. Up to 25 passages were performed until cells were used for RNA purification as already described for primary hippocampal neurons.

3.8 RNA/DNA purification and cDNA synthesis

3.8.1 RNA purification from cells

In order to prepare cells for RNA purification, they were cultured on 6 well plates with a density of 50.000-100.000 cells/cm^2 as described previously, washed in 1 ml cold 1 x DPBS and subsequently removed from wells by cell scrapers in a second wash of cold 1 x DPBS. Cells in DPBS were centrifugated at 3500 x g for 5 minutes and supernatant was removed. The cell pallet was snap-frozen in liquid nitrogen and stored at -80°C until further use. RNA isolation was performed using the RNeasy Mini Kit and according to the manufacturer´s instructions. Shortly, pellets were solved in 350 µl RLT buffer containing 2-mercaptoethanol and homogenized by QIAshredder columns. After adding 350 µl 70% ethanol to the homogenized lysate and a centrifugation step in a RNeasy spin column, DNAse digestion was performed using the RNAse-Free DNAse Set and according to the manufacturer´s instructions. Shortly, 80 µl DNAse I incubation mix was added to the spin column followed by an incubation time of 15 minutes. RNA purification was now performed by several washing steps with different solutions and consecutive centrifugation. Finally, the purified RNA was eluted in 50 µl RNAse-free water and total RNA content was measured by the NanoDrop.

3.8.2 cDNA synthesis

0.1 ng - 5000 ng RNA per 11 µl RNAse free water were reverse transcribed using the RevertAid cDNA Synthesis Kit. All steps were performed according to the manufacturer´s instruction. Shortly, 1µl random hexamer primer was added to the isolated RNA followed by 5 minutes of incubation at 65°C. Subsequently, a mixture containing Reaction Buffer, RNase inhibitor RiboLock, 10mM dNTP mix and RevertAid H Minus M-MuLV Reverse Transcriptase was added. This mixture was incubated for 5 minutes at 25°C, 60 minutes at 42°C and finally 5 minutes at 70°C. Total cDNA concentration was measured by the NanoDrop. cDNA concentration was adjusted by dilution with RNAse free water to the sample containing the lowest amount of cDNA.

3.9 Quantitative real-time polymerase chain reaction

For qPCR, the cDNA was diluted 1:10 or 1:5. All steps were performed according to the manufacturer´s instruction. Summarized, one PCR reaction mix for each gene of interest was prepared containing 2 x TaqMan Gene Expression Master Mix, RNAse free water and the respective TaqMan Gene Expression Assay. 8µl PCR reaction mix was added to each well of a 96-well or 384-well plate followed by 2 µl cDNA. Triplicates or Duplicates were used for each gene of interest and each cDNA sample. As a negative control, duplicates of each reaction mix and RNAse free water instead of cDNA were added to a respective number of wells. The qPCR was performed with the SDS 2.4 software for 96-well plates or the QuantStudio™ Real-Time PCR software for 384-well plates. The thermal profile was adjusted as followed. Initially, the plates incubated for 2 minutes at 50°C followed by a denaturation step for 10 minutes at 95°C. The annealing step was carried out for 1 minute at 60°C. Up to 45 cycles were performed. The mean CT value from the triplicates of each gene of interest was calculated and analyzed by the ΔΔCt method. mRNA expression was normalized to the housekeeping gene TATA-binding protein *(Tbp).*

3.10 Statistical analysis

Graphs were created and statistics performed with Prism 8. All data are presented as mean values ± standard error of the mean (SEM). Parametric data were tested for normality by the Shapiro-Wilk normality test. Differences between normally distributed, parametric data sets were analyzed by the unpaired, two-tailed Student´s t-test. Non-parametric and parametric but not normally distributed data were analyzed by the two-tailed Mann-Whitney test. For analysis of the Kaplan-Meyer curve, the log-rank (Mantel-Cox) test was used. $P < 0.05$ was considered as significant (*) and $P < 0.01$ as highly significant (**).

4 Results

4.1 Characterization of hippocampal changes in EAE

The overall aim of this work is to define neuroprotective properties of an exercise intervention potentially attenuating the EAE disease course. Of particular interest is the HC with its various and essential functions. In EAE mice, spatial memory deficits are present even before motor symptoms occur even though EAE is known to primarily injure the spinal cord (Dutra et al., 2013, Kim et al., 2012). This could be related to a hippocampal impairment, but the nature and extent of these changes are still poorly and differentially described in the literature **(see Chapter 1.2.3)**. For the matter of comparability, it is necessary to define the hippocampal changes resulting from the EAE protocol illustrated above to evaluate protective effects of an exercise intervention. Therefore, the degree of inflammation and neurodegenerative alterations were analyzed at different time points of EAE via IHC to visualize distinct proteins. **Figure 4.1** illustrates the experimental design and timepoints of hippocampal analysis.

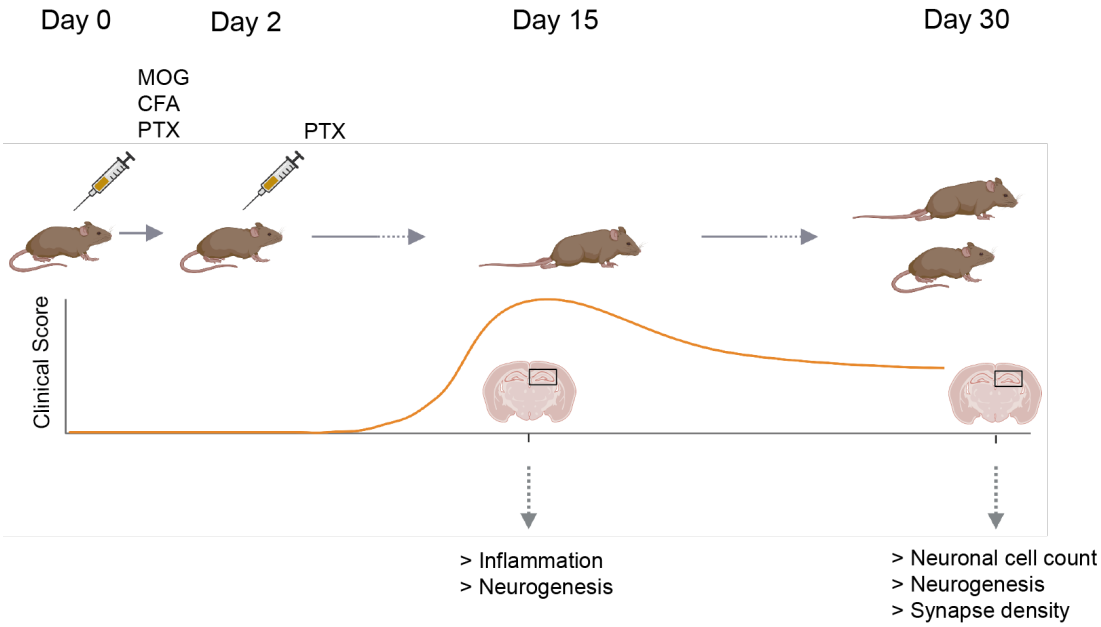


Figure 4.1 Characterization of hippocampal changes in EAE

Experimental design to identify hippocampal alterations during the EAE disease course. EAE was induced in mice with MOG_{35-55} peptide in complete CFA containing *M. tuberculosis* and PTX. A second dose of PTX was injected at day 3 postimmunization. Mice were sacrificed at either day 15 (acute EAE) or day 30 (chronic EAE) postimmunization to analyze the parameters shown underneath the graph via IHC. The graph represents the EAE disease course measured by a clinical scoring system schematically over 30 days starting at day 0 as the day of immunization. The clinical symptoms of mice are also shown by the hind limb and tail paralysis of mice on top of the graph (Created with BioRender.com).

4.1.1 Hippocampal inflammation in acute EAE

To confirm an inflammatory response in the HC in our EAE model, immunohistochemical staining for T lymphocytes and microglia was performed on hippocampal slices from mice at day 15 postimmunization and healthy mice. Day 15 is considered as the acute phase of EAE with the main part of immune cell infiltration and activation. Clinically, mice are rapidly starting to develop symptoms around day 12 postimmunization, starting with tail and hind limb weakness. To detect potential immune cell infiltration in the HC, wildtype mice were immunized with MOG_{35-55} peptide as described above and killed at day 15 postimmunization simultaneously with healthy controls of the same age. All mice were perfused and the brain was processed in order to use it for immunohistochemical staining as previously described. Thereafter, coronal paraffin sections containing the hippocampal formation were stained for CD3 to visualize T lymphocytes **(Figure 4.2A)** according to the DAB staining protocol. The numbers of $CD3^+$ cells were then manually counted in meninges and hippocampal parenchyma. T lymphocytes showed to predominantly infiltrate the hippocampal meninges in EAE mice and there was a significantly increased count of T lymphocytic infiltration detectable in the meninges and hippocampal parenchyma compared to healthy controls, where rarely one $CD3^+$ cell was detectable **(Figure 4.2B)**. To analyze activation of resident immune cells in the HC, microglia were visualized by staining of IBA1 **(Figure 4.2C)**. Subsequently, the IBA1-stained area was quantified and is presented as the percentage of the whole hippocampal region. In acute EAE, microglia occupied a significantly larger area in comparison to heathy state **(Figure 4.2D)**.

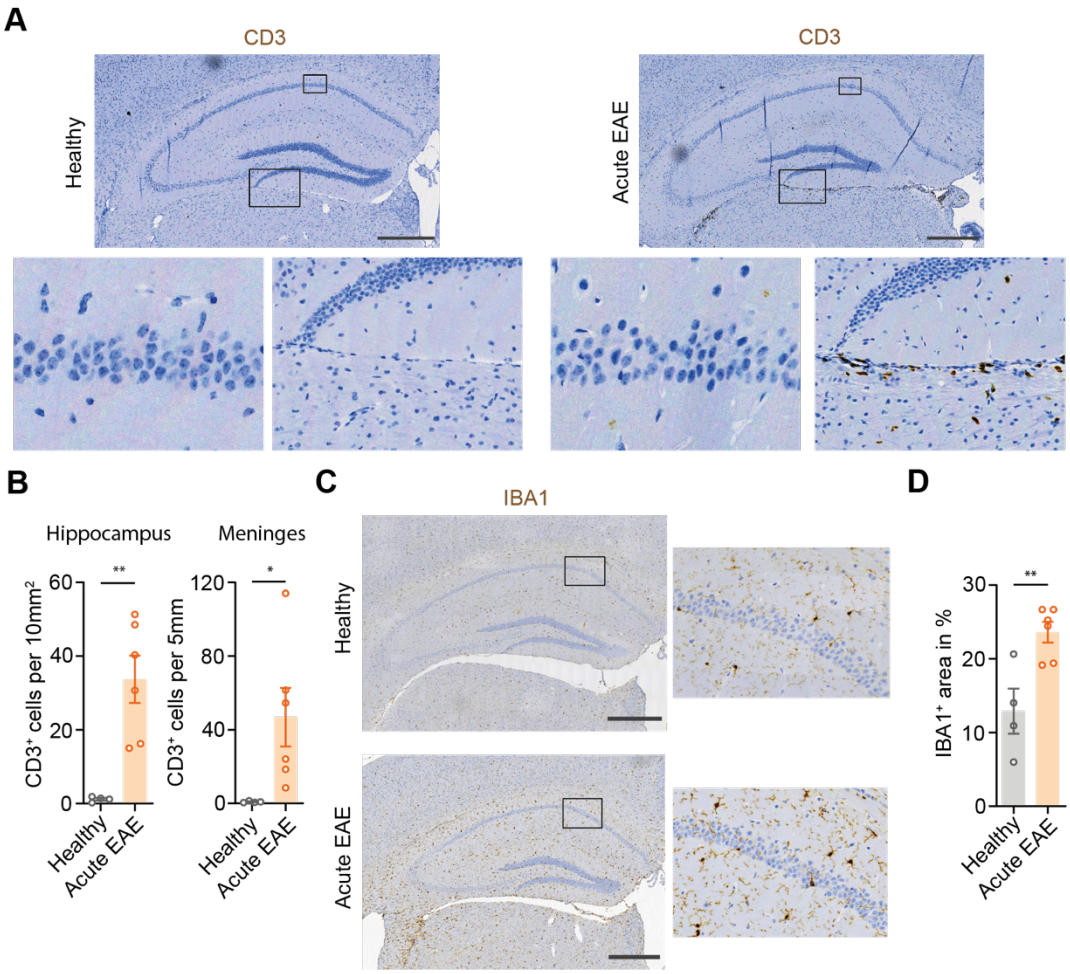


Figure 4.2 Hippocampal inflammation in acute EAE

A, Representative immunohistochemical DAB-stainings of T lymphocytes (CD3) in HC and meninges of mice with acute EAE (15 days postimmunization) compared to healthy mice performed by UKE Mouse Pathology Facility. Scale bar, 1000µm. **B**, Quantification of T lymphocytes (CD3) in HC and meninges of mice with acute EAE compared to healthy mice (two-tailed Student´s t-test; n = 4 healthy versus n = 6 acute EAE mice; HC, t(10) = 4.043, P = 0.0037; meninges, t(10) = 2.314, P = 0.0494). **C**, Representative immunohistochemical DAB-stainings of microglia (IBA1) in the HC of mice with acute EAE compared to healthy mice performed by UKE Mouse Pathology Facility. Scale bar, 500µm. **D**, Quantification of the IBA⁺ area in % of the whole hippocampal formation of mice with acute EAE compared to healthy mice (two-tailed Student´s t-test; n = 4 healthy versus n = 6 acute EAE mice; t(10) = 3.572, P = 0.0073). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

4.1.2 Reduction of adult hippocampal neurons in chronic EAE

With knowledge that the HC is affected by immune cell infiltration and activation during EAE, further immunohistochemical analysis were performed in order to define potential hippocampal neurodegenerative processes during the EAE disease course. Of particular interest is the question whether the number of mature neurons is altered in EAE. Wildtype mice were again immunized with EAE reagents and sacrificed at day 30 postimmunization together with healthy

controls of the same age. This is considered to represent the chronic phase of EAE. Clinically, mice are partly recovering after the acute phase in terms of their clinical symptoms. Nevertheless, neurodegenerative processes can be studied best in the chronic phase (Voskuhl and MacKenzie-Graham, 2022). After sacrificing and perfusing the animals, brains were dissected from skull and processed in order to perform IHC on cryosections containing the hippocampal formation. Mature neurons were visualized using a primary ab binding NeuN. After adding the secondary ab and additional staining of all nuclei (DAPI), $NeuN^+$ cells were visualized using a laser scanning microscope. Representative images were taken from the CA1 as well as CA3 part of the HC **(Figure 4.3A)** and $NeuN^+$ cells were counted within regions of defined size and comparable localization for each mouse **(Figure 4.3B)**. Interestingly, there was a highly significant reduction of $NeuN^+$ cells detectable within the CA1 region of the HC exclusively. Additionally, the thickness of CA1 was measured and compared between healthy and EAE animals. Nevertheless, this effect could not reach significance **(Figure 4.3C)**.

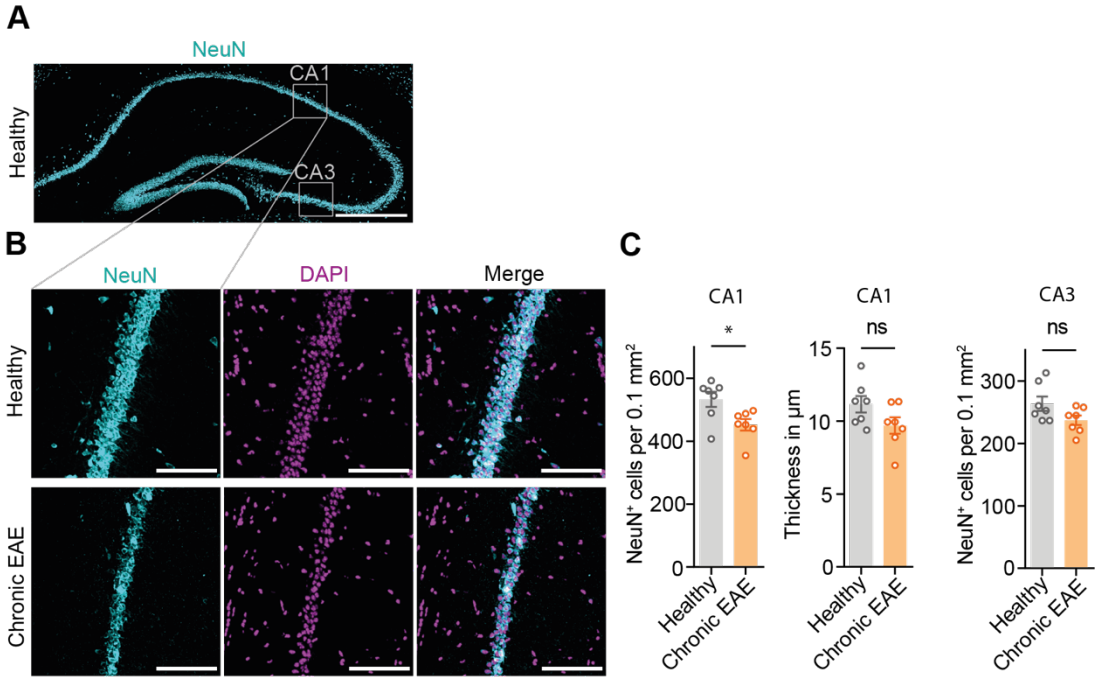


Figure 4.3 Hippocampal cell count in chronic EAE

A, Immunohistochemical staining of adult neurons (NeuN) in the HC. To analyze changes in neuronal cell count, neurons in the labelled regions of CA1 and CA3 of the HC were counted in healthy and mice with chronic EAE (30 days postimmunization). Scale bar, 500µm **B**, Representative immunohistochemical stainings of adult neurons (NeuN) and neuronal nuclei (DAPI) to distinguish neuronal cell bodies in CA1 of mice with chronic EAE compared to healthy mice. Scale bar, 50µm. **C**, Quantification of adult neurons in CA1 and CA3 and average thickness of the CA1 region of mice with chronic EAE compared to healthy mice (two-tailed Student´s t-test; n = 7 healthy versus n = 7 chronic EAE mice; neurons CA1, $t(14) = 2.675$, $P = 0.0202$; thickness CA1, $t(14) = 1.827$, $P = 0.0926$; neurons CA3, $t(14) = 1.923$, $P = 0.0785$). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

4.1.3 Impairment of hippocampal neurogenesis in chronic EAE

Neurogenesis is an extremely interesting phenomenon especially in terms of an exercise intervention. Due to divergent opinions in the literature **(see Chapter 1.2.3)**, the analysis of neurogenesis dynamics during EAE was indicated. One of the questions addressed by this investigation is whether the neuronal cell loss during EAE can be explained by neurodegeneration only or whether impaired neurogenesis also contributes to this observation. DCX was used as a marker for newborn and NeuN as a marker for adult neurons. EAE-induced wildtype mice were sacrificed either on day 15 (acute EAE) or day 30 (chronic EAE) postimmunization together with age-matched healthy controls respectively. Fluorescence-based IHC was used to visualize these markers on cryosections of the mouse brain. Since neurogenesis takes place in the DG of the HC, representative images were taken from this region **(Figure 4.4A)**. DCX^+ cells which did not show any NeuN positivity were counted within the images compared between healthy and EAE animals **(Figure 4.4B)**. There was no difference detectable between healthy mice and mice with acute EAE whereas mice with chronic EAE showed a significant decline of newborn neurons **(Figure 4.4C)**. This indicates, that chronic EAE somehow impairs neurogenesis in the DG of wildtype mice.

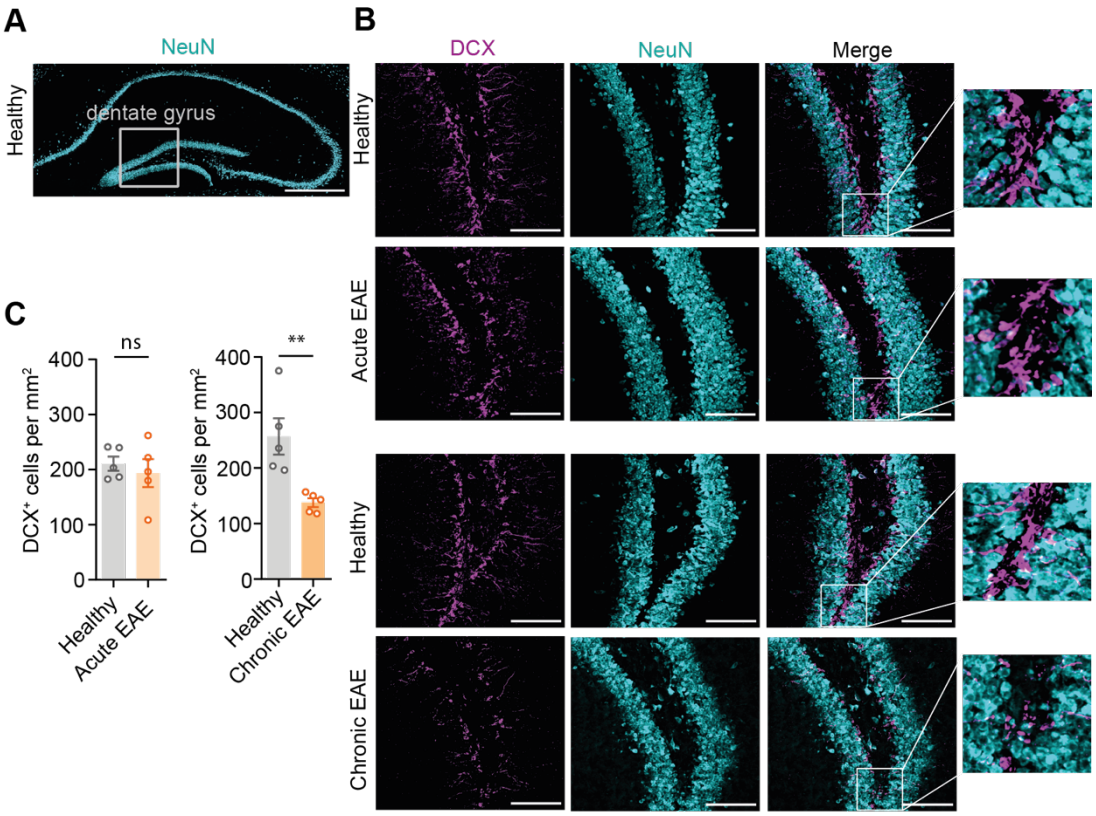


Figure 4.4 Hippocampal neurogenesis in acute and chronic EAE

A, Immunohistochemical staining of adult neurons (NeuN) in the HC. To analyze changes in neurogenesis, newborn neurons in the labelled region (DG) of the HC were counted in healthy mice and mice with acute EAE (15 days postimmunization) and chronic EAE (30 days postimmunization). Scale bar, 500µm. **B**, Representative immunohistochemical stainings of newborn neurons (DCX) and adult neurons (NeuN) in the DG in healthy mice and mice with acute and chronic EAE. Scale bar, 100µm. **C**, Quantification of newborn neurons in the DG of mice with acute and chronic EAE compared to healthy mice (two-tailed Student´s t-test; n = 5 healthy versus n = 5 acute EAE mice, t(10) = 0.6009, P = 0.5645; n = 5 healthy versus n = 5 chronic EAE mice, t(10) = 3.561, P = 0.0074). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

4.1.4 Reduction of hippocampal synaptic density in chronic EAE

In order to investigate possible alteration of cell-cell interaction, the synaptic density was determined in wildtype mice with chronic EAE compared to healthy mice. EAE was induced as described before and animals were perfused at day 30 of EAE together with age-matched healthy mice. Fluorescence-based IHC on cryosections was used to detect presynapse and postsynapse. An ab against Synapsin 1/2 was used to visualize the presynapse and Homer 1/2/3 as a marker for the postsynapse. Images were taken from comparable regions in the stratum radiatum in CA1, CA2 and CA3 **(Figure 4.5A)** and automatically analyzed by the puncta analyzer from ImageJ (Fiji). First, the count of exclusively Synapsin $1/2^+$ particles as well as exclusively Homer $1/2/3^+$ particles was determined for each area. Interestingly, there was no difference detectable in CA1, CA2 and CA3 regarding Synapsin $1/2^+$ and Homer $1/2/3^+$

particles **(Figure 4.5C-D)**. Furthermore, synaptic contacts were quantified defined as the overlap of Synapsin 1/2^{+} and Homer 1/2/3^{+} particles. Only in CA1 there was a significant reduction of synaptic contacts detectable **(Figure 4.5E).**

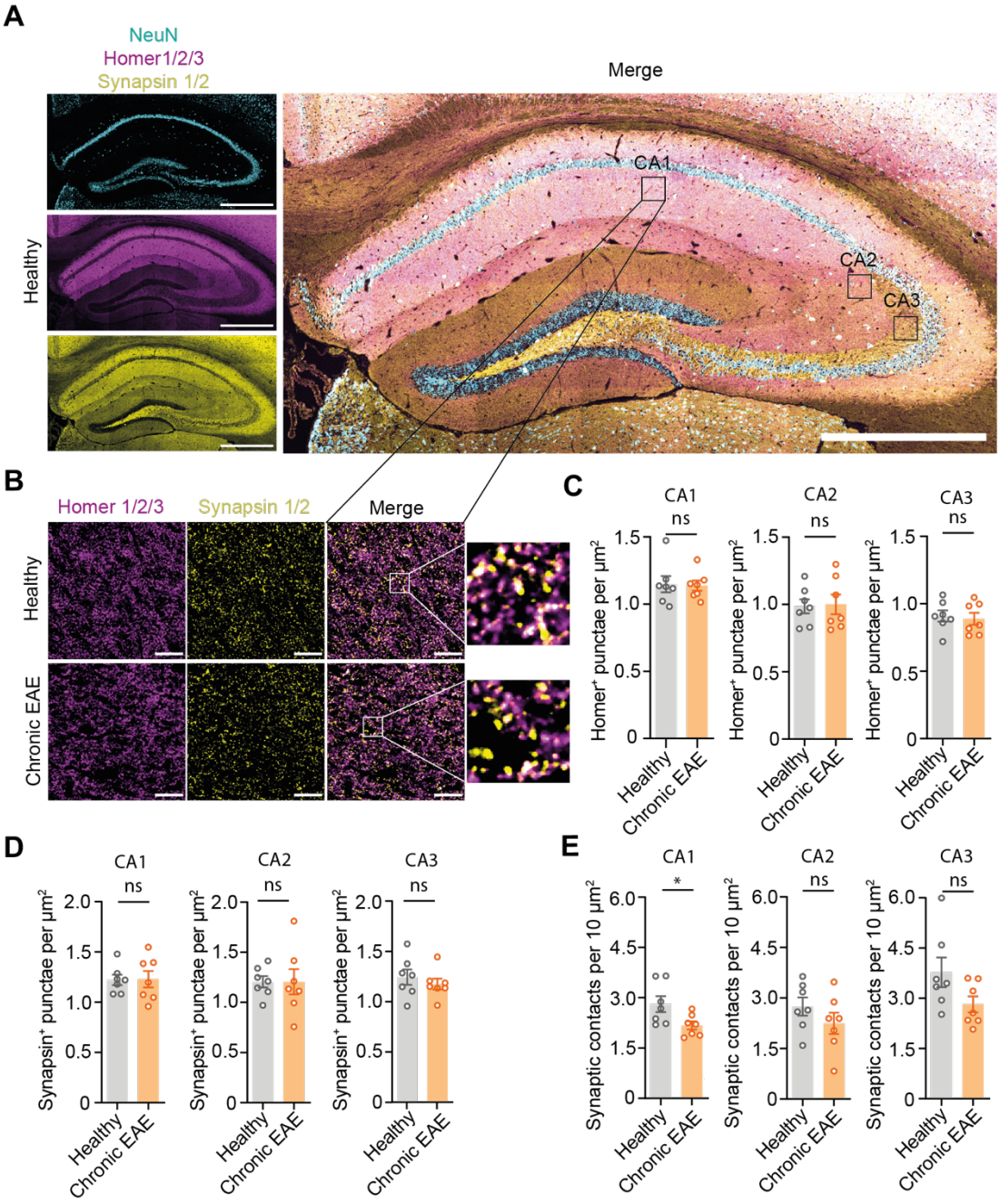


Figure 4.5 Hippocampal synaptic denstity in chronic EAE

A, Immunohistochemical staining of presynapse (Synapsin 1/2), postsynapse (Homer 1/2/3) and neurons (NeuN) in the HC. To analyze changes in synaptic density, synapses in the labelled regions (CA1, CA2 and CA3) of the HC were counted in healthy mice and mice with chronic EAE (30 days postimmunization). Scale bar, 500µm. **B**, Representative immunohistochemical stainings of presynapse (Synapsin 1/2) and postsynapse (Homer 1/2/3) and visualization of synaptic contacts by merging the stainings in healthy mice and mice in chronic EAE. Scale bar, 10µm. **C-E**, Quantification of postsynapses

(C), presynapse (D) and synapses (E) in CA1, CA2 and CA3 in mice with chronic EAE compared to healthy mice (two-tailed Student´s t-test; n = 7 healthy versus n = 7 chronic EAE mice; postsynapse CA1, t(14) = 0.1583, P = 0.8769; postsynapse CA2, t(14) = 0.1585, P = 0.8767; postsynapse CA3, t(14) = 0.3460, P = 0.7353; presynapse CA1, t(14) = 0.06394, P = 0.9501; presynapse CA2, t(14) = 0.001213, P = 0.9991; presynapse CA3, t(14) = 0.7423, P = 0.4722; synapses CA1, t(14) = 2.391, P = 0.0341; synapses CA2, t(14) = 1.191, P = 0.2566; synapses CA3, t(14) = 1.898, P = 0.0820). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

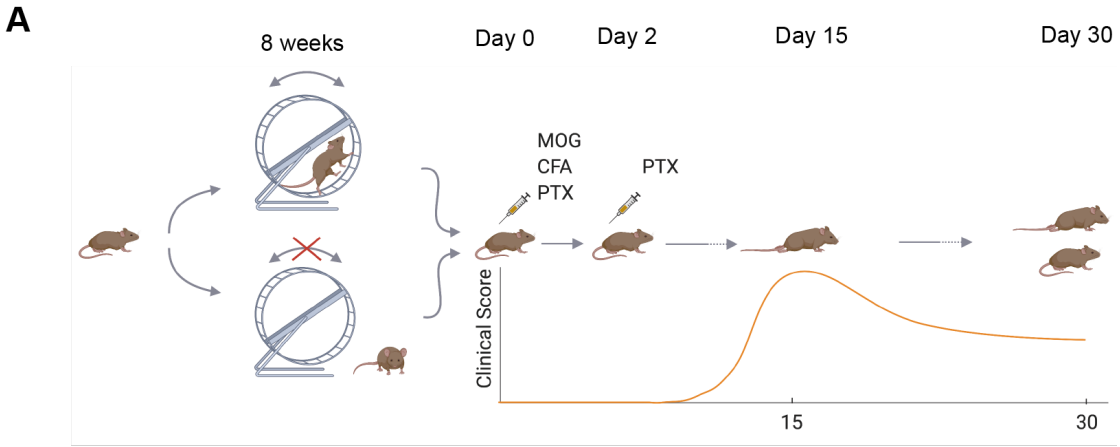
Taken together, the HC presents to be negatively affected by EAE. In acute EAE, there is a significant increase in immune cell infiltration and activation detectable. During chronic EAE, neurodegenerative processes can be seen characterized by impaired hippocampal neurogenesis as well as a decrease in neuronal cell count and synaptic density in the CA1 region exclusively. This provides the basis for the further experiments investigating potential protective effects of an exercise intervention with focus on the CA1 region.

4.2 Impact of exercise on the EAE disease course

4.2.1 Exercise ameliorates the EAE disease course

To investigate whether a chronic exercise intervention ameliorates the clinical EAE disease score, EAE was induced subsequent to an exercise versus sedentary intervention. For this, mice were individually housed in cages with either a blocked or functional running wheel for eight weeks. To avoid a stressful exercise environment, mice in the exercise EAE cohort could use their running wheel voluntarily during the whole experiment including the 30 day of EAE **(Figure 4.6A)**. Functional running wheels were connected to a counting instrument to control and record the running distance twice a week **(Figure 4.6B)**. One mouse was excluded due to a markedly small running distance. After this period of eight weeks, EAE was induced in exercise and sedentary animals by injection of MOG_{35-55} peptide in complete Freund´s adjuvant containing *Mycobacterium tuberculosis* together with pertussis toxin. Mice were sacrificed at day 30 postimmunization. The Kaplan-Meier graph illustrates EAE disease progression in the exercise and sedentary cohort shown as the percentage of healthy mice during EAE disease course **(Figure 4.6C)**. Two mice in the exercise EAE cohort did not develop any EAE symptoms and had therefore to be excluded for further statistical analysis because reasons for the failure of EAE induction other than the exercise intervention could not be ruled out. Altogether, the exercise EAE cohort presented with a significantly milder disease course calculated as the cumulative score over 30 days of EAE **(Figure 4.6D)**. Furthermore, the maximum score which is normally reached in the acute EAE was significantly decreased in mice with exercise intervention **(Figure 4.6E)**. However, analysis of the day of disease onset, meaning the day on which mice initially showed typical EAE symptoms, did not reveal significant differences between groups **(Figure 4.6F)**. A cardinal symptom in mice with EAE is weight loss often starting before clinical symptoms such as tail weakness. Mice were weighed

every day starting at day six postimmunization. Analysis of the normalized weight development revealed that animals in the exercise EAE cohort suffered from a significantly lower weight loss. They furthermore recovered from initial weight loss and regained the weight from day of immunization. This was not the case for the sedentary EAE cohort **(Figure 4.6G)**. Taken together, mice with eight weeks of voluntary exercise intervention preceding EAE induction showed an ameliorated disease course regarding clinical score and weight development.


A
8 weeks
Day 0
Day 2
Day 15
Day 30
MOG
CFA
PTX
PTX
Clinical Score
15
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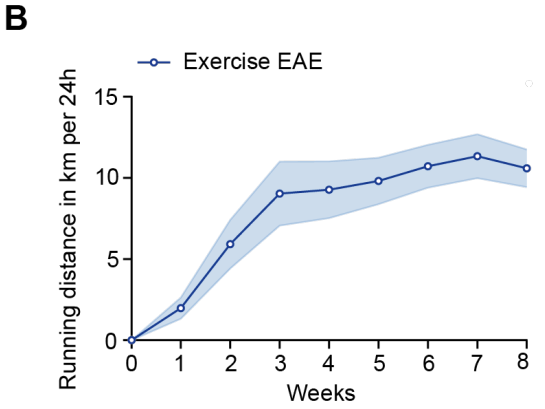
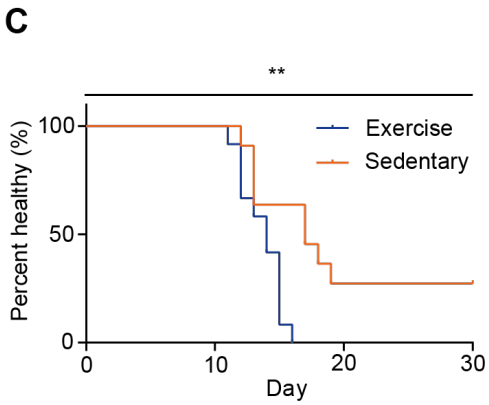
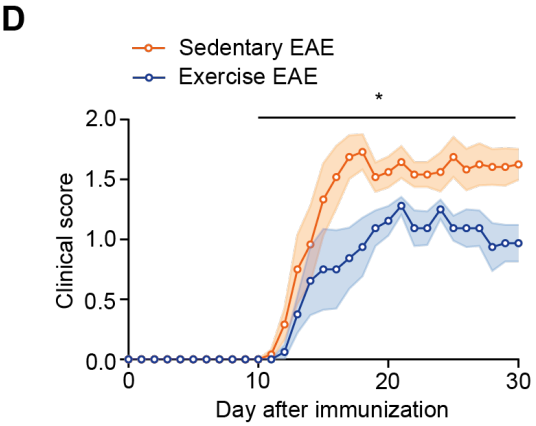
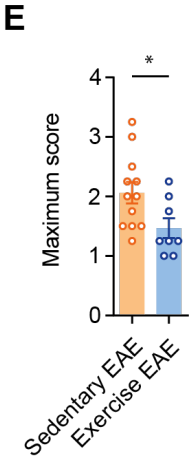
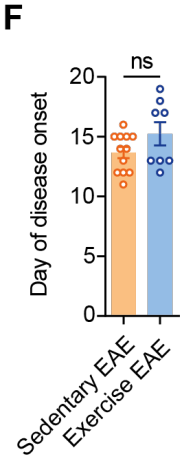

B
Exercise EAE
Running distance in km per 24h
Weeks


C
**
Exercise
Sedentary
Percent healthy (%)
Day


D
Sedentary EAE
Exercise EAE
*
Clinical score
Day after immunization


E
*
Maximum score
Sedentary EAE
Exercise EAE


F
ns
Day of disease onset
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Exercise EAE

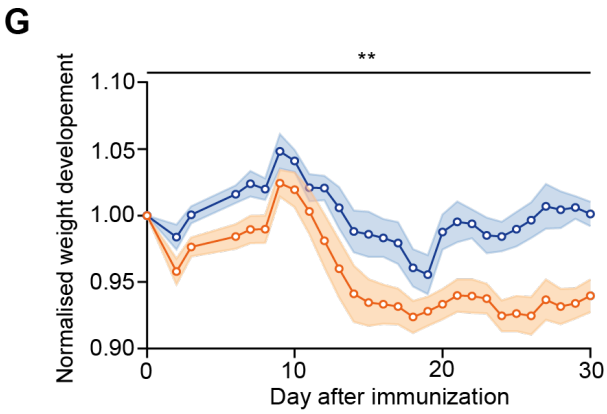
G
**
Normalised weight developement
Day after immunization

Figure 4.6 Experimental setup and impact of exercise on the EAE disease course

A, Experimental design to identify alterations in the clinical EAE disease course in exercise mice compared to sedentary mice. Mice were randomly assigned to either the exercise or sedentary cohort. After eight weeks, all mice were induced with EAE as described before and sacrificed at day 30 postimmunization (Created with BioRender.com). **B**, Demonstration of the running distance of exercise mice during the eight weeks preceding EAE induction measured by a counting instrument connected to the running wheels (n = 23). **C**, Kaplan- Meier graph illustrating EAE disease progression in the exercise and sedentary cohort shown as the percentage of healthy mice during EAE disease course (log-rank (Mantel-Cox) test; n = 11 exercise versus n = 12 sedentary mice; Chi square = 7.535, P = 0.0061). **D**, Clinical scores of exercise and sedentary mice undergoing EAE (Mann Whitney test of cumulative EAE score; n = 8 exercise versus n = 12 sedentary mice, Man-Whitney U = 21, P = 0.0387). **E-F**, Maximum score (E) and day of disease onset (F) of exercise and sedentary mice undergoing EAE (two-tailed Student´s t-test; n = 8 exercise versus n = 12 sedentary mice; maximum score, t(20) = 2.288, P = 0.0345; day of disease onset, t(20) = 1.624, P = 0.1218). **G**, Normalized weight development of exercise and sedentary mice undergoing EAE from day of immunization until day 30 postimmunization (Mann Whitney test of normalized weight development; n = 8 exercise versus n = 12 sedentary mice, Man-Whitney U = 15, P = 0.0096). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

4.3 Exercise-induced hippocampal changes

4.3.1 Morphological hippocampal changes due to exercise

Due to the multiple and complex effects of exercise on the EAE disease course, the analysis of exercise-induced changes in the HC without EAE was conducted to concentrate on neuroprotective effects in the absence of inflammatory impacts. For this, immunohistochemical analysis was performed on brain slides from mice after an exercise intervention compared to sedentary mice. As described before, mice were randomly assigned to a cage with either a functional or blocked running wheel and individually housed for eight weeks **(Figure 4.7A)**. Mice were sacrificed and cryosections of the HC were analyzed for microglial activation as described before to identify potential influences of exercise on hippocampal inflammation. There was no significant difference detectable between groups **(Figure 4.7B)**. Another cohort of mice underwent the same experimental protocol and was sacrificed in order to determine changes in neuronal cell count, amount of neurogenesis and synaptic density via fluorescence-based IHC. Interestingly, only hippocampal neurogenesis was significantly enhanced **(Figure 4.7C)** while there was only a trend for the numbers of adult neurons. The synaptic density was not altered by the exercise intervention **(Figure 4.7D-G)**.

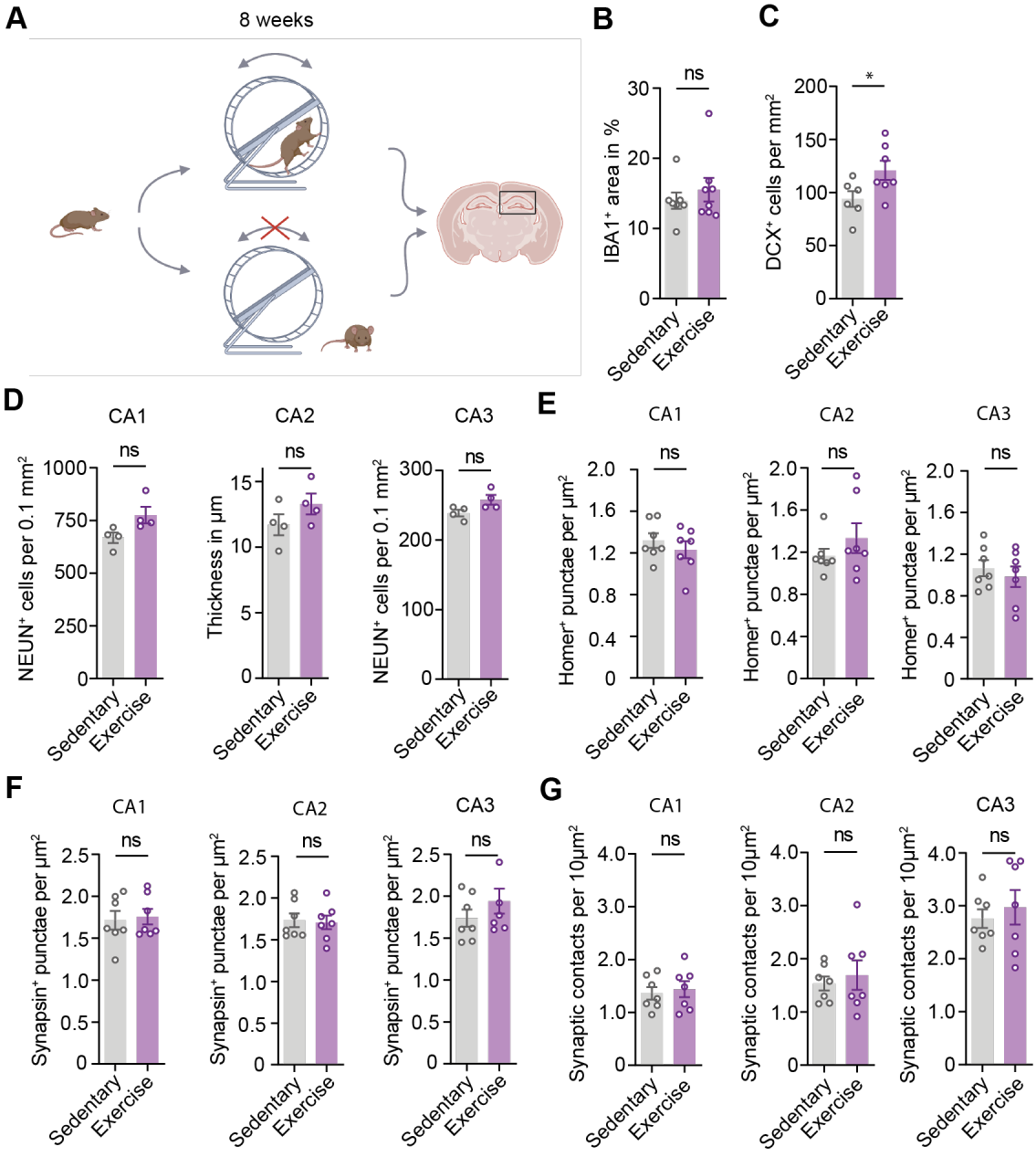

Figure 4.7 Hippocampal changes due to exercise

A, Experimental design to identify hippocampal alterations due to an exercise intervention. Mice were randomly assigned to either the exercise or sedentary cohort for eight weeks and sacrificed to analyze the HC via IHC. **B**, Quantification of the IBA$^+$ area in % of the whole hippocampal formation of exercise compared to sedentary mice (Mann Whitney test; n = 8 exercise versus n = 7 sedentary mice, Man-Whitney U = 27, P = 0.9551). **C**, Quantification of newborn neurons in the DG of exercise compared to sedentary mice (two-tailed Student´s t-test; n = 7 exercise versus n = 6 sedentary mice, t(13) = 2.261, P = 0.0450). **D**, Quantification of adult neurons in CA1 and CA3 and average thickness of the CA1 region of exercise compared to sedentary mice (two-tailed Student´s t-test; n = 4 exercise versus n = 4 sedentary mice; neurons CA1, t(8) = 2.321, P = 0.0593; thickness CA1, t(8) = 1.406, P = 0.2093; neurons CA3, t(8) = 2.286, P = 0.0623). **E-G**, Quantification of postsynapses (E), presynapses (F) and synapses (G) in CA1, CA2 and CA3 in exercise compared to sedentary mice (two-tailed Student´s t-test; n = 7 exercise versus n = 7 sedentary mice; postsynapse CA1, t(14) = 0.8219, P = 0.4272; postsynapse CA2,

t(14) = 1.067, P = 0.3068; postsynapse CA3, t(14) = 0.6556, P = 0.5244; presynapse CA1, t(14) = 0.2862, P = 0.7796; presynapse CA2, t(14) = 0.2103, P = 0.8369; presynapse CA3, t(14) = 1.113, P = 0.2876; synapses CA1, t(14) = 0.3945, P = 0.7001; synapses CA2, t(14) = 0.5183, P = 0.6137; synapses CA3, t(14) = 0.5755, P = 0.5756). Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

4.3.2 Hippocampal gene expression after exercise

In addition to the analysis of morphological exercise-induced hippocampal alterations, investigation of changes in neuronal gene expression in hippocampal neurons due to exercise was conducted. For this purpose, the bacTRAP method was used to isolate the cell-type specific mRNA of exclusively hippocampal neurons as described above (Heiman et al., 2014). Briefly, mRNA was isolated from hippocampal tissue from *Lypd1-L10-eGFP* mice after eight weeks of voluntary exercise compared to sedentary mice. In this mouse line, the ribosomal protein L10 is transcribed in fusion with eGFP under the promoter *Lypd1* which is specifically expressed in hippocampal neurons. Via isolation, cell-specific ribosome-bound mRNA of hippocampal neurons could be analyzed and compared between groups by overrepresentation analysis of biological process gene ontology (GO) terms. BacTrap experiment and evaluation was performed by Lukas Raich and results are not shown in detail at this point. Comparison of gene expression profiles visualized in heat maps revealed interesting candidate genes up- or downregulated in hippocampal neurons following the exercise intervention. First, validation of these genes via qPCR was conducted to confirm mRNA expression in hippocampal neurons, hippocampal tissue and N2A cells (experiments were performed with help of Lukas Raich).

Validating the expression of candidate genes in hippocampal neurons via qPCR

BacTRAP is a new and specific method to analyze mRNA expression. To validate gene expression of interesting candidate genes showing altered expression due to exercise in primary hippocampal neurons, embryos were collected from the uterus of pregnant mice at embryonic day 16.5. and sacrificed. HC were dissected from brain and hippocampal cells were dissociated and dissolved. Neurons were seeded in 6 well plates for RNA purification using the RNeasy Mini Kit according to the manufacturer´s instructions. Next, RNA was transcribed into cDNA and used for qPCR. To verify gene expression, respective TaqMan Gene Expression Assays for *Cd93, Btk, Fzd9, Gstk1, Pax2, Trim59, Tex9, Kif26a, Capn1* and *Actn4* were added and expression was normalized to the housekeeping gene *Tbp* as demonstrated in **Figure 4.8**. Furthermore, mRNA from hippocampal neurons isolated from HC of *Lypd1-L10-eGFP* mice during the bacTRAP experiment performed by Lukas Raich as well as mRNA form cultured N2A cells was isolated, measured and transcribed into cDNA. Again, gene expression of the same genes mentioned above was quantified via qPCR and relative expression was normalized to *Tbp* **(Figure 4.8)**. Interestingly, *Actn4*, *Capn1* and *Trim59* were the most highly

expressed candidate genes in hippocampal neurons from *Lypd1-L10-eGFP* mice and N2A cells **(Figure 4.8)**. As literature research revealed interesting neuronal functions as discussed later in this work, these three genes were selected in order to identify neuroprotective properties potentially contributing to the ameliorated EAE disease course after an exercise intervention.

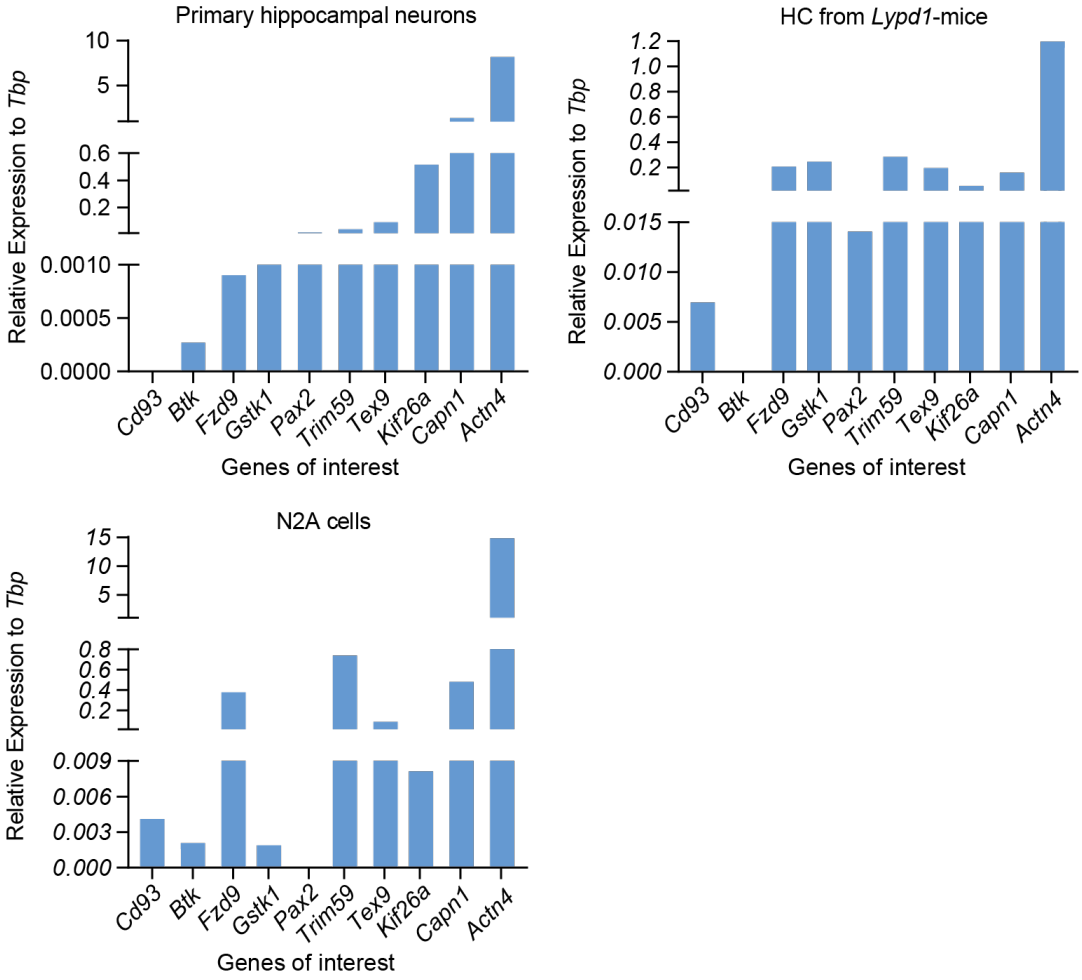

Figure 4.8 Validation of bacTRAP candidates via qPCR

Gene expression of *Cd93, Btk, Fzd9, Gstk1, Pax2, Trim59, Tex9, Capn1, Actn4* in relation to the housekeeping gene *Tbp*. Relative expression was measured in primary hippocampal neurons, hippocampal tissue *Lypd1*-mice and N2A cells.

Validating the expression of candidate protein in hippocampal neurons via IHC.

To identify whether expression of *Actn4*, *Capn1* and *Trim59* was not only altered on a mRNA level after chronic exercise, IHC was performed on frozen brain sections from *Lypd1-L10-eGFP* mice containing the hippocampal formation in order to detect changes in protein expression. Mice were sacrificed subsequent to eight weeks of an exercise versus sedentary intervention as described before together with mice for bacTRAP experiment performed by

Lukas Raich. Mice were perfused and brains were processed in order to use them for fluorescence-based IHC on cryosections. Slides were stained with primary antibodies against ACTN4, CAPN1 and TRIM59 followed by visualization via secondary antibodies. Images were taken from comparable regions in CA1 **(Figure 4.9A)**. To quantify protein expression, the MFI of respectively ACTN4, CAPN1 and TRIM59 staining in all neurons was determined and compared between exercise and sedentary mice. Furthermore, the MFI of the same proteins was measured in exclusively GFP^+ neurons in order to analyze cells which were isolated and analyzed in the bacTRAP. Similar to changes in the bacTRAP (results not shown), the expression of TRIM59 derived from the fluorescence intensity presented to be downregulated in the HC from exercise mice whereas the expression of CAPN1 was upregulated after an exercise intervention even though differences could not reach significance. Interestingly, the expression of ACTN4 did not show any differences between groups **(Figure 4.9E-G)**.

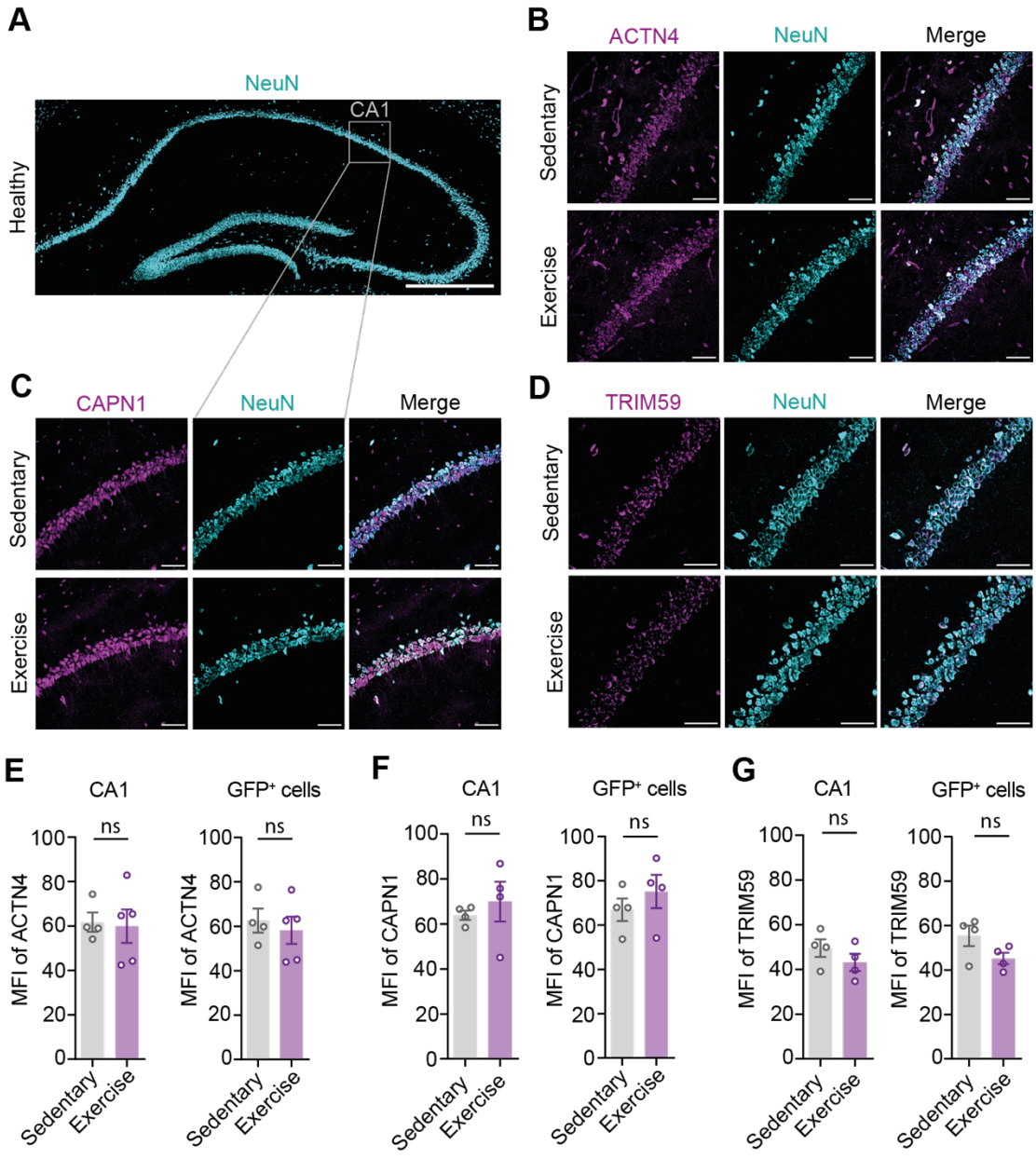

Figure 4.9 Protein expression of candidate genes in hippocampal neurons

A, Immunohistochemical staining of neurons (NeuN) in the HC. To analyze changes in protein expression, the MFI of ACTN4, CAPN1 and TRIM59 was quantified in neurons in the labelled region of CA1 in exercise versus sedentary mice. Scale bar, 500µm. **B-D,** Representative immunohistochemical stainings of neurons (NeuN) and ACTN4 (B), CAPN1 (C) and TRIM59 (D) in the CA1 region in exercise and sedentary mice. Scale bar, 100µm. **E-G**, Quantification of the MFI of ACNT4 (E), CAPN1 (F) and TRIM59 (G) in CA1 neuronal cells and only GFP⁺ cells in exercise versus sedentary mice (two-tailed Student´s t-test; n = 5 exercise versus n = 4 sedentary mice; MFI ACNT4 all cells, t(9) = 0.1921, P = 0.8532; MFI ACNT4 only GFP⁺ cells, t(9) = 0.5228, P = 0.6173; n = 4 exercise versus n = 4 sedentary mice; MFI CAPN1 all cells, t(8) = 0.6675, P = 0.5293; MFI CAPN1 only GFP⁺ cells, t(8) = 0.8998, P = 0.4029; n = 4 exercise versus n = 4 sedentary mice; MFI TRIM59 all cells, t(8) = 1.166, P = 0.2880; MFI TRIM59 only GFP⁺ cells, t(8) = 1.909 P = 0.1049. Results are presented as mean value +/- s.e.m. *P < 0.05. **P < 0.01.

5 Discussion

5.1 Hippocampal changes in EAE

5.1.1 EAE as a model for neurodegeneration

EAE is a widely used model to study MS pathology. As with any animal model, results cannot be directly transferred to humans. When investigating neurodegenerative changes in EAE, the pathophysiology must be considered. Monophasic EAE is triggered by the induction of a peripheral immune response that secondarily spreads to the CNS. Compared to the pathophysiology of MS, it corresponds most closely to the *outside-in* model. The immune response in EAE is directed against CNS structures, more specifically MOG, a component of myelin sheaths (Stromnes and Goverman, 2006). The destruction of myelin and thus demyelination is a central process in MS, which allows a certain comparability with EAE (Compston and Coles, 2008). Nevertheless, antigen presentation and thus subgroup of activated T cells differ. In EAE $CD4^+$ lymphocytes dominate the inflammation due to antigen presentation on major histocompatibility complex (MHC) class II molecules, whereas in MS antigens are mainly presented on MHC class I molecules and therefore activate $CD8^+$ lymphocytes.

Neurodegeneration is defined by neuronal and axonal damage and neurodegenerative processes play a decisive role in EAE (Friese et al., 2014). Even though there is evidence for early neurodegeneration even before motor symptoms occur (Mancini et al., 2017), the chronic phase of EAE, in this study defined as day 30 postimmunization, is particularly suitable for investigating neurodegenerative processes. This phase can be compared with SPMS, in which neurodegenerative processes also define the progressive disability (Constantinescu et al., 2011). The knowledge of many neurodegenerative mechanisms of MS **(see Chapter 1.1.2)** derives from EAE studies. It must be mentioned that in some studies different time points postimmunization were chosen for the analysis of neurodegenerative changes, which could limit the comparability of the results. For example, Giannakopoulou et al. (2017) analyzed hippocampal alterations in chronic EAE at day 30 postimmunization while Novkovic et al. (2015) chose day 40-45 postimmunization. In accordance to previous studies, the infiltration of immune cells was investigated at day 15 postimmunization as time of maximum symptom severity. Most studies, including this work, see symptom onset around the day 12 postimmunization with peak symptom severity around the day 15, which can be characterized as acute EAE (Constantinescu et al., 2011). Simplified, the pathophysiology at this point can

be compared to an acute relapse of RRMS with acute neuroinflammation mediated by infiltrating and resident immune cells.

Due to these observations, EAE is considered as a suitable model for further studies investigating inflammatory induced neurodegenerative processes even though the aforementioned aspects must be considered.

5.1.2 Hippocampal damage

Inflammation

The first aim of this work was to define a hippocampal phenotype during EAE that can provide the basis for subsequent investigations. In acute EAE, the HC was shown to be affected by inflammation compared to healthy mice **(Figure 4.2)** although in comparison to the spinal cord immune cell infiltration is much lower. This confirms the involvement of the HC in acute CNS inflammation. Interestingly, the inflammation is characterized by both lymphocyte infiltration and increased microglial activation. Most lymphocytes, visualized by staining of the lymphocytic marker CD3, were localized around the meninges adjacent to the HC, consistent with the pervious observation that the CNS, which is normally immune privileged, is infiltrated by lymphocytes due to a disruption of the BBB (Minagar and Alexander, 2003). In addition and as described by Mori et al. (2014), the parenchyma also showed a lymphocytic infiltration, which was not seen in other studies (Ziehn et al., 2010). A possible explanation for the deviating results could be that Ziehn et al. (2010) only examined a section of the CA1. Kyran et al. (2018) performed a subregional analysis and described lymphocytic infiltration mainly in the meninges but also in specific hippocampal areas with omission of the CA1 region. Microglial activation was examined by quantification of IBA1 staining **(Figure 4.2C)**. It is known that microglia fulfill diverse functions in the CNS and have both pro- and anti-inflammatory influences. A distinction between M1 microglia, which are more pro-inflammatory, and M2 microglia, with anti-inflammatory character, has not been made at this point (Guo et al., 2022b). However, it is known that chronic activation of microglia all in all maintains progressive neurodegeneration in MS and EAE (Di Filippo et al., 2016, Colasanti et al., 2016). In line with previous studies (Ziehn et al., 2010, Di Filippo et al., 2016, Di Filippo et al., 2013), microglia showed an activated morphology with a significantly larger expansion area **(Figure 4.2D)**. This chronic inflammation could be one factor driving progressive hippocampal neurodegeneration.

Neurodegeneration

As already described above, particularly CA1 and CA3 are essential components of the trisynaptic signaling pathway and thus the basis of memory formation **(see Chapter 1.2.1)**. For this reason, these two regions were examined more closely with regard to

neurodegenerative changes, more precisely alterations of neuronal cell count and synaptic density in chronic EAE. Subregional analysis revealed a significant loss of $NeuN^+$ cells in the CA1 region at day 30 postimmunization. In CA3, no significant alterations were seen **(Figure 4.3)**. Previous studies reported a significant volume loss (Ziehn et al., 2010) and a 40% loss of pyramidal cells in the CA1 region (Kurkowska-Jastrzębska et al., 2013). However, Kurkowska-Jastrzębska et al. (2013) used a different EAE model with injection of reactive lymphocytes, so comparability is limited. Neuronal cell loss may be associated with existing results showing increased apoptosis in the HC (Kim and Sung, 2017). Another aspect could be reduced neurogenesis, this connection is discussed later in this chapter. The successful demonstration of hippocampal cell loss in CA1 may explain the observations of cognitive impairment in EAE mice (Novkovic et al., 2015). Further studies could correlate the amount of CA1 neuronal loss with severity of the cognitive impairment to further strengthen the link between these two findings. LTP and LTD as functional markers of synaptic connections have already been investigated many times with differing results (Di Filippo et al., 2018). However, there is a lack of evidence to what extent the number of synapses and thus the connectivity between neurons is altered. Synaptic contacts, measured as the overlap of Synapsin and Homer as pre- and postsynaptic proteins, were significantly reduced, again in the CA1 region **(Figure 4.5)**. Interestingly, the individual pre- and postsynapses were not significantly altered. It appears that EAE causes processes that prevent the formation of synaptic contacts. All and all and consistent with previous studies, it was also demonstrated here that the CA1 region in particular is affected by neurodegeneration (Rocca et al., 2018).

Neurogenesis

It is known to be difficult to study AHN, as markers are very susceptible to tissue processing and neuronal stem cells can develop into different cell types, making it difficult to predict the maturation process (Moreno-Jimenez et al., 2019). Studies investigating AHN in EAE show very different results, which is why an investigation of the process in the EAE model used here was indicated. In this work, the microtubule-associated protein DCX was chosen for studying AHN due to its specificity for newly generated neurons and expression during a range of differentiation stages (Couillard-Despres et al., 2005, Moreno-Jimenez et al., 2019). Interestingly, and partly in contrast to previous results, hippocampal neurogenesis was significantly reduced in chronic EAE, whereas no significant changes were observed in acute EAE **(Figure 4.4)**. The idea that increased AHN could exist as an attempt to compensate for the damaging processes, particularly in acute EAE, has existed for some time. Giannakopoulou et al. (2013) did indeed observe an increased number of $BrdU^+$ cells in the hippocampal DG in acute EAE, but further revealed that the subgroup of DCX^+ cells tended to be lower than in healthy mice, although this observation did not reach significance. The proliferating cells therefore do not appear to develop into neurons, which could indicate a

malfunction of maturation in this direction. However, the underlying mechanisms causing this observation are still unknown, which makes it difficult to develop therapeutic strategies. One explanation for the insufficient increase in neurogenesis in acute EAE could be early neurodegenerative processes in the HC, which impair the sensitive process of AHN. Furthermore, inflammation has also been shown to have a detrimental effect on hippocampal neurogenesis (Monje et al., 2003). Contrary to the reduction in newborn neurons in chronic EAE described here, Giannakopoulou et al. (2017) saw an increase in DCX^+ neurons at day 30 postimmunization. This could at least partly be explained by different EAE induction protocols, as Giannakopoulou et al. (2017) applied significantly fewer amounts of MOG and did not inject any *Mycobacterium tuberculosis*. It is possible that neuroprotective mechanisms can counteract neurodegeneration in this mild EAE. The results of other studies support the observation that neurogenesis is reduced in chronic EAE (Aharoni et al., 2005, Guo et al., 2010). In addition to suboptimal environmental conditions in EAE, a possible cause could also be a lack of neurotrophins such as BDNF. BDNF was shown to primarily be elevated in acute EAE, maybe counteracting neurodegenerative processes, with subsequent decline in brain tissue (Bernardes et al., 2013). Addressing neurogenesis therapeutically to potentially counteract neuron loss in the HC would be an interesting approach and provides a link to the therapeutic benefits of endurance exercise.

5.2 Exercise and EAE disease course

To date, there are no established therapeutic options to counteract neurodegeneration and the resulting symptoms of MS, such as cognitive impairment. Due to its neuroprotective properties, excise offers an interesting approach **(see Chapter 1.3.1)**. Exercise subsumes both endurance and strength as well as acute and chronic physical activity with chronic endurance exercise in particular having shown potential neuroprotective effects such as accumulation of BDNF and neurogenesis in the past (Liu and Nusslock, 2018). High-intensity exercise, achieved by forced models in the animals, can have negative effects through the stimulation of oxidative stress, while moderate exercise interventions tend to have an antioxidant and therefore a neuroprotective effect (Radak et al., 2016). In this study, a voluntary exercise intervention prior to EAE induction was performed and disease course was compared to sedentary mice **(Figure 4.6)**. Motor symptoms of the EAE mice were evaluated here, which are primarily due to damage to the spinal cord. However, future studies could investigate the influence of a prior exercise intervention on cognitive aspects of EAE to demonstrate the neuroprotective properties of exercise on the HC. As described above, acute EAE is primarily characterized by the acute infiltration of immune cells into the CNS. Chronic EAE, on the other hand, is a good model for the investigation of neurodegenerative processes (Voskuhl and MacKenzie-Graham, 2022). A significantly milder acute EAE, as seen here,

could therefore be due to both exercise-induced anti-inflammatory effects and increased resilience of CNS structures, which is difficult to distinguish at this point **(Figure 4.6)**. The milder course of chronic EAE, which is also reflected in an almost complete recovery of body weight, supports the theory that exercise counteracts progressive neurodegeneration. That endurance exercise has a positive effect on EAE has already been described in previous studies (Einstein et al., 2018, Bernardes et al., 2016, Pryor et al., 2015, Rizzo et al., 2021, Rossi et al., 2009, Souza et al., 2017). However, these studies used widely different exercise models. Some forced animals to exercise via treadmill (Einstein et al., 2018, Souza et al., 2017) or swimming (Bernardes et al., 2016) interventions. Forced exercise could trigger stress in animals, an stress is a known inductor of neurodegeneration through, among other things, increased neuroinflammation and impaired energy metabolism due to mitochondrial dysfunction (Peña-Bautista et al., 2020). Cognitive impairment and hippocampal alterations has also been associated with stress (Sandi, 2004). Nevertheless, those forced exercise interventions consistently showed to ameliorate acute and chronic EAE. In the past, voluntary exercise models were usually started at the same time as the EAE induction (Pryor et al., 2015, Rizzo et al., 2021, Rossi et al., 2009). However, in order to maximize the neuroprotective mechanisms of exercise, it was considered to be best to start the exercise intervention before the EAE induction. This could allow the neuroprotective properties to be fully utilized before neurodegeneration due to EAE begins. Rizzo et al. (2021) described, as also shown in this study, an ameliorated EAE disease course without significant alteration of time of disease onset. Given this course, largely unchanged immune cell infiltration and activation could be assumed, with the neuroprotective mechanisms of exercise showing their effect during the course of the disease. An unaltered severity of acute EAE with attenuated chronic EAE consistent with this thesis could not be shown here. At this point, it is important to note that the underlying mechanisms by which exercise exerts positive effects on EAE symptoms are still unknown. It has been postulated before, that exercise exclusively alters the immune system without direct neuroprotective effects (Einstein et al., 2018). This statement resulted from a study in which mice, adjacent to a treadmill intervention or sedentary mice, were injected with autoreactive T cells from mice immunized with EAE. No change in the course of EAE was observed here. With regard to comparability, it must be said that a different EAE protocol with myelin proteolipid protein instead of MOG was used, which appears to show a milder disease and most important a relapsing-remitting course predominantly driven by recurring CNS inflammation (Einstein et al., 2018, Badawi and Siahaan, 2013). In contrast, voluntary exercise was shown to have no effect on immune cell infiltration to the spinal cord of EAE mice while synaptic and dendritic abnormalities were significantly attenuated supporting the hypothesis of a predominantly neuroprotective and less anti-inflammatory phenotype (Rossi et al., 2009).

Examination of the HC showed a restorative effect of exercise on synaptic plasticity impaired by EAE among significant reduction of microglial activation (Rizzo et al., 2021). Exercise was also shown to exhibit immunomodulatory alterations with downregulation of the immune response in the brain and also hippocampal tissue (Souza et al., 2017, Rizzo et al., 2021). Kim and Sung (2017) saw a restoration of AHN in EAE due to exercise together with enhanced BDNF levels. Induction of BDNF thus represents one of many possible exercise-induced neuroprotective mechanisms. Summarized, exercise appears to combine both anti-inflammatory, which could generally attenuate the entire course of EAE, and direct neuroprotective mechanisms that individually or in combination lead to the observation presented here that exercise attenuates the course of EAE. Accordingly, it was decided to first evaluate purely neuroprotective mechanisms of exercise in HC without EAE induction.

5.3 Exercise-induced hippocampal changes

To rule out the complex influences of exercise on the immune system and therefore EAE disease course, exercise-induced hippocampal changes were examined without prior EAE induction. Initially, the same hippocampal structures were examined as in the characterization of the EAE. Although extreme exercise interventions can have harmful and pro-inflammatory properties (Cerqueira et al., 2019), moderate to intense exercise shows mainly anti-inflammatory properties such as a decrease in pro- and increase in anti-inflammatory cytokines (Liu et al., 2019, de Sousa et al., 2017). Previous studies reported a decrease in hippocampal microglial count after eight weeks of moderate exercise (Wang et al., 2021, Mee-Inta et al., 2019) even though a strong dependence of the effect on the age and sex of the rodents was reported (Kohman et al., 2013). However, no significant alteration of microglial activation could be seen in this study **(Figure 4.7B)**. Further studies could differentiate pro-inflammatory M1 and anti-inflammatory M2 microglial cells to identify possible changes in the ratio of these subgroups.

Significantly more DCX^+ neurons could be detected in exercise compared to sedentary mice **(Figure 4.7C)**. Since exercise has already been shown to be an inducer of AHN (van Praag et al., 1999, Liu and Nusslock, 2018, Ma et al., 2017), this result indicates that the voluntary exercise model used here is suitable for the investigation of neuroprotective mechanisms. Interestingly, a forced exercise intervention, unlike voluntary exercise, did not show an increase in AHN, which indicates an increased neuroprotective influence of the voluntary intervention (van Praag et al., 1999). The induction of AHN could result from exercise-improved mitochondrial metabolism and altered expression of neurotransmitters and neurotrophins, with BDNF as one of the best-studied representatives (Burtscher et al., 2021, Liu and Nusslock, 2018). The FNDC5/BDNF pathway represents a link between increased

muscle activity and the induction of BDNF in the CNS (Wrann et al., 2013). A crucial and difficult to investigate question is whether the newly formed neurons are integrated into existing neuronal networks of the HC and thus offer the potential to counteract the reduced number of neurons due to neurodegeneration. Exercise has been shown to increase the survival rate of progenitor cells and promote differentiation into neuronal cells (van Praag et al., 1999). This could potentially counteract the observation that neurodegenerative processes in EAE inhibit differentiation into a neuronal phenotype (Giannakopoulou et al., 2013). To address this question, hippocampal neurons expressing NeuN were counted in representative regions of CA1, CA2 and CA3. There was a trend towards higher numbers of neurons in exercise mice compared to sedentary mice, although this observation did not reach significance **(Figure 4.7D)**. This could be due to a relatively small number of mice in this study, which is probably not sufficient to detect even small changes in the number of neurons. Therefore, the experiment should be repeated on a larger group of animals in the future. Other studies reported an increase in hippocampal cell count as a result of an exercise intervention especially in the DG, CA1 and CA3 region (Wang et al., 2021, Uysal et al., 2005). Interestingly, the increase in hippocampal cell count was not associated with changes in apoptosis frequency (Uysal et al., 2005), so increased neurogenesis may provide an explanation.

Synapses and their dynamic formation form the basis of the plasticity of the HC alongside neurogenesis (Neves et al., 2008). Synapses can be subdivided in different ways. Functionally, LTP and LTD can be investigated. Morphologically, studies have quantified the density of dendritic spines and pre- and postsynaptic markers. Exercise was shown to induce LTP (Loprinzi, 2019) and increase the densitiy of synaptic spines (Eadie et al., 2005). In this study, synaptic contacts were quantified as already described above, as this method allows a prediction about the actual connectivity of neurons. In fact, no significant change in the number of synapses could be detected **(Figure 4.7G)**.

In summary, except for increased neurogenesis, no significant histological changes of the HC in exercise compared to sedentary mice could be demonstrated. This may indicate that changes in these analyses were below the detection threshold. Future studies of a bigger cohort and quantification of larger hippocampal areas would be useful to detect marginal effects.

5.3.1 Hippocampal gene expression

Exercise exhibits several neuroprotective properties potentially counteracting neurodegenerative processes **(see Chapter 1.3.1)**. Clinical and morphological exercise-induced alterations are described in the literature and could partly be shown in this work. However, underlying mechanisms are largely unknown. One possible cause of the exercise-

induced changes is an alteration in protein expression with the potential to make neurons more resilient to neurodegeneration. A relatively new method, the so-called bacTRAP, enables the isolation of the currently transcribed mRNA from specific cell populations (Heiman et al., 2014). The major advantage of this method is that it avoids possible contamination by proteins from other cell types, such as immune cells. The bacTRAP method has already been used to analyze the changes in mRNA expression in exclusively motor neurons in acute EAE (Schattling et al., 2019). This method was subsequently established to extract mRNA from hippocampal neurons using the *Lypd1* promoter **(Figure 5.1)**. These analyses revealed several candidate-genes with altered expression following an exercise intervention (results not shown). The expression of promising candidates with potential neuroprotective properties was then validated by qPCR from hippocampal neurons, N2A cells and hippocampal tissue. Three genes (*Actn4, Capn1, Trim59*) showed particularly high expression and therefore protein expression was further analyzed in exercise and sedentary mice using IHC **(Figure 4.9)**.

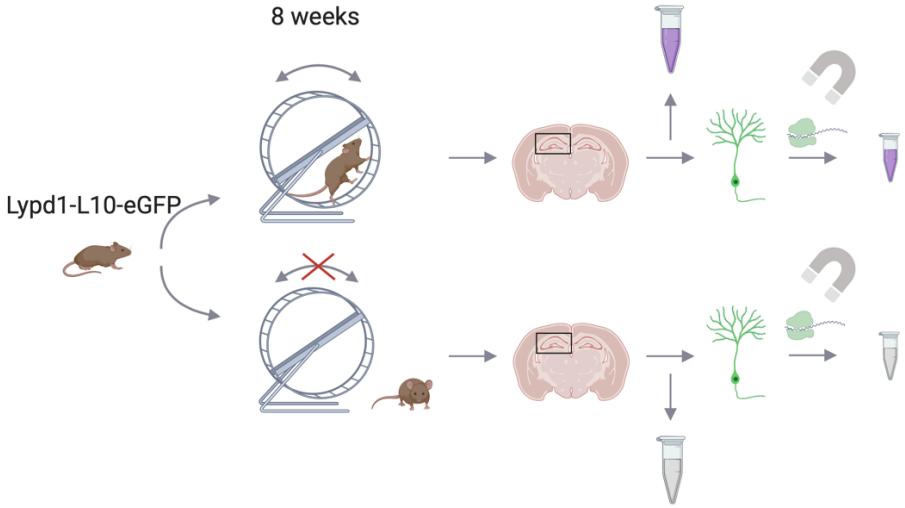

Figure 5.1 Hippocampal BacTRAP

Experimental design to identify alterations in hippocampal gene expression in exercise mice compared to sedentary via bacTRAP. *Lypd1-L10-eGFP* mice were randomly assigned to either the exercise or sedentary cohort. After eight weeks, all mice were sacrificed and the HC dissected in order to extract the mRNA from the hippocampal neurons using the bacTRAP method (illustrated by the magnet). The samples obtained were then subjected to further analysis (Created with BioRender.com).

ACTN4

The hippocampal bacTRAP revealed an upregulation of *Actn4* in the HC of exercise mice (results not shown). Actin-binding proteins are cytoskeletal proteins of the spectrin family (Broderick and Winder, 2005). In non-muscle cells actin-binding proteins connect actin to the cell membrane, in muscle cells it anchors myofibrillar actin filaments. ACTN4 does not appear to be expressed in muscle cells (Otey and Carpen, 2004). Interestingly, ACTN4 is largely expressed in the brain and more specifically in the HC (Kalinowska et al., 2015). The protein

has been widely studied in the context of tumorigenesis (Tentler et al., 2019). In brain tumors, for example, the protein was found to be upregulated (Fukushima et al., 2014). High levels of ACTN4 in tumors have been linked to their invasiveness, which is primarily associated with increased tumor cell motility (Tentler et al., 2019). Another interesting feature is the involvement in spine remodeling. If ACTN4 is missing, spine elongation and turnover are restricted. Overexpression, on the other hand, showed an elongation of the spine heads (Kalinowska et al., 2015). ACTN4 could therefore contribute to increased synaptic plasticity. Exercise has been shown to increase the amount of extracellular vesicles containing ACTN4, even when not performed in brain tissue (Eichner et al., 2018). ACTN4 is an interesting protein, that may contribute to exercise-induced enhancement of synaptic plasticity and neuroprotection. Using IHC, the upregulation by exercise seen in the bacTRAP could not be confirmed so far **(Figure 4.9E)**. Since ACTN4 is enriched in excitatory synapses and colocalizes with PSD95, a synaptic protein, quantification of expression in synapses after exercise would be an interesting next step (Kalinowska et al., 2015).

CAPN1

The *Capn1* gene was also found to be upregulated in exercise mice in the hippocampal bacTRAP and expressed in hippocampal neurons **(Figure 4.8)**. CAPN1 is a calcium-regulated protease ubiquitously expressed in the body including the brain (Su et al., 2020). Calpain activity was detected in the HC of mice, although it was lower than in other brain regions such as the cerebellum (Baudry et al., 1986). CAPN1 appears to have neuroprotective properties, as a lack of the protein leads to increased susceptibility of the brain to traumatic brain injury (Wang et al., 2018). The protease contributes to synaptic plasticity and reconstruction and the maturation and maintenance of neurons by CAPN1 has also been described (Gan-Or et al., 2016). Various studies have investigated the impact of CAPN1 knockout. Interestingly, an impairment of hippocampal LTP and HC-dependent learning and memory was observed (Liu et al., 2016). CAPN1 appears to regulate several genes that influence various brain functions, so that an involvement in the genesis of brain disorders such as AD has already been concluded (Su et al., 2020). Remarkably, a recently published study showed that exercise not only improved cognition in a rat model of Parkinson's disease, but also increased the expression of CAPN1 in the brain of the rats (Guo et al., 2022a). However, there are opposing opinions that consider CAPN1 as a promoter of neurodegeneration and propose the inhibition of CAPN1 as a neuroprotective strategy, although an activity-related change in behavior is also being discussed (Gan-Or and Rouleau, 2016). In this study, an increased protein expression in the HC of exercise mice could be detected, but this effect did not reach significance **(Figure 4.9F)**. A further study with a larger cohort of mice would be useful. In addition, an investigation of the HC in CAPN1 knockout mice would be interesting to further analyze the neuroprotective properties.

TRIM59

Trim59 appeared to be downregulated in the HC of exercise mice (results not shown). It has been described that the protein is localized in the endoplasmic reticulum of cells and plays a role in multiple processes, including cell proliferation, apoptosis and cell differentiation (Chen et al., 2019). *Trim59* is classified as an oncogene because it accelerates proliferation in various tumors, including neuroblastoma, prostate carcinoma and cervical carcinoma, by interfering with the cell cycle (Chen et al., 2019, Aierken et al., 2017, Lin et al., 2016). Hypermethylation of *Trim59* was previously associated with AD with alterations of the cell cycle and proapoptotic signaling indicating neurodegenerative properties (Wezyk et al., 2018). Very little is known about the function of TRIM59 in the CNS and even more specifically in the HC. A downregulation in the HC, albeit not significant, could be shown at protein level **(Figure 4.9G)**. Repeating the experiment with a larger cohort of mice could provide more evidence. Further studies are needed to understand the function of TRIM59 in the CNS and potential neurodegenerative properties of the protein.

5.4 Outlook and transferability to MS patients

Histological changes of the HC in exercise compared to sedentary mice showed an increased neurogenesis and a trend for higher neuronal numbers **(Figure 4.7)**. Further experiments could be conducted with a higher number of mice per group in order to better identify even slight changes. Furthermore, it would be useful to investigate the integration of newborn neurons into the HC. The exact changes in the local hippocampal immune response to exercise should be investigated. This would allow better differentiation of potentially protective mechanisms of exercise intervention in terms of immunomodulation or direct neuroprotection. Subsequently, comparison of the HC of EAE mice with and without exercise intervention could be performed. The interesting candidate proteins CAPN1, ACTN4 and TRIM59 need to be further validated and their molecular function investigated. The development of knockout or overexpressing mice would be a suitable method to investigate the neuroprotective or neurodegenerative properties in more detail.

Hippocampal alterations, as also seen here in chronic EAE, functional impairments and atrophy of the HC have been demonstrated in MS and could explain the cognitive impairment of MS patients (Damjanovic et al., 2017, Papadopoulos et al., 2009, Planche et al., 2017). Exercise had a positive effect on symptoms and disease progression at various disease stages in terms of primary, secondary and tertiary prevention (Dalgas et al., 2019). Exercise-induced neurogenesis was detected in this study and represents an interesting neuroprotective mechanism **(Figure 4.7C)**. AHN in adult humans is a controversial topic, although there is evidence to support it (Boldrini et al., 2018, Eriksson et al., 1998, Manganas et al., 2007,

Moreno-Jimenez et al., 2019). Induction of neurogenesis in patients with MS could preserve or improve hippocampal functions by increasing plasticity and neuronal cell count. To date, there are no drugs treating neurodegeneration as the cause of symptoms such as cognitive impairment. Proteins like ACTN4, CAPN1 and TRIM59, whose expression is altered by exercise, could underlie neuroprotection. In the future, these proteins could be used to establish drug approaches that also address symptoms such as cognitive impairment in patients with MS who are not able to undergo exercise intervention.

6 Summary

A cardinal symptom in patients with multiple sclerosis (MS) is cognitive impairment, which correlates best with early and progressive neurodegeneration of the central nervous system (CNS). This has a major impact on quality of life and is one of the main reasons for early retirement of MS patients, resulting in a large socioeconomic burden. Cognitive impairment may be due to hippocampal injury during disease progression, although the extent and underlying biological mechanisms are unclear. Thus, uncovering these mechanisms is an important step in treatment development. To date, all therapeutic approaches only address the inflammatory component of MS, but neurodegeneration is not affected. Notably, exercise can delay the first symptoms of MS and ameliorate disease progression, suggesting neuroprotective properties. To test this, first of all the extent of hippocampal damage was examined in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS. Significant neuronal cell loss and reduction of synaptic density was detected in the CA1 region of the hippocampus (HC). Furthermore, hippocampal neurogenesis was significantly impaired. It was further shown, that a voluntary exercise intervention prior to EAE induction significantly attenuated disease progression, particularly in the chronic phase, which correlates best with progressive neurodegeneration. However, the acute EAE was also attenuated, suggesting anti-inflammatory impacts of exercise. To identify the purely neuroprotective influences, the HC was examined in exercise and sedentary mice. Neurogenesis was found to be significantly stimulated. An increase in the number of neurons or synapse density did not reach significance. A relatively new method, the so-called bacTRAP, has already enabled the detection of promising changes in gene expression in hippocampal neurons. In this work, these results were further validated and analyzed for expression at the protein level in the HC. Further experiments are needed in the future to identify the potential neuroprotective effects of these genes that may underlie the positive effects of exercise. Furthermore, they may provide a basis for drug approaches addressing symptoms caused by neurodegeneration such as cognitive impairment.

7 Zusammenfassung

Ein Kardinalsymptom bei Patienten mit Multipler Sklerose (MS) ist kognitive Beeinträchtigung, die am besten mit einer frühen und fortschreitenden Neurodegeneration des zentralen Nervensystems (ZNS) korreliert. Dies hat erhebliche Auswirkungen auf die Lebensqualität und ist einer der Hauptgründe für eine frühzeitige Rente von MS-Patienten, was eine große sozioökonomische Belastung darstellt. Kognitive Beeinträchtigungen können auf eine Schädigung des Hippokampus (HK) während des Krankheitsverlaufs zurückzuführen sein, obwohl das Ausmaß und die zugrunde liegenden biologischen Mechanismen unklar sind. Daher ist die Aufdeckung dieser Mechanismen ein wichtiger Schritt bei der Entwicklung von Therapien. Bislang zielen alle therapeutischen Ansätze ausschließlich auf die entzündliche Komponente der MS ab, die Neurodegeneration wird jedoch nicht beeinflusst. Bemerkenswert ist, dass körperliche Betätigung die ersten Symptome der MS hianuszögern und das Fortschreiten der Krankheit abmildern kann, was auf neuroprotektive Eigenschaften schließen lässt. Um dies zu prüfen, wurde in dieser Arbeit zunächst das Ausmaß der Schädigung des HK bei der experimentellen autoimmunen Enzephalomyelitis (EAE), einem Mausmodell der MS, untersucht. In der CA1-Region des HK konnte ein signifikanter neuronaler Zellverlust und eine Verringerung der synaptischen Dichte festgestellt werden. Darüber hinaus war die Neurogenese im Hippokampus deutlich beeinträchtigt. Es konnte ferner gezeigt werden, dass eine freiwillige sportliche Betätigung vor der Induktion der EAE das Fortschreiten der Krankheit deutlich abschwächte, insbesondere in der chronischen Phase, die am besten mit der fortschreitenden Neurodegeneration korreliert. Aber auch die akute EAE zeigte sich abgeschwächt, was auf eine entzündungshemmende Wirkung der Sport-Intervention hindeutet. Um die rein neuroprotektiven Einflüsse zu ermitteln, wurde der HK bei sportlich aktiven im Vergleich zu ruhenden Mäusen untersucht. Es wurde festgestellt, dass die Neurogenese durch die körperliche Aktivität signifikant stimuliert war. Eine Untersuchung der Zunahme der Zahl der Neuronen oder der Synapsendichte erreichte keine Signifikanz. Mit einer relativ neuen Methode, der sogenannten bacTRAP, konnten bereits vielversprechende Veränderungen der Genexpression in hippokampalen Neuronen nachgewiesen werden. In dieser Arbeit wurden diese Ergebnisse weiter validiert und auf die Expression auf Proteinebene im HK untersucht. In Zukunft sind weitere Experimente erforderlich, um die potenziell neuroprotektiven Eigenschaften dieser Gene zu ermitteln, die möglicherweise den positiven Auswirkungen des Sports zugrunde liegen. Darüber hinaus könnten die Gene eine Grundlage für medikamentöse Ansätze zur Behandlung von durch Neurodegeneration verursachten Symptomen wie kognitiven Beeinträchtigungen bilden.

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10 Curriculum vitae

Der Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

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