EXERCISE-INDUCED BRAIN PLASTICITY

– potential roles of lactate receptor HCA_1 in neurogenesis and mood regulation, and neurotrophic factors in stroke

Linda Thøring Øverberg

OsloMet Avhandling 2023 nr 19

OSLO METROPOLITAN UNIVERSITY
STORBYUNIVERSITETET

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Dissertation for the Degree of Philosophiae Doctor (PhD)
PhD programme in Health Sciences
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Spring 2023

OsloMet Avhandling 2023 nr 19

ISSN 2535-471X (trykt)
ISSN 2535-5414 (online)
ISBN 978-82-8364-486-9 (trykt)
ISBN 978-82-8364-508-8 (online)

OsloMet – storbyuniversitetet
Universitetsbiblioteket
Skriftserien
St. Olavs plass 4,
0130 Oslo,
Telefon (47) 64 84 90 00

Postadresse:
Postboks 4, St. Olavs plass
0130 Oslo

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"If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes"

Richard J. Goss [1].

Acknowledgements

I have read that doing a PhD is like running a marathon. In some ways, I agree. In the beginning you feel good and in control, but it is a long stretch, and you feel the pain both mentally and physically. The final year can be compared with the last 10K and is especially demanding. You start to doubt whether you will ever cross the finish line.

With motivation and stamina, I have kept going. Four years have passed since I started. A year longer than planned, because I volunteered as an intensive care nurse during the COVID-19 pandemic. It is so good to have finally succeeded. However, unlike a marathon which is very much an individual effort, this PhD would not have been possible without the help, support, and guidance of many, to whom I am so grateful.

First, I would like to express my deepest appreciation to my main supervisor, Professor Cecilie Morland. She saw the potential in me and opened my eyes to the neuroscience field. For me this was unknown territory. With my nursing background, it has not been a walk in the park to study complex brain processes, handle laboratory mice and analyze blood samples. Thank you for never losing faith, for the continuous support, encouragement and patience throughout the whole project.

Special thanks also go to my co-supervisors: Professor Ole Morten Rønning, Dr. Bente Thommessen and Professor Birgitta Langhammer. They willingly shared their expertise in stroke and rehabilitation, provided plasma and clinical data from the stroke patients, and helped me in finalizing my third article.

I am very grateful for the support and help of Gezime, my brilliant and always positive PhD-colleague, and all the other inspiring members in the Morland group. The work of master students Elise and Emma, who analyzed blood samples and participated in the forced swim test experiments, respectively, was of major importance for my projects. Much appreciated has also been the assistance of former head engineer Mona Gaarder, for handling the blood samples gathered in my human project, and for all the good chats over a morning coffee. I am also grateful to Thor-Øistein Endsjø for valuable help with the recruitment of participants to the healthy control group, and for being a mentor and teaching me how to safely conduct high-intensity interval training for elderly people.

I would like to extend my sincere thanks to Oslo Metropolitan University, the Faculty of Health Sciences and Department of Behavioral Sciences for giving me the opportunity to accomplish this PhD. I am also deeply indebted to the University of Oslo and the Department of Pharmacy for providing office space, making me feel included and sharing their knowledge.

I am also grateful to Tone and Hilde, my friends, former colleagues, and academic role models. To Marte, my dear running companion, friend, and personal therapist. Thank you for listening, advising, and understanding and for all our fantastic runs over the years. To Brendan, for being so kind to read and suggest changes to my thesis.

Finally, to my beloved family. We have gone through the toughest of times with two heart-breaking losses. My dear father (1951-2018) and kindest little brother Glenn (1977-2022). We miss you so much! A special appreciation to my altruistic sister Ellen, for all the love and care you gave when our brother was sick, and for always taking care of our brave and caring mother. Without you, I would never have been able to complete this PhD. I am eternally grateful. I would also like to thank my encouraging husband Ole Kristen and our three wonderful daughters Oline, Kristine and Johanne for supporting me and accepting an absent wife and mother for so many evenings, weekends and even holidays. I hope my hard work motivates you for your future studies and careers. I love you!

Oslo, 11 May 2023

Linda Thøring Øverberg

Sammendrag av avhandlingen

Hjernen er svært plastisk. Den reagerer på endringer i miljøet ved å omorganisere nervebaner, skape nye forbindelser, eller styrke eksisterende, samt ved å danne nye blodårer og nerveceller. Hjerneplastisitet er en viktig del av normal hjernefunksjon, læring, regulering av stemningsleie og en rekke andre prosesser. Plastisitet er også viktig i beskyttelsen mot sykdom i hjernen, som for eksempel hjerneslag, og i tilheling etterpå. Måter å regulere nevroplastisitet på er derfor et viktig tema innen nevrovitenskap. Trening har enorm effekt på fysisk og mental helse og øker nevrogenese og andre former for hjerneplastisitet. Vår forskergruppe har tidligere vist at treningsindusert angiogenese (nydannelse av blodårer) i hjernen delvis skjer via laktatavhengig aktivering av hydroksykarboksylsyrereseptor 1 (HCA_1). For å undersøke om intervalltrening med høy intensitet (HIIT) effektivt økte nevrogenese, og i så fall om dette skjedde via laktatavhengig aktivering av HCA_1, sammenlignet vi HCA_1 knockout (KO) mus og villtype (WT) mus.

I artikkel 1, viste vi at nevrogenese reguleres forskjellig i de to viktigste nisjene for nevrogenese i hjernen, den subgranulære sonen (SGZ) i hippokampus og den subventrikulære sonen (SVZ) ved de laterale ventriklene. Vi viste at nevrogenese ble regulert av HCA_1-aktivering i SVZ, siden økt nevrogenese ble observert som respons på HIIT i WT-mus, men ikke i HCA_1 KO-mus. Videre økte L-laktatbehandling nevrogenese i SVZ i WT-mus mer enn hva HIIT gjorde, og igjen var effekten fraværende i HCA_1 KO-mus. I SGZ, derimot, fant vi at HIIT økte nevrogenese like mye i begge genotyper, mens behandling med L-laktat ikke økte nevrogenese i noen av genotypene. Dette indikerer at treningsindusert nevrogenese i denne nisjen var uavhengig av laktatsignalering gjennom HCA_1.

Siden depresjon har vært knyttet til redusert nevral plastisitet og hippocampal nevrogenese, og trening er kjent for å ha antidepressive effekter som ligner på det man ser ved antidepressive legemidler, undersøkte vi om de antidepressive effektene av trening ble indusert via HCA_1-aktiveringen. I artikkel 2, viser vi at to uker med HIIT eller intervalltrening med medium intensitet (MIIT), begge reduserte depresjonslignende atferd hos WT-mus, men ikke hos HCA_1 KO-mus. Etter seks ukers trening ble en reduksjon i depresjonslignende oppførsel observert kun som respons på MIIT, og ikke HIIT. Interessant nok nådde denne effekten statistisk signifikans bare i WT-mus og ikke i HCA_1 KO-mus, noe som antyder at HCA_1-aktivering har en rolle i å formidle denne antidepressive effekten, og derfor kan representere et nytt mål for

antidepressiv terapi. Nevrogenese og andre former for hjerneplastisitet er i stor grad regulert av vekstfaktorer, og er viktige i tilheling etter akutt iskemisk hjerneslag.

I artikkel 3, hadde vi som mål å finne ut om plasmanivåene av viktige vekstfaktorer var forskjellige hos akutte hjerneslagpasienter sammenlignet med friske kontroller med samme alder og kjønn. Vi fant at plasmanivåene av 'brain-derived' nevrotrofisk faktor (BDNF) og epidermal vekstfaktor (EGF) var signifikant lavere hos slagpasienter enn hos kontroll-personene, mens nivåene av 'basic' fibroblast vekstfaktor (bFGF) og myokinet irisin ikke var forskjellige mellom gruppene. Disse funnene indikerer et redusert nivå av vekstfaktorer, og dermed mulig redusert potensial for plastisitet og tilheling ved hjerneslag der slike mekanismer ville være nødvendig for å forhindre permanent hjerneskade. Interessant nok ble det funnet store individuelle forskjeller i nivåene av alle vekstfaktorer og irisin. Ytterligere forskning er nødvendig for å fastslå om disse forskjellene i vekstfaktornivåer, målt i den akutte fasen av hjerneslag, kan brukes til å forutsi grad av tilheling etter hjerneslag.

Alt i alt tyder dataene som er presentert i denne avhandlingen på at HCA_1-aktivering bidrar til å øke nevrogenese i SVZ og medierer noen av de antidepressive effektene av trening. Hvorvidt HCA_1-aktivering ved endrogen laktat i den akutte fasen av hjerneslag, eller behandlinger med HCA_1-agonister, vil bidra til økt tilheling etter hjerneslag gjenstår å undersøke.

Summary of thesis

The brain is highly plastic. It responds to changes in the environment by reorganizing pathways, creating new connections, or strengthening existing ones, rewiring itself, as well as by creating new blood vessel and neurons. Brain plasticity is an important part of normal brain function, learning, mood regulation, and a myriad of other processes, and is important in the protection against, and the recovery from, brain diseases such as stroke and mood disorders. Finding ways to regulate neuroplasticity is therefore a hot topic in neuroscience. Exercise has immense effect on physical and mental health and induces neurogenesis and other forms of brain plasticity. Our research group has previously reported that exercise-induced angiogenesis (growth of new blood vessels) in the brain occurs partly via lactate-dependent activation of the hydroxycarboxylic acid receptor 1 (HCA_1). To investigate if high-intensity interval training (HIIT) efficiently induced neurogenesis and, if so, whether this occurred via lactate-dependent activation of HCA_1, we compared HCA_1 knockout (KO) mice and wild-type (WT) mice.

In paper 1, we showed that neurogenesis is regulated differently in the two main neurogenic niches in the brain, the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. We reported that neurogenesis was regulated by HCA_1-activation in the SVZ, since enhanced neurogenesis was observed in response to HIIT in WT mice but not in HCA_1 KO mice. Furthermore, L-lactate treatment enhanced neurogenesis in the SVZ of WT mice even more than HIIT, and again the effect was not apparent in HCA_1 KO mice. In the SGZ, on the other hand, HIIT induced neurogenesis equally in both genotypes, and L-lactate treatment did not induce neurogenesis in either genotype. This indicates that exercise-induced neurogenesis in this niche was independent of lactate signaling through HCA_1.

Since depression has been linked to reduced neuroplasticity and hippocampal neurogenesis, and exercise is known to have antidepressant effects similar to those of antidepressant drugs, we investigated whether the antidepressant effects of exercise were mediated via the HCA_1-activation. In paper 2, we showed that two weeks of HIIT or medium-intensity interval exercise (MIIT), equally reduced depression-like behavior in WT mice but not in HCA_1 KO mice. After six weeks of exercise, a reduction in depression-like behavior was observed only in response to MIIT, not to HIIT. Interestingly, this effect reached statistical significance only in the WT mice and not in the HCA_1 KO mice, implying that HCA_1-activation plays a role in mediating this antidepressant effect, and may represent a novel target for antidepressant therapy.

Neurogenesis and other forms of brain plasticity are to a large degree regulated by growth factors and are important in the recovery after acute ischemic stroke. In paper 3, we aimed to determine if the plasma levels of key growth factors differed between acute stroke patients and healthy age and gender matched controls. We found that the plasma levels of brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) were significantly lower in stroke patients than in the healthy controls, while the levels of basic fibroblast growth factor (bFGF) and the myokine irisin did not differ. These findings indicate a reduced level of growth factors, presumably consistent with a reduced potential for plasticity and repair, in the condition of stroke where such mechanisms would be needed to prevent permanent brain damage. Interestingly, large individual differences were found in the levels of all growth factors and irisin. Further research is needed to determine whether these differences in growth factor levels, measured in the acute phase of stroke, can be used to predict post-stroke recovery.

All in all, the data presented in the present thesis suggest that HCA_1-activation contribute to enhancing neurogenesis in the SVZ as well as to the antidepressant effects of exercise. Whether HCA_1-activation by intrinsically released lactate in the acute phase of stroke, or treatments with HCA_1-agonist would contribute to enhanced recovery after stroke remains to be investigated.

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L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1.
Acta Physiologica, 231(3), https://doi.org/10.1111/apha.13587

II. Øverberg, L.T.*, Bjørkeng, E. K, Lambertus, M, Geiseler, S, and Morland C.,
The antidepressant effect of exercise is HCA_1-dependent.
(Unpublished manuscript)

III. Øverberg, L.T.*, Lugg E. F., Gaarder, M., Langhammer, B., Thommessen, B., Rønning, O.M., Morland, C., (2022).
Plasma levels of BDNF and EGF are reduced in acute stroke patients.
Heliyon 8, https://doi.org/10.1016/j.heliyon.2022.e09661

Abbreviations

4-CIN	Alpha-cyano-4-hydroxycinnamic acid
Ahus	Akershus University Hospital
AIS	Acute ischemic stroke
Barthel ADL	Barthel activities of daily living
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CSF	Cerebrospinal fluid
DCX	Doublecortin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FNDC5	Fibronectin type III domain containing protein 5
FST	Forced swim test
GF	Growth factor
GHRP-6	Growth hormone releasing-peptide-6
HCA_1	Hydroxycarboxylic acid receptor 1
HIT	High-intensity training
HIIT	High-intensity interval training
HPA-axis	Hypothalamic-pituitary-adrenal-axis
HRpeak	Peak heart rate
HS	Hemorrhagic stroke
IF	Immunofluorescence
Ki-67	Marker of proliferation
KO	Knockout
LDH	Lactate dehydrogenase
mBDNF	Mature brain derived neurotrophic factor
MCAO	Middle cerebral artery occlusion
MCT	Monocarboxylate transporter
MECT	Maximal exercise capacity test

MICT	Moderate-intermittent continuous training
MIIT	Medium-intensity interval training
MMSE	Mini-mental state examination
mRS	modified Rankin scale
MT	Mechanical thrombectomy
NGF	Nerve growth factor
NIHSS	National institute of health stroke scale
NPC	Neuronal progenitor cell
NSC	Neural stem cell
NSPC	Neuronal stem/progenitor cell
PA	Physical activity
PE	Physical exercise
proBDNF	Precursor brain derived neurotrophic factor
RCT	Randomized controlled trials
REC	Regional committee for medical and health research
S100β	Cytoplasmic calcium-binding protein
SPT	Sucrose preference test
TIA	Transient ischemic attack
TMT A-B	Trail making test A and B
tPA	Tissue plasminogen activator
TST	Tail suspension test
VAL66Met	Methionine substitution for valine at codon 66
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
SGZ	Subgranular zone
SVZ	Subventricular zone
VO_2max	Maximal oxygen uptake
VO_2peak	Peak oxygen uptake
V-SVZ	Ventricular-subventricular zone
WT	Wild-type

Introduction

1. Brain plasticity

Starting with the discovery of the axonal growth cone by 'the father of modern neuroscience', Santiago Ramon Y Cajal, the neuroscience pioneers of the early 20th century understood that the immature brain was malleable and that changes in the brain could occur during infancy and early childhood. Nevertheless, they believed that by early adulthood, the physical structure of the brain was permanent. Consequently, the belief was that the brain was incapable of any regeneration after damage or injury. These early neuroscientists also believed that vertebrates, including humans, were born with one fixed set of brain cells that could not be replaced or renewed [2,3]. Over the last four decades, however, this 'old dogma' has gradually been challenged with strong evidence that structural changes occur in the brain throughout life. The ability of the mammalian brain to generate new neurons was first reported in rodents by Altman in 1962 [4,5]. Later Eriksson and Gage reported that adult neurogenesis also occurred in the human brain [6,7]. Similarly, the connections between neurons were also originally believed to be fixed. In 1968, however, Michael Merzenich found evidence of neuroplasticity in primates when he mapped out the neural circuitry of mature monkey brains, using micro-electrodes [8].

Today, the ability of the brain to change in response to experience and use is termed 'brain plasticity' and has become a leading field of study [9]. It is now generally accepted that the brains of adult vertebrates are flexible. In a broader view, 'plasticity' refers to the brain's ability to adapt to its environment by creating, altering, developing, or forming new connections and pathways or neural maps, with complex electrochemical connections. The brain is a product of experiences, because it modifies, adapts, and is constantly changing and rewiring itself, making stronger or weaker connections, depending on how it is used as the individual experiences new things [8,9]. Remarkably, the changes that occur in the adult brain are influenced by the activities an individual engages in, as well as the environment in which an individual lives, works, and plays. Generally, the connections in the brain form more easily and tend to be more sensitive and responsive to experiences in young brains than in older brains [9]. The brain is capable of plastic adaptation to damage caused by events, like stroke or traumatic injury, including some degree of structural reorganization [10,11]. Brain plasticity can be divided into functional and structural plasticity. Functional plasticity is the brain's ability to 'move functions' from a damaged area of the brain to other undamaged areas [12]. Structural plasticity is the brain's

ability to change its physical structure as new things are learned or new memories are formed. The latter form of plasticity involves alterations in the strength of the synapses between neurons, the generation of new neurons (neurogenesis) [7], and the generation of other structural elements, including the growth of new blood vessels (angiogenesis) [13].

1.1 Adult neurogenesis

The development of the mammalian central nervous system (CNS) is a process that evolves from a small number of multipotent cells, called neuronal stem cells (NSCs). NSCs have the ability to proliferate and generate identical NSCs progeny through symmetric cell division, and to differentiate and become specialized brain cells such as neurons, oligodendrocytes or astrocytes [14-17]. During the embryonic and early postnatal stages most neurons are generated from neural progenitors and stem cells, located in all regions of the neural tube. However, a smaller number of neurons will continue to be generated persistently throughout life, although at a much lower rate [18]. This process is called adult neurogenesis [19].

The field of neurogenesis was pioneered by Joseph Altman who, in 1962, provided the first evidence of neurogenesis in the adult rat after traumatic brain injury [4]. A few years later, he reported the presence of newly generated dentate granule cells in the adult rat hippocampus, a brain region known to play a vital role in learning, memory, and spatial navigation [20,21]. Later Altman reported that neurogenesis also occurred in the ventricular-subventricular zone (V-SVZ). He discovered that the newly generated subventricular zone (SVZ) cells migrated rostrally from the lining of the lateral ventricles to the olfactory bulb, and hence he called the path ‘the rostral migratory stream’ [5]. At the time Altman made his discoveries, the concept of adult neurogenesis was unthinkable for most neuroscientists. As a result, this groundbreaking experimental research was largely overlooked by the neuroscience field for decades. This ignorance slowly started to change in the 1980’s when Paton and Nottenbohm published their first clear evidence of functional integration of new neurons in the adult CNS in another class of vertebrates, namely songbirds [22]. Although showing that neurons were constantly being renewed, also after the birds reach adulthood, the researchers still concluded that there was no evidence for neurogenesis occurring in humans or other primates [23]. Not until Kuhn and colleagues confirmed the occurrence of neurogenesis in the adult rat brain using bromodeoxyuridine (BrdU) labeling, did the field of adult neurogenesis take off [24]. In 1998, Eriksson and colleagues demonstrated that neurogenesis also occurs in the human dentate gyrus throughout life [6]. Adult neurogenesis may therefore be regarded as the extreme of a continuum

of CNS development. The process of neurogenesis is not occurring ubiquitously in the brain. In fact, active adult neurogenesis continues to occur in mainly two distinct neurogenic niches of the adult mammalian brain, namely the SVZ along the walls of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation [7,21,25,26] (**Figure 1**).

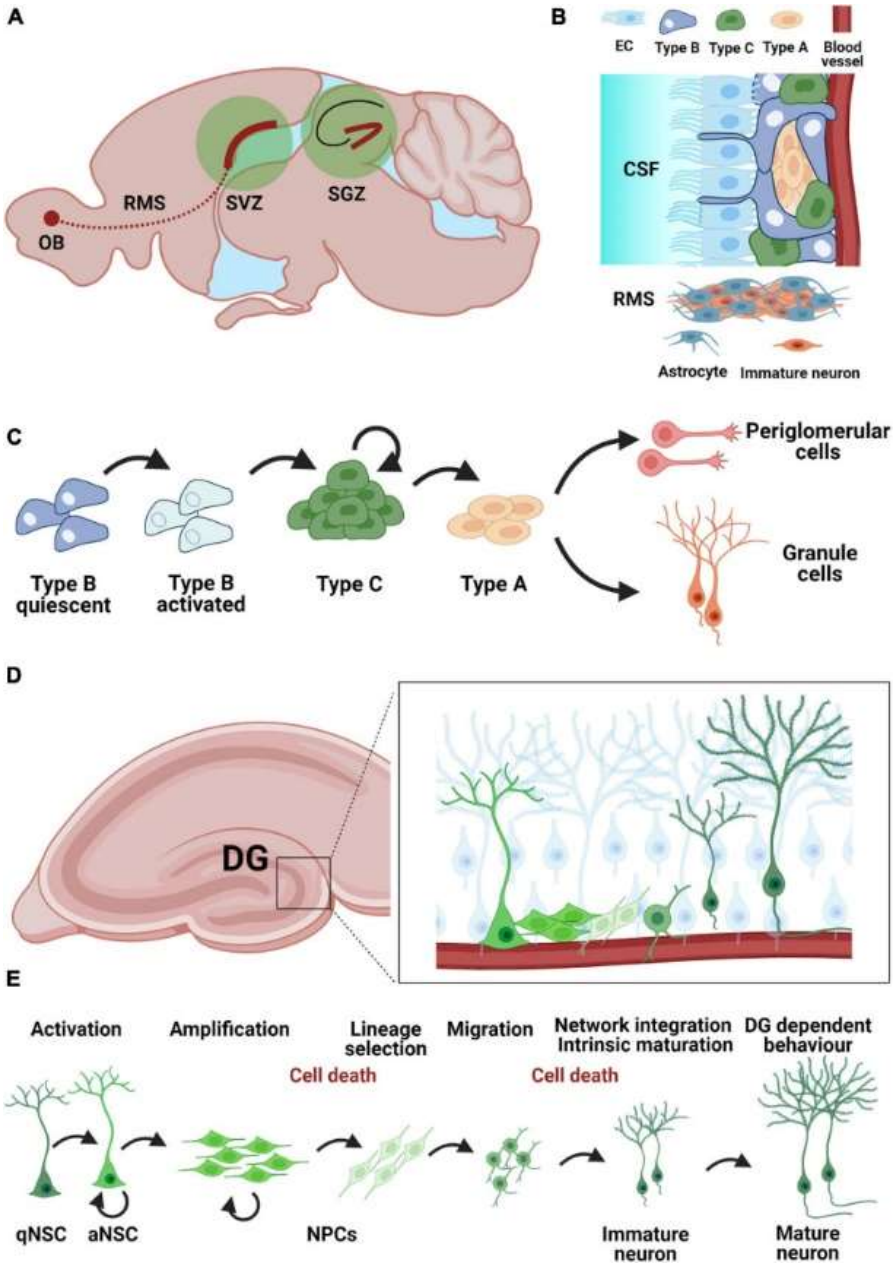

Figure 1: Adult neurogenesis**.** (A) The neurogenic niches in the adult mouse brain includes the SVZ and the SGZ of the hippocampal dentate gyrus. (B) Organization of the SVZ neurogenic niche. (C) Developmental stages during neurogenesis in the SVZ. (D) Adult neurogenesis in the SGZ neurogenic niche. (E) Developmental stages during neurogenesis in the SGZ. Figure from [27] reprinted with permission.

The SVZ is a C-shaped cavity located deep within the cerebrum, along the lateral ventricles, and is lined with a large number of NSCs (type B1 cells), which differentiate into neurons that populate the olfactory bulb. Despite being the largest of the two niches, the functional significance of SVZ neurogenesis and the neurogenic process of this niche is less well known and characterized. Newly generated neurons of the SVZ are, however, suggested to be involved in complex processes such as olfactory memory formation, odor discrimination and social interactions [11,14]. The SGZ is located at the interface between the hilus and the granular layer of the hippocampus. In the hippocampus, adult neurogenesis is thought to play a role in both mood regulation and cognition [21]. Animal studies have recently found that new neurons are also present in several other brain regions, such as the neocortex, hypothalamus, striatum, amygdala, and to a limited degree the substantia nigra [28] (**Figure 2**).

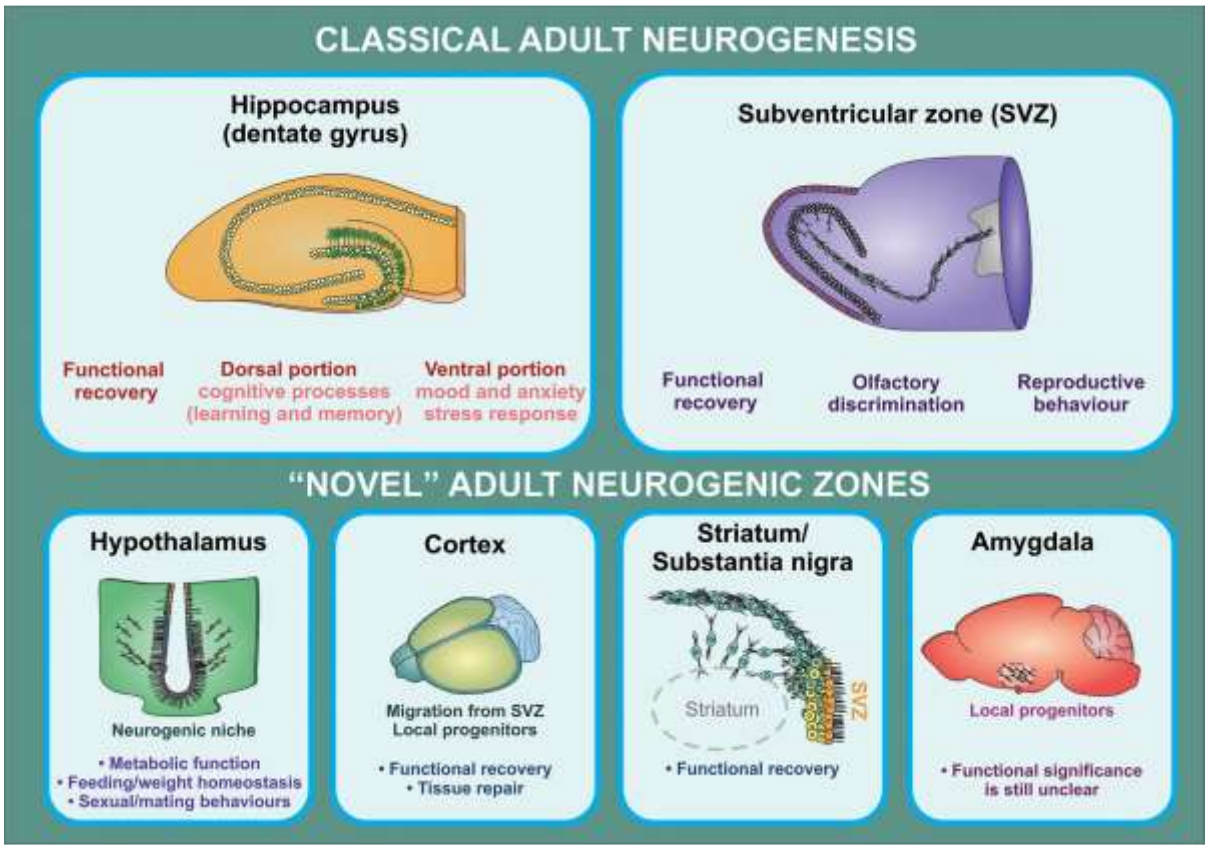

Figure 2: Functional implications of adult neurogenesis in classical and novel adult neurogenesis. Figure from [21] reprinted with permission.

The process of neurogenesis is complex and highly regulated, and often divided into several stages [29]. The first stage is called the proliferation phase and occurs 1-3 days after birth of the stem cell. Thereafter, the cells enter the differentiation phase, during which they transform and become different cell types with specialized functionalities. At this stage the neuronal

progenitors depart from the cell cycle and are devoted to the neuronal lineage. The next stage is known as the migration phase and occurs approximately two to three weeks after birth of the stem cell. At this stage post-mitotic neurons start to extend their axonal projections, and the dendritic growth starts. Finally, the cells mature into functional neurons and establish their synaptic contacts, integrating into pre-existing circuits. In total, it takes about 2-4 months for an adult-born neuron to fully integrate with surrounding cells and incorporate into the neural circuits [11,15,21].

Adult neurogenesis is a well-established phenomenon in animals. Whether and to what degree it occurs in the human brain, however, has recently been debated. In two seminal papers published in 2018 opposite findings were presented, despite the authors using similar methods. While Sorrells and colleagues found a sharp drop in human hippocampal neurogenesis during childhood, reaching undetectable levels in adults [30]. Boldrini and colleagues reported that neurogenesis occurred in adult human hippocampus throughout aging [31]. One thing remains certain, however, during development the human brain has the capacity for neurogenesis. This opens for the possibility that this process may, if the conditions are right, be induced again. Hence, studying the regulation of adult neurogenesis in animal models may suggest mechanisms that can be used to prevent the age-related decline and/or to induce adult neurogenesis in humans, either endogenously or therapeutically.

1.2 Regulation of adult neurogenesis

Hippocampal dentate gyrus is especially vulnerable to aging [32]. Normal aging, age-related neurodegenerative disorders, and depression are associated with reduced neurogenesis in this region [33]. Other forms of plasticity are also reduced in response to these conditions [34]. Hence, means to prevent the drop in plasticity have gained focus as possible treatments to delay the process of brain ageing and cognitive decline. From studies in animals both acute and chronic stress are known to decrease hippocampal neurogenesis [35]. Furthermore, cerebral hypoperfusion, especially of the hippocampus, has been suggested as an early event of Alzheimer's disease [36]. Increased hippocampal angiogenesis could possibly slow down the cognitive decline in this age-related neurodegenerative disorder. Angiogenesis also directly affects neurogenesis, as neurogenesis has been shown to occur primarily adjacent to newly formed capillaries [37] which support the migration and survival of NSCs [38,39]. Therefore, increased cerebral angiogenesis may be beneficial for improved availability of nutrients and oxygen to the brain *per se*, but may also be a way to enhance other forms of plasticity. Learning,

exercise, psychotherapy, and antidepressant drugs have all been shown to positively influence neurogenesis [40-44]. In rodents, an enriched environment containing cardboard houses, plastic tubes, and/or shredded paper has been shown to promote the survival of newborn neurons and their successful integration into the existing hippocampal network [45], especially if the enrichment includes a running wheel [46,47]. Exercise enhances hippocampal neurogenesis as well as angiogenesis [47-49], while stress does the opposite (**Figure 3**).

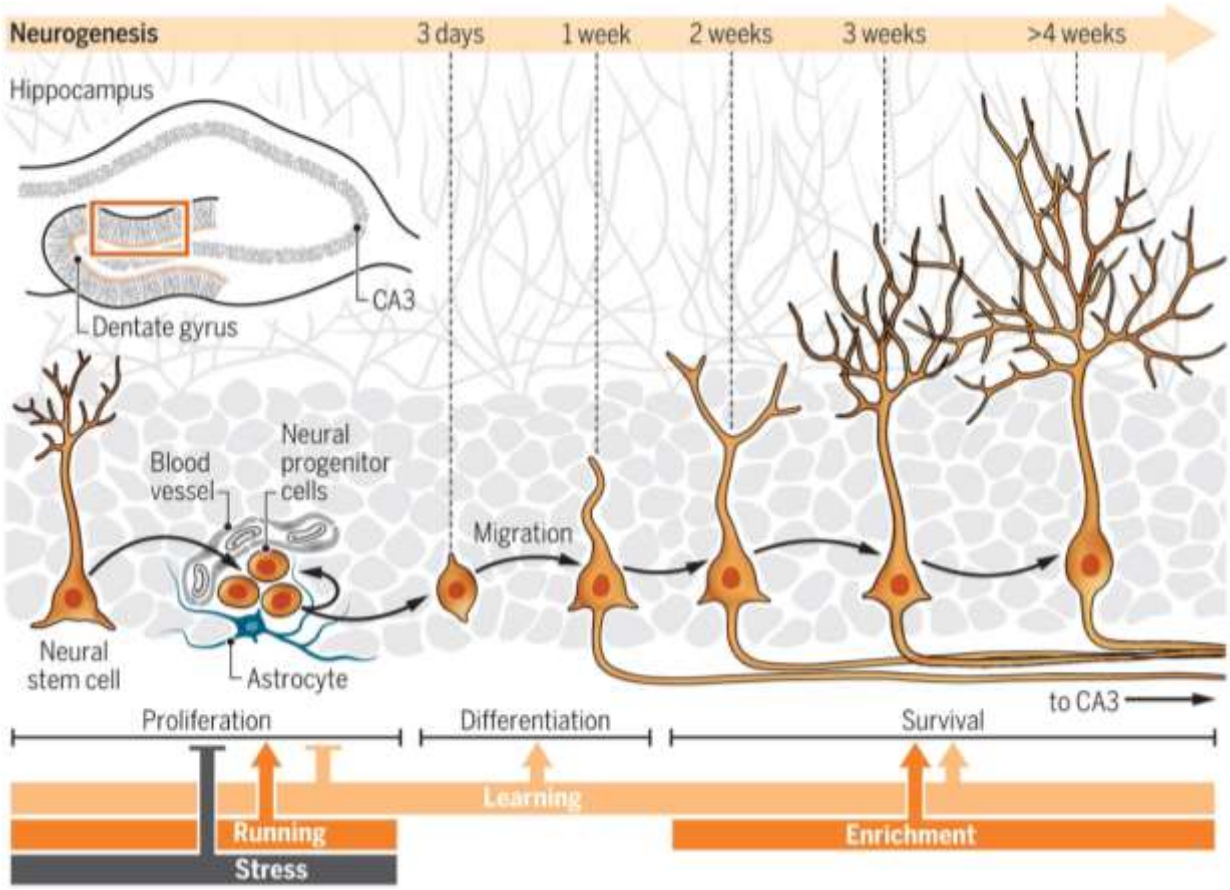

Figure 3: Overview of mammalian neurogenesis in the hippocampal dentate gyrus in the SGZ and the regulation of different behaviors. Running potentially increases neurogenesis and promotes proliferation of the neural progenitor cells. An enriched environment has a complementary effect by enhancing the survival of neurons during their maturation. In contrast, experiencing stress conceals the proliferation of neural progenitor cells. The learning effect are more complex, suppressing neurogenesis at some stages and increasing it at others. Figure from [50] reprinted with permission.

Evidence for the involvement of neurogenesis, angiogenesis, and other forms of plasticity in brain ageing, depression, and stroke will be presented below, along with how exercise interventions may affect brain plasticity in these conditions. Special emphasis will be put on regulation of neurogenesis. In addition, altered levels of soluble mediators like growth factors (GFs), neurotrophins, cytokines, or hormones seem to be involved in the mechanisms leading to enhanced neurogenesis and angiogenesis [33]. GFs are a group of peptides/proteins which

stimulate cell survival, growth, proliferation and differentiation [51], neural plasticity, including angiogenesis and neurogenesis [37], synaptogenesis and synaptic strengthening [52]. GFs exert their stimulation through endocrine, paracrine, or autocrine mechanisms [53]. Some of the most relevant GFs and irisin, a novel exercise-induced myokine, their roles in stroke and depression, and their regulation by exercise is presented below.

2. Acute ischemic stroke

Stroke is a clinical medical state caused by a sudden death of brain cells due to lack of oxygen when the blood supply to a particular part of the brain is halted. Acute ischemic stroke (AIS) is the most common type of stroke and accounts for 85% of all cases. AIS is caused by a partial or total occlusion of a cerebral artery, by either a thrombosis or an embolism [54]. Hemorrhagic stroke (HS), accounts for approximately 15% of all stroke cases. HS is caused by the rupture of a blood vessel, resulting in bleeding in or around the brain. Both stroke types cause cerebral ischemia, which if left untreated, will rapidly result in tissue necrosis and acute brain injury, with the consequent loss of brain functions, permanent disabilities, or death [55]. Transient ischemic attack (TIA) is caused by the same mechanism as an ischemic stroke, but the occlusion is transient, and the patients often only have temporary stroke symptoms lasting less than 24 hours [56,57]. In this thesis the term ‘stroke’ will be used for acute ischemic stroke unless otherwise specified.

The Global burden of disease study 2019 [58] estimates that there are over 12.2 million new strokes each year, and of those, 7.6 million are ischemic strokes. In addition, 3.3 million died from ischemic stroke, and over 77 million people currently living have experienced ischemic stroke. This makes stroke the second-leading cause of death and one of the leading causes of long-term disability [59]. In Norway, 9158 acute stroke cases were reported to the Norwegian stroke registry (NSR) in 2021 [60]. Although the number of strokes and stroke mortality is declining in high-income countries, there is a disproportional rise in the numbers in low-and middle-income countries [61]. Interestingly, 90% of all stroke cases have been suggested to be linked to one or more of ten modifiable risk factors (e.g., hypertension, current smoking, poor diet, high body mass index, hyperlipidemia, high fasting glucose, kidney dysfunction, alcohol consumption, air pollution, and physical inactivity) indicating that awareness, preventive treatments, and healthy life-style choices may prevent many of the stroke cases [59,61,62]. The recovery after a stroke is multifactorial, and the prognosis depends on the patient`s age, stroke severity, stroke etiology, infarct location, as well as comorbidities and the effect of acute therapy. The degree of functional loss in response to stroke can vary from no symptoms to different degrees of impaired motor function, speech deficit and reduced cognitive function, and severe disabilities [63,64]. The severity of impairment is also linked to the patient's functional status, e.g., cognitive and physical function before the ischemic event. However, patients with the same stroke size and location show large differences in post-stroke outcome

and disabilities, which cannot be explained by their pre-stroke functional status alone. Hence, other factors also contribute to the degree of recovery, but these are only partly known.

Many post-stroke survivors require long-term follow-up and rehabilitation. Stroke therefore imposes an extensive economic and social burden on society, as well as on the patients and their families. The estimated global cost of stroke per year is over US$ 891 billion [59,65,66]. For the last decades, thrombolytic therapy with intravenous injections of tissue plasminogen activator (tPA; alteplase) has been, together with treatment in dedicated stroke units, the main treatment in acute ischemic stroke. Later mechanical thrombectomy (MT) was introduced. The reperfusion therapy revolutionized the ischemic stroke therapy. However, some drawbacks limit their use. Most importantly, there is a short therapeutic time window to perform these treatments (in general; 4.5 hours for tPA and up to 6 hours for MT from stroke symptom onset), making only a small fraction (3-5%) of patients eligible for treatment [67]. MT is currently not available in all countries, and only at specialized hospitals. Hence, MT is offered to only a small subset of patients, globally. In contrast, in Norway approximately 20% received trombolytic therapy and 5.9% MT in 2021 [60]. Today, there is no treatment available that directly increases neuroprotection or tissue repair. Finding new treatment options for stroke, as well as novel biomarkers for stroke prediction, diagnosis, or prognosis is therefore a focus area in stroke research.

2.1 The role of growth factors and neuroplasticity in stroke

GFs and other soluble neurotrophins are key regulators of neural plasticity, and the combined action of GFs regulates angiogenesis, neurogenesis, and neuroprotection [37,68], as well as the migration of neuronal stem cells into the ischemic stroke area, and their proliferation into functional neurons [69-71]. In the two following sections, the soluble neurotrophins most relevant for the work presented in the present thesis will be introduced, along with their role in acute ischemic stroke and mood disorders (**Table 1**).

Table 1: General description of relevant growth factors and irisin in stroke and major depressive disorder

Growth factor	Receptor	General description	Effects	References
VEGF	VEGFR-1 VEGFR-2	VEGF has two tyrosine kinase receptors: VEGFR-1 (prevent VEGF binding to VEGFR-2), and VEGFR-2 (which increases angiogenesis). VEGF promotes angiogenesis, vasculogenesis, endothelial cell growth, migration, permeabilization of blood vessels and tissue perfusion.	Neurotrophic and neuroprotective as well as angiogenic properties	[72-75]
BDNF	TrkB	BDNF is mainly expressed in the hippocampal neurons, but also in cerebellum, cerebral cortex, and amygdala. BDNF signaling pathways acts via MAPK and PI3K/Akt regulating neuronal survival, development, function, and plasticity. As well as dendric sprouting and long-term potentiation.	Neurotrophic effect through multiple pathways	[76-83]
EGF	EGFR/ErbB1/ Her1	EGF is broadly expressed in the brain, including in neurons, astrocytes, and microglia, and the levels are highest in children. EGF has a multifactorial neuroprotective role by stimulating proliferation, differentiation, survival, and migration of neural progenitor cells, enhance angiogenesis and reduce apoptosis. The EGF signaling pathways include Ras/MAPK and PI3K. Changes in EGFR trafficking and signaling may be beneficial in regeneration and counteract neurodegeneration.	Likely neuroprotective effect	[84-87]
bFGF	FGFR 1-4	bFGF is a heparin-binding protein which binds to FGFR 1-4, signaling through Ras/MAPK and AKT. Involved in the migration, proliferation, differentiation, and survival of different cell types, gliogenesis, axonal outgrowth, myelination, and memory consolidation, as well as being a potent angiogenic factor. The FGFR receptors are found in neurons, glia, and myelin sheets.	Neurotrophin with pleiotropic effects	[88-97]
Myokine				
Irisin	FNDC5-like-receptors	Irisin is a myokine secreted by exercise, activates the PGC-1α-FNDC5/irisin pathway stimulating many effects of exercise in muscle, including adipose browning, thermogenesis, energy metabolism, and has a protective role against type 2 diabetes mellitus and in the cardio-cerebrovascular system. Irisin induces cognitive benefits, acts as a behavioral antidepressant in mood regulation and may have neuroprotective effects.	Pleiotropic effects	[98-106]

Abbreviations:
GFs: Vascular endothelial growth factor (VEGF); Brain-derived neurotrophic factor (BDNF); Epidermal growth factor (EGF); basic Fibroblast growth factor (bFGF).
Receptor's: Vascular endothelial growth factor receptor 1 and 2 (VEGFR 1-2): Tyrosine protein kinase (TrkB); Epidermal growth factor receptor (EGFR); basic fibroblast growth factor receptor (bFGFR); Fibronectin type III domain containing 5-like receptor (FNDC5-like receptor).
Signaling pathways: Mitogen-activated protein kinases (MAPK); Phosphoinositide 3-kinase (PI3K); Protein kinase B (Akt); Peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α).

Vascular endothelial growth factor (VEGF): The VEGF family consists of five members: VEGF-A, -B, -C, -D, and placental growth factor. VEGF-A is the most studied and seem to be responsible for many of the actions of VEGFs. Hence in the remaining text the term VEGF will be used when referring to VEGF-A specifically or VEGFs in general. There are incongruent results emerging in the research on VEGF levels in stroke patients. While some studies report that serum VEGF levels are significantly elevated in acute ischemic stroke patients compared to healthy controls [75,107], others report the opposite results [108]. A recent meta-analysis of 14 case-control studies reported that no difference in VEGF levels could be found between stroke patients and controls. The authors underlined that VEGF serum levels are time-independent, and could not be significantly associated with an ischemic stroke diagnosis [109]. When it comes to the correlation between VEGF levels and stroke outcome (long-term prognosis), studies have found that patients with the more severe outcomes have higher plasma and serum VEGF levels compared to patients with mild strokes or healthy controls, and that VEGF levels are significantly correlated with the long-term prognosis of ischemic stroke [75,110]. Another study reported that serum VEGF levels in acute and chronic stages of ischemic stroke patients, with small vessel disease or large vessels disease, are independently correlated with infarct volume and clinical disability [111].

The conflicting findings regarding the effects of VEGF on the clinical outcome after stroke may be due to methodological differences, stroke type, and severity etc. [75,107]. Timing also seems to be of the essence, as VEGF has opposite effects on stroke outcome depending on the time of the VEGF increase after stroke onset (for review see [109]). Increased VEGF during the acute phase of a stroke promotes blood-brain barrier (BBB) breakdown which lead to disturbed homeostasis, the invasion of peripheral immune cells, and edema, that inevitably accompanies vascular growth, leading to worsened brain damage [72]. These effects are transient, and increased VEGF after the acute phase has a neuroprotective effect. In this phase, VEGF enhances the growth of blood vessel and glial cells, whilst also providing direct neuroprotection. VEGF is expressed in neurons, astrocytes, macrophages, and vascular endothelial cells in ischemic and hypoxic brain tissues after cerebral ischemia [112]. In ischemic stroke both hypoxic and inflammatory mediators are present and VEGF and its receptors (VEGFR-1 and VEGFR-2) are upregulated [113] as early as one hour after stroke onset, with a peak between 24 and 48 hours after stroke onset [114]. Increased VEGFR levels have been observed in the ischemic core as well as in the penumbra along with a significant upregulation

of VEGF [115]. Activation of VEGFR2 promotes angiogenesis through different processes [73], (for review see [116]).

Brain-derived neurotrophic factor (BDNF): BDNF belongs to the neurotrophin family of growth factors and is also one of the most extensively studied neurotrophins in the mammalian brain. BDNF is synthesized predominantly in neuronal compartments like axons and dendrites as the precursor BDNF (proBDNF), which undergoes cleavage to produce the mature BDNF (mBDNF) protein [117]. In many processes, proBDNF and mBDNF show opposite effects [118], but since the mature form usually is released at higher concentrations, the net effect is often that of mBDNF. The term BDNF will, in this thesis, be used when describing the net effects of both forms of BDNF or of mBDNF alone. Individuals carrying the methionine substitution for valine at codon 66 (Val66Met) mutation in the BDNF gene exhibit decreased levels of BDNF along with decreased hippocampal volume, deficits in episodic memory function as well as increased anxiety and depression [99]. BDNF decreases with age and is associated with age-related neuronal loss. Plasma BDNF has been suggested as biomarker for impaired memory and cognitive function, and it is known to boosts neuroplasticity throughout life [119,120].

Animal studies have shown that BDNF is upregulated in the brain in response to stroke. Increased levels were seen already four hours after stroke onset [82,121] and the increase was reported to be sustained for up to seven days [122]. Secretion of BDNF, and the subsequent activation of the tyrosine receptor kinase B (TrkB) following stroke have multiple protective roles. For instance, BDNF plays a central role in the regulation of neurite outgrowth and neurogenesis [123,124]. Human studies have demonstrated lower BDNF levels in stroke patients compared to heathy controls [125,126]. Furthermore, low levels of circulating BDNF within 24 hours after stroke onset was found to be associated with poor stroke recovery [125-128].

Epidermal growth factor (EGF): EGF is the founding member of the EGF family [68] and it has multiple effects in the CNS. Studies of plasma or serum EGF levels in post-stroke patients, are lacking. However, based on the known physiological effects of EGF, one may expect that secreted EGF after stroke may stimulate neurogenesis and promote recovery in the areas surrounding the injury, hence stimulating the replacement of the injured neurons and promoting the regeneration of the axons [85]. In situations like this, upregulation of epidermal growth factor receptor (EGFR) on astrocytes would be expected to endorse mitosis which again promotes the astrocyte proliferation necessary to reestablish the damaged BBB, a crucial process in post-

stroke recovery [129]. Additionally, activation of EGFR is important for the ability of astrocyte to help in the regulation of differentiation and functions of neurons and neuroglia [130].

In a series of experiments, Garcia del Barco-Herrera and colleagues have shown that treatment with a combination of EGF and growth hormone releasing-peptide-6 (GHRP-6) has a neuroprotective effect in experimental models of global and focal brain infarction. The authors reported a reduction in the number or the severity of neurological symptoms and infarct volume, along with an increased preservation of neuronal density and increased survival of the animals with infarct as compared to vehicle treated control animals [131]. Later, the same group demonstrated that the EGF /GHRP-6 could be administrated up to four hours after stroke induction [132], which is about the same time window as for sandard thrombolytic treatment. The effectiveness of the combined EGF /GHRP-6 treatment was further found to be as comparable to that of hypothermia [133]. The treatment has reached clinical trials and a US patent application has been formed based on these results [134]. The EGF/GHRP-6-treatment seems to enhance cell survival mechanisms, thereby providing protection against a broad range of pathologic processes [87,135] also in the clinical setting of humans with ischemic stroke.

Fibroblast growth factor (FGF): Altogether 22 members of the FGF family have been identified. Fibroblast growth factor-2 (FGF-2) is also known as basic FGF (bFGF). The term bFGF will be used further in this thesis. bFGF is a heparin-binding protein. In post-stroke injury both bFGF and fibroblast growth factor receptor 1 (FGFR1) are upregulated in the brain, predominantly in the penumbra. Accumulated evidence suggests that bFGF has neuroprotective effects in animal models and may be effective in the treatment of stroke [97,136,137]. Additionally, bFGF has shown to be a potent angiogenic inducer enhancing mitogenesis of endothelial cells, and also stimulating the release of VEGF. bFGF appears, however, to have an angiogenic effect in itself, as the combination of VEGF and bFGF induces more angiogenesis than VEGF alone [138]. bFGF has also been shown to enhance functional recovery associated with increased neuronal sprouting in rodents following cerebral ischemia [139]. Studies of human stroke patients have reported significantly higher serum bFGF levels compared to the control groups, and high levels correlate to improvement in clinical outcomes post-stroke [140-142]. Despite the progress in understanding the role of bFGF in the CNS, therapeutic interventions involving the administration of bFGF have not yet been applied for treatment of human patients.

Irisin: Irisin is a hormone-like myokine with neurotrophic effects. Irisin increases in the blood, during exercise when the irisin-precursor fibronectin type III domain-containing protein (FNDC5) in the periphery is cleaved, resulting in the release of irisin. After brain injury, such as ischemic stroke, two independent research groups have reported that plasma irisin concentration and intramuscular FNDC5 protein expression are decreased, and that a low concentration of plasma irisin is associated with poor prognosis on the functional outcome in patients after stroke [143,144]. The underlying mechanisms, however, need to be investigated further. It is confirmed that exercise can increase the expression of FNDC5 and promote the secretion of irisin from hippocampal neurons. This suggests that FNDC5/irisin may be related to energy metabolism in brain tissue [100], as well as to neuroprotective effects induced through BDNF-dependent mechanisms [145]. Another study reported that increased levels of irisin may protect the BBB from disruption after focal cerebral ischemia/reperfusion in rats [146]. Additionally, irisin may induce and stimulate the beneficial effects on post-stroke outcome by preventing post-ischemic inflammation, reducing oxidative stress and improving mitochondrial function [145], inhibiting neuronal apoptosis and inducing NSC differentiation after cerebral ischemia [143]. Indirect, long-term effects on stroke protection are also possible, as high irisin levels prevent obesity and improve glucose homeostasis, thereby lowering key risk factors for stroke [98].

3. Major depressive disorder

Depression is characterized by a low mood, with symptoms such as: anhedonia or reduced interest in enjoyable activities, feelings of guilt and lack of value, apathy and fatigue, reduced concentration, appetite changes, agitation, sleep instabilities, and/or sometimes suicidal thoughts, which are present for at least two weeks [147]. There are several types of depression, for instance: depression as part of a bipolar disorder, perinatal depression, psychotic depression, and clinical depression, also called major depressive disorder (MDD). The terms MDD or 'depression' will be used interchangeably further in the thesis.

The World Health Organization (WHO) estimates that 280 million people, equivalent to 3.8% of the world's population, suffers from depression at any given time [148]; this correlates to a lifetime prevalence of 16.2% [149]. The etiology of MDD is complex and multifactorial with biological, genetic, psychosocial and environmental factors contributing [150]. For decades the pharmacological treatment of MDD has been based on the monoamine hypothesis, which states that depressive symptoms result from low levels of the neurotransmitters serotonin and noradrenaline in parts of the brain [151]. While many antidepressants change monoamine levels within a few hours, the clinical effects of these drugs on mood appear after several weeks of treatment. This mismatch in time is hard to explain based on the primary pharmacological mechanism of actions-inhibition of neurotransmitter reuptake. Alternative hypotheses have therefore emerged to account for this time-lag in the clinical effect. In fact a recent review claims there is no convincing evidence that depression is associated with, or caused by, lower serotonin activity [152]. Nevertheless, antidepressant treatments increase monoamine signaling and alleviate depressive symptoms in many patients. In animal models of depression there is evidence of reduced levels of neurogenesis in the SGZ of the hippocampus [153] which contributes to depression-like behavior [154]. Additionally, a decrease in hippocampal volume is observed in people experiencing depression [155,156]. Experimentally, both central monoamines and growth factors have been shown to modulate hippocampal progenitor proliferation and cell survival [157]. Hence, antidepressant drugs may counteract this effect by increasing the number of adult-born neurons [158,159]; these neurons take at least two to four weeks to completely mature, and continue to mature and develop synaptic connections thereafter [160]. Hence, neurogenesis in the hippocampal SGZ may be a key downstream result of antidepressant drugs, and a key mechanism that adjusts and regulates mood [161,162].

Chronic stress increases the risk for MDD and is associated with structural brain changes such as loss of dendritic spines and synapses, as well as reduced dendritic arborization, together with neuronal loss in general and impaired hippocampal neurogenesis [163]. In the case of prolonged stress, the hypothalamic-pituitary-adrenal axis (HPA-axis) is pathologically activated resulting in elevated levels of the stress hormone cortisol to the circulation. In the brain, cortisol may cause depletion of hippocampal neurogenesis [164], and augmentation of the amygdala's reaction to fear [165]. Hence, hyperfunction of the HPA-axis is thought to be connected to the pathophysiology of depression, (for review see [166]) which may, at least partly, be explained by reduced neurogenesis. Since reduced neurogenesis appears to be involved in the etiology of MDD, and antidepressant drugs increase neurogenesis and other forms of plasticity, GFs are important regulators of depressant and antidepressant mechanisms.

3.1 The role of growth factors and neuroplasticity in MDD

VEGF: Growing amount of evidence implies that VEGF plays a role in the pathophysiology of MDD, but results are conflicting. For instance, exposure to unpredictable stress has shown to decrease the expression of VEGF, which in turn leads to degeneration of limbic structures, decreased hippocampal neurogenesis, and depressive symptoms [167,168]. Furthermore, treatment with antidepressants counteracted the effects of stress, leading to increased hippocampal VEGF levels and prevention of the degeneration of limbic structures [169]. Along the same lines, infusion of VEGF into the lateral ventricles has been shown to generate an antidepressant-like effect in mice [168]. Somewhat surprisingly, a meta-analysis, comparing peripheral VEGF levels between MDD patients and controls, including 16 articles and over 1700 patients and control persons, demonstrated significantly higher blood levels of VEGF in patients with MDD than in healthy controls, suggesting that VEGF levels may serve as a biomarker for depression [170]. However, a recent study highlights that this conclusion should be cautiously interpreted, due to a significant degree of heterogeneity across studies and lack of information in the meta-analysis [171].

BDNF: The most investigated growth factor in MDD is BDNF, and most previous studies report a negative association between mental illness and level of BDNF. The 'neurotrophic hypotheses' of depression suggests that reduced BDNF levels in the brain causes depression, and that antidepressant drugs reduce depressive symptoms by increasing BDNF levels [168,172]. This effect of antidepressants on BDNF levels have been demonstrated both in animal models [173] and in MDD patients [174]. However, a few studies have reported the opposite [175,176].

Reduced BDNF levels in depression can be explained by the hyperactivation of the HPA-axis described above, as BDNF levels are reduced in response to stress and cortisol [177,178]. BDNF is a main regulator of neurogenesis, and reduced BDNF levels in the hippocampus in response to stress likely results in reduced neurogenesis. In line with this, increased BDNF levels, for instance in response to effective antidepressant treatment, is believed to increase adult neurogenesis and synaptogenesis, and prevent depressive symptoms [179]. Animal studies have shown a correlation between stress, reduced BDNF expression in the brain and depressive-like behavior [180]. In contrast, human studies, particularly those with a longitudinal design, suggest that the reduction in serum BDNF is a consequence of the depression rather than a cause [181].

EGF: Decreased levels of EGF has been associated with improvements in symptoms of stress and mood disorders [182]. However, whether the levels of circulating EGF is increased or reduced in mental illnesses is uncertain. In a longitudinal study of young patients with mood disorders (52 with unipolar depression, 27 with bipolar disorder, and 10 with a mixed diagnose) a significantly disease-dependent elevation of EGF was reported, compared to healthy controls [183]. On the other hand, Tian and colleagues reported significantly lower EGF levels in MDD patients compared to the controls [184], while Wu and colleagues found no difference in EGF levels in older adults with MDD compared to age matched healthy controls [185]. EGF does, however, appear to enhance neurogenesis [186]. Suggesting a mechanistic link between antidepressant drugs and EGF-induced neurogenesis, dopamine appears to increase the expression of EGF, which is a major contributor to SVZ proliferation [186].

bFGF: In a recent review article, it was found that the expression levels for several of the FGF family members were altered in MDD patients, indicating a role for these GFs in depression. [187]. However, contradictory findings are also reported. In humans, downregulation of bFGF in the hippocampus [188] and in serum has been reported in patients with MDD, while the serum level of bFGF has been found to increase in response to antidepressant therapy [189]. In contrast, another study reported that MDD patients have increased peripheral levels of bFGF [190]. The latter result is supported by a recent meta-analysis which found significantly higher peripheral bFGF levels in MDD patients compared to healthy controls, although no significant difference in the central bFGF levels were detected between the two groups [191]. Animal studies verify the connection between bFGF and depression, as increased depression-like behaviors with bFGF deficiency have been reported in rats [192]. Furthermore, a study reported that KO mice lacking bFGF showed extended immobility times in the forced swim test (FST) and decreased

preference for sugar in the sucrose preference test (SPT), indicating depression-like behavior [193]. In a study where bFGF was administered peripherally in a mouse model of chronic unpredictable mild stress, depressive symptoms were found to be relieved, suggesting a potential clinical use of bFGF in the treatment of depression [194]. The antidepressant effects of bFGF may be mediated by various intracellular signaling pathways cumulating in neuronal remodeling, including neurogenesis, alteration of synaptic connections and other forms of plasticity, and involving monoamine neurotransmitter system regulation [84,187].

Irisin: The roles and mechanisms of irisin in depression are still largely unknown. It is likely, however, that irisin, by increasing cerebral BDNF, mediates many of the same effects of depression as described for BDNF above. Indirectly suggesting an antidepressant role for irisin, decreased muscle mass (sarcopenia) leads to reduced release of irisin and correlates with depressive behavior [195,196]. In addition, irisin has been suggested to be a novel independent prognostic marker, improving the currently used systematic risk mapping tools of stroke patients, and that reduced serum irisin levels is a powerful biological marker for predicating risk of developing post-stroke depression [197]. In animals it has been suggested that irisin affects neuronal function in the prefrontal cortex, activates the peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) pathway in the hippocampus, promote the expression of BDNF, and reduces the surface expression of EGFR; some of these mechanisms may contribute to alleviating depressive symptoms [98,99,105]. Another study indicates that activation of irisin, BDNF signaling could effectively reduce stress-induced depression [198].

In sum, growth factor activity in the brain is hypothesized to be critical in mood disorders. However, to date there are still uncertainties regarding to what extent they influence MDD. As mentioned above, antidepressant drugs are efficient in only a subset of patients, and physical exercise has emerged as an alternative or supplementary treatment, showing similar effectiveness as the common antidepressants.

4. Physical exercise

The terms physical activity (PA) and physical exercise (PE) are often used interchangeably in common language. Nevertheless, there are substantial differences between PA and PE: While PA includes all energy demanding bodily movements initiated by skeletal muscles, PE refers to planned, structured, repetitive, and purposive activities done to improve or maintain physical fitness [199,200]. The present thesis focusses on PE, and the term 'exercise' will be used further in the thesis. In cases where PA is discussed, this will be explicitly stated.

A sedentary lifestyle is associated with high risk of physical and mental illness [201], represents an extensive public health challenge, and is considered the leading risk factor for non-communicable diseases [202] such as: stroke, depression, metabolic syndrome, type 2-diabetes, high blood pressure, and dementia to name a few [203,204]. In order to promote exercise-induced health benefits in the population, the WHO has published global guidelines for exercise and PA [205]. For adults (age 18-65), the recommendation is at least 150-300 minutes of moderate-intensity aerobic exercise or 75-150 minutes of vigorous-intensity aerobic exercise per week, in addition to complementary muscle-strengthening exercise two or more days a week [200]. Worldwide, one in four adults, and three in four adolescents (aged 11-17 years) do not meet the recommended exercise levels set by WHO, and as countries develops economically, levels of inactivity increases [206]. In line with this, almost half of the global population suffers from at least one chronic disease, which could be relieved or prevented by exercise [207-209].

In the vast research field of exercise, a plethora of different exercise regimes, protocols and intensities are used, but with similar names and abbreviations, complicating the research navigation, as well as comparison of the results. One often divides exercise intensities into three levels which progress from low to moderate to high intensity. For the sake of simplicity, in this thesis, exercise will be categorized as either medium-intensity (representing intensities below the anaerobic threshold) or high-intensity exercise (where at least parts of the exercise is above the anaerobic threshold). It is well documented that moderate-intensity exercise (MIT), both moderate-intensity interval exercise (MIIT) and moderate-intensity continuous exercise (MICT), improves cardiorespiratory fitness (CRF) and reduces the risk of cardiovascular disease, as well as reducing all-cause mortality across all ages [210]. Quite recently, high-intensity interval exercise (HIIT) has gained interest as a more time-effective means to increase cardiovascular fitness. Exercise has been shown to have neuroprotective benefits and to improve cognitive function, but most of this research has focused on exercise of medium

intensity [211]. Hence, it is not known if one of these exercise intensities is superior to the other when it comes to improving various brain functions. Thus, the search for the optimal exercise mode, duration and frequency remains unresolved.

4.1 High-intensity exercise

High-intensity interval training (HIIT) was introduced as an alternative, more time efficient training regime than MICT, and is today known as one of the most effective means of improving cardiorespiratory, metabolic function, and systemic vascular adaption [212,213]. In high intensity exercise the energy demand in the muscles exceeds what can be produced in oxidative metabolism, usually because the uptake of oxygen is insufficient compared to supporting the energy production. Consequently, the body is forced to rely more on anaerobic processes, predominantly glycolysis. There are various forms of HIIT, for instance repeated short (<45 sec.) to long (2–4 min.) bouts of rather high, but not maximal-intensity exercise, or short (<10 sec.) or long (>20–30 sec.) all-out sprints, interspersed with recovery periods. The intensity of the interval bouts is often aimed close to HRmax (~85% to 95%) [212].

Traditionally, MICT has been recommended for older people [214]. However, HIIT has received increasing attention in the recent decade because of its benefits to cardio metabolic parameters, also in elderly. Most data on HIIT, however, is from people with heart diseases. In a systematic review and meta-analysis including 273 patients with coronary artery disease, heart failure, hypertension, metabolic syndrome, and obesity a significantly higher increase (almost double) in oxygen uptake during peak exercise (VO_2peak) was reported after HIIT compared to MICT [215]. Similarly, a randomized study by Wisløff and colleagues, which included 27 patients with stable post-infarction heart failure who received either MICT (70% of peak heart rate (HRpeak), aerobic interval training (95% of HRpeak) or standard advice regarding physical activity (control group) for 12 weeks, reported that exercise intensity was an important factor for the beneficial effects of exercise on the heart, as well as the patients quality of life [216]. Additionally, results from a systematic review and meta-analysis comparing the mean difference in the change in vascular function assessed via brachial artery flow-mediated dilation from baseline to post-intervention between HIIT and MICT found in a population with vascular endothelial dysfunction that 4 x 4 minutes of HIIT (three times per week for 12-16 weeks, performed at 85 to 95% HRpeak, separated by 3 minutes active recovery or low intensity at an intensity of ~ 50 to 70% HR) is capable of enhancing vascular function, more than other prescriptions of HIIT or MICT may provide the optimal stimulus [217]. Another review by

Lucas and colleagues reported that HIT confers similar if not improved metabolic, cardiac, and systemic vascular adaptation compared with traditional MICT. However, the authors calls for more research to optimize exercise outcomes in brain-related health and disease prevention [209].

Further, in a study investigating adherence, enjoyment, and cardio metabolic outcomes, 17 overweight or obese young adults were randomized to HIIT or MICT for eight weeks. The study reported that exercise enjoyment and adherence were high with no differences between the groups. However, treatment with HIIT resulted in a larger decrease in low-density lipoprotein cholesterol and a larger increase in VO_2peak than MICT. HIIT was also associated with an increase in inflammation, with increased levels of CRP and IL-6 in HIIT and decreased levels in MICT in this population [218], which may be a result of obesity, a risk factor able to trigger several inflammatory alterations [219]. However, opposite findings are reported in sedentary males [220]. Physical exercise is an important strategy for reduction of inflammatory established process. While some research suggested that the benefits gained from HIIT (in health parameters, and cardiovascular disease factors) are not due to its intensity, but its intermittent characteristics [221], others suggests that the benefits could actually be from the intensity itself [222,223]. Despite the rapid escalation of the interest in HIIT, there is still a lack of evidence regarding which HIIT protocol is the most effective and safe, and for which population groups and outcome measures [224].

5. Exercise-induced brain plasticity

It is well documented that aerobic exercise improves cognitive and motor function as well as mood. Some researchers claim that the brain may be the organ most susceptible to beneficial effects of exercise, and that exercise is one of the most promising methods for positively affecting learning and memory throughout life, and also postpone the risk of age-related cognitive decline [225,226]. Exercise is now accepted as an important strategy to counteract the risk factors related to neurodegenerative and chronic diseases [227]. As a result, exercise-induced cognitive effects related to various exercise intensities have been investigated in several studies. In the coming sections some of these studies will be elucidated.

5.1 Cognitive effects of high-intensity exercise

While most studies have focused on effects of MICT [228], the effects of HIT to induce neurogenesis and beneficial cognitive effects have been the focus of more recent research. For instance, a study comparing 69 older adults performing HIIT, MICT and resistance training (RT) for six weeks found that despite similar improvements in cardiorespiratory fitness, HIIT, not MICT or RT, improved cognition [229]. In another study, the time to complete a stroop 'color word' test (a neuropsychological test used to measure multiple cognitive functions) [230] was significantly lower in the HIIT session when compared with that of the control session [231]. In contrast, a recent study comparing the effects of MICT and HIIT on cognition and serum BDNF levels in middle-aged and overweight men found no significant difference in improvements in cognitive test scores, and BDNF levels in the MICT vs. the HIIT group [228]. Conflicting results as to whether low and/or moderate intensities, and acute (a single bout of exercise) or chronic (repeated bouts of exercise over a short or long-term period) exercise best promote cognition and memory are also reported. In a current longitudinal study by Manning and colleagues it was found that different exercise intensities seem to affect memory in different ways thus, suggesting that exercising at any intensity improves cognitive performance and benefits mental health. These findings also open for the possibility that specific exercise intensity regimens could be tailored to boost different types of cognitive performance, and reduce symptoms of depression and anxiety [232]. In a larger meta-analysis of 50 studies investigating the effects of acute and chronic [233] cardiovascular exercise on memory found that acute exercise produced moderate to large effects on long-term memory and more moderate effects on short-term memory. On the other hand, chronic exercise was, in the same study, not found to have significant effect on long-term memory and produced only small improvements

in short-term memory [234]. In a recent extensive systematic review, the association between moderate-to-vigorous intensity exercise and cognitive functions was reported to be moderate. The same review, however, showed strong evidence for acute bouts of moderate-to-vigorous exercise having transient effects on cognition, and strong evidence that higher amounts of exercise are associated with a lower likelihood of developing cognitive impairment (e.g., Alzheimer's disease) [235,236].

Studies differ due to various study design, irregularities in the exercise regimes and parameters such as intensity, frequency, different cognitive test-scoring metrics (e.g., clock-drawing test, mini-mental state examination (MMSE), Montreal cognitive assessment (MoCA), or trail making test A and B (TMT A-B) to name a few [237], and participant characteristics [238]. To date, there is no established agreement as to whether moderate or high exercise intensity generates the greatest benefits.

5.2 Other effects of exercise on brain plasticity

While a hippocampal volume increase has been consistently observed in animal models in response to aerobic exercise, the evidence from human studies is equivocal. A systematic review and meta-analysis examining the effect of aerobic exercise on the hippocampal volumes in 737 humans found that aerobic exercise had positive effects on left hippocampal volume in comparison to control conditions [239]. Another systematic review and meta-analysis investigating 631 participants suggests that aerobic exercise may have positive effects on the right hippocampus and potentially beneficial effects on the hippocampus overall [240]. Furthermore, in people over 60 years of age: greater volumes of the hippocampus, prefrontal cortex, and basal ganglia, as well as greater functional brain connectivity, white matter integrity, brain activity and executive and memory function were found to be associated with a higher degree of PA and objective measures of fitness level [241,242]. However, a critical systematic review casts doubt on this by claiming that the reported effect of exercise on whole grey matter brain volume is based on spars and inconclusive data [243].

More recently, some researcher argue that benefits of exercise may in fact vary due to for instance genetic and dietary factors, as well as the age of the study participants which can be the reason for the heterogeneity in the effect sizes reported in the literature [236]. In addition, some animal and human studies have found an association between maternal physical activity before and during pregnancy and improved learning and memory, including the academic performance in the offspring, especially in males [244,245]. Even though there is a consensus

from animal and human research that exercise benefits brain function, additional research is required to search for the neurobiological mechanisms facilitating the benefits of exercise on cognition, behavior, and neurodegenerative diseases [41].

5.3 Exercise-induced regulation of key growth factors

The regulation of GFs is likely to contribute to the beneficial effects of exercise. For example, **VEGF** is known to be upregulated by both anaerobic and aerobic exercise, which may underlie the angiogenic effect of exercise in the brain [246,247]. However, both exercise-induced increase and decrease in the VEGF levels are observed in elderly people [248]. VEGF does not cross the BBB, but nevertheless, peripheral VEGF may induce brain plasticity effects through a variety of other mechanisms [120]. Neeper and colleagues were the first to show an exercise-induced elevation in neurotrophins like **BDNF** and nerve growth factor (NGF) in the hippocampus of adult male rats exposed to voluntary wheel running. To date, most studies report increased BDNF levels after exercise, and the magnitude of the increase seems to be dependent on exercise intensity [249,250]. A few studies, however, fail to report changes, or even show a decrease in BDNF concentrations [251]. All evidence taken together, however, strongly demonstrates that BDNF is a crucial mediator of exercise-induced neuroplasticity, increasing adult neurogenesis, synaptogenesis and preventing neuronal loss in healthy humans [250,252,253]. In line with this, pharmacological blocking of the BDNF signaling in the hippocampus of rodents has been found to reduce the neuroplasticity effects of exercise [254]. The regulation of **EGF** in response to exercise is not completely clear. A study of 30 sedentary young men with or without obesity reported significantly higher levels of EGF in normal weight subjects than in obese subjects before exercise. A significant reduction of EGF serum levels was seen after exercise in both weight groups, although more evident in severely obese subjects. A possible explanation for the decrease in EGF levels in response to exercise, is the role of this growth factor in the defense against oxidative stress [255]. In a study evaluating the effects of aerobic-resistance training on plasma levels of **bFGF** in patients after coronary artery bypass graft, increased serum levels of bFGF were reported, and that this GF may initiate angiogenesis leading to increasing capillary density [256]. Another study, however, found no significant changes in plasma bFGF levels in healthy human subjects in response to treadmill exercise [257]. Furthermore, pre-exercise bFGF levels have been shown to be significantly higher in individuals with a higher fitness level compared to individuals with lower fitness levels [258]. Several reviews and meta-analyses report that acute aerobic exercise increases the levels of circulating **irisin** (a myokine), and that resistance and high-intensity exercise protocols are more

effective than aerobic and low-intensity exercise in this respect. Furthermore, chronic exercise regimes seem not to affect the circulating irisin levels [259,260]. In support, Jandova and colleagues investigated 33 studies and found that the long-term effect of PA and exercise on irisin blood levels increased in 23 studies and decreased in 10 studies [261].

5.4 Effects of exercise on neurogenesis in the SGZ and SVZ

The first evidence of adult neurogenesis in the SGZ of mice was reported by Kempermann and colleagues in response to enrichment of the cage housed mice. The increased neurogenesis was correlated with improved performance in a hippocampus-dependent spatial learning task [45]. Later it was discovered that neurogenesis was enhanced only when the mice were subjected to voluntary wheel running, suggesting that exercise was the critical factor mediating adult hippocampal neurogenesis [46,262,263]. Voluntary running has shown to double the number of surviving newborn cells, demonstrating that voluntary exercise is sufficient for enhanced neurogenesis in the SGZ of adult mice [46]. Neurogenesis in the other main neurogenic niche, the SVZ, may be involved in olfactory learning and discrimination [264] and sexual behavior [40]. However, whether exercise actually increases neurogenesis in the SVZ is controversial. While some studies have shown exercise-induced neurogenesis in the SVZ, others do not. In 2003, Brown and colleagues [265] reported that neither enriched environment or voluntary wheel running for 12 days resulted in neurogenesis in the SVZ, while both treatments produced a doubling in the amount of new hippocampal granule cells in the SGZ. Later several studies have reported that exercise does, in fact, induce neurogenesis in the SVZ. For instance, Bednarczyk and colleagues conducted a six-week experiment of voluntary wheel running and found an increase of proliferating cells in the SVZ compared with standard-housed control mice. In addition, they found that the number of newly born neurons correlated with the total running distance [266]. Mastrorilli and colleagues also conducted a voluntary exercise regime over 12 days and reported an increase of immature neurons, but not of proliferating neurons on the SVZ [267]. Interestingly, Niwa and colleagues found that rats doing voluntarily wheel running showed increased neurogenesis in other smaller neurogenic niches in the brain (e.g., the hypothalamus and ependymal lining of the third ventricle). The latter finding has been interpreted as part of a recovery of homeostatic functions after brain injury [268]. One question, which still remains regarding the regulation of neurogenesis by exercise, is the initial signal that starts the process. Some of the neurotrophic factors mentioned above may represent initial signals, while others may be secondary signals downstream of a yet unknown initiator. One

interesting candidate, which may explain why high or moderate exercise intensities may affect the brain differently, is lactate.

5.5 Lactate – a regulator of brain plasticity?

In the beginning of the 20th century, lactate was considered solely a waste product of aerobic and anaerobic glycolysis and a marker of pathology [269]. Today, however, lactate is known to display pleiotropic effects in the brain where it operates as an energy source, as well as a signaling molecule [270,271]. Through these mechanisms, lactate has been suggested to regulate adaptive functions, including memory, and to have a neuroprotective factor [42,272]. There are two isomers of lactate, L-lactate, and D-lactate. Both are produced from and metabolized to pyruvate by the action of the enzyme lactate dehydrogenase (LDH). In the mammalian cells, however, only LDH is expressed, and consequently the lactate produced is almost exclusively L-lactate [273]. Hence, in this thesis, the term 'lactate' will be used when referring to L-lactate or both isoforms together. The plasma concentration of lactate is usually within the reference range of 0.5-1.5 mmol/L at rest but can rise to greater than 20 mmol/L during intense exercise [273,274]. Lactate is formed through glycolysis in situations where the availability of oxygen is too low to fuel complete oxidative metabolism of lactate in the Krebs' cycle and the electron transport chain. This typically occurs in the skeletal muscles during high-intensity exercise, when the oxygen uptake from the lungs to the blood is insufficient to support complete oxidation of lactate. Consequently, the flux of lactate and pyruvate through oxidative metabolism is lower than the glycolytic flux, and thereby lactate accumulates and is released to the circulation [272,274]. Lactate crosses the BBB via the endothelial monocarboxylate transporters (MCTs) and can be used to fuel brain cells. Alternatively, lactate may bind to and activate the hydroxycarboxylic receptor 1 (HCA_1 or HCAR1, previously known as G-protein-coupled receptor 81 (GPR81). The role of this receptor in adipose tissue is to inhibit lipolysis, and thereby to promote lipid storage in adipocytes [275]. This receptor is, however, active in the mammalian brain [247] where it is highly enriched in pial fibroblast-like cells that line the vessels supplying blood to the brain, and in pericyte-like cells along intracerebral micro vessels. HCA_1 is also reported in the choroid plexus as well as along the lining of the third ventricle [276]. HCA_1 is a G_i-coupled receptor, and hence activation of the receptor by the main endogenous agonist lactate leads to the inhibition of adenylyl cyclase and a reduction in cyclic adenosine monophosphate (cAMP). Through activation of HCA_1, lactate has been suggested to act as a signaling molecule that links neuronal activity, cerebral blood flow, energy metabolism, and energy substrate availability, including a glucose-and glycogen-saving response [277,278].

The activation of HCA_1 by lactate produced during exercise was discovered to stimulate angiogenesis in the brain [247] and to modulate neuronal firing [279]. Wang and colleagues, on the other hand, have reported that chronically elevated lactate levels in the hippocampus resulted in reduced neurogenesis in the SGZ [280]. Whether these effects of lactate are mainly metabolic, or whether activation of HCA_1 also affects other plasticity measures in the brain was an open question at the time when this doctoral thesis was started. Especially, if HCA_1 signaling was involved in the regulation of neurogenesis of the SGZ and the SVZ was unknown.

5.6 Effects of exercise in major depressive disorder

Animal studies have shown that exercise can counteract hippocampal structural changes under depression. Along with this, human studies suggests that exercise can protect the brain structure of patients with schizophrenia and prevent depression symptoms [21,281,282]. Even though numerous studies have reported exercise-induced effect on depression, there are still many unresolved questions regarding the mechanisms through which this effect occurs. Exercise can, amongst other things, trigger the secretion of endorphins [283], but the extent to which this contributes to trigger the effect of exercise on brain plasticity is not known. BDNF levels increases in response to exercise in both animals [100] and humans [284], and is known to affect many brain processes, such as early cell survival, boosting the nerve cells and synapses formation [21,285] as presented above. Hippocampal neurogenesis is one of the secondary effects seen in antidepressant drug therapy, and a BDNF elevation may therefore be an imperative exercise-induced antidepressant effect [286]. In accordance with this, a known mutation in the *bdnf* gene, the Val66Met gene variant, has been suggested to affect the exercise-induced effect of BDNF on the brain. Studies of human *BDNF* Val66Met is found in approximately 25% of the Caucasian population, and almost double in those with Asian ethnicity, it is also more prevalent in females [287]. While some studies suggests that BDNF Val66Met polymorphism appears to be implicated in a number of psychiatric conditions including major depression, anxiety, schizophrenia and eating disorders [288,289], other studies highlight the opposite. In a mini review of eight studies, it was reported that the Val66Met polymorphism in the BDNF gene was not associated with MDD or hippocampal volume in MDD patients, as decreased plasma/serum levels were found in acute MDD, and the polymorphisms were associated with the treatment response. Additionally, both antidepressant treatment and electroconvulsive therapy increased BDNF plasma and serum levels compared to healthy controls [290-292]. Ieraci and colleagues found that exercise resulted in reduced immobility time in the FST in control mice with normal BDNF gene compared to BDNF KO

mice indicating that BDNF protects against depression-like behavior. Furthermore, a reduction in hippocampal neurogenesis was found in mice with the BDNF Val66Met mutation, this was not seen in the normal gene mice [285]. Research has also shown that the HPA-axis is important in stress responses and affects hippocampal BDNF levels. In agreement with this, a study reported that the negative effects of unpredictable stress was reversed by exercise, and exercise-induced behavioral changes and/or unpredictable stress were associated with the hippocampal BDNF levels [293]. This is also in line with a more recent study confirming the antidepressant effect of exercise as well as an increase in BDNF expression in rats, suggesting the involvement of BDNF in the amelioration of depression by exercise [294].

Like other forms of antidepressant treatment, the effectiveness of exercise is not apparent in all patients, and the mechanisms underlying the positive effects of exercise action have not been fully revealed. Nevertheless, different exercise interventions have been shown to effectively improve depression in patients with MDD. For instance, in one study, 16 weeks of aerobic exercise was found to be equally effective as antidepressants in reducing depression among aged patients with MDD [295]. In line with this, a single-blinded, controlled clinical trial of 57 MDD patients, randomized either to a four-week aerobic (4 x per week) intervention or no activity (control group) reported that the exercise group required a lower dose of the antidepressant drug, sertraline, compared to the group who did not receive exercise intervention [296].

In addition, it has been shown that chronic exposure to adrenal glucocorticoids decreases the synaptic density and function in the prefrontal cortex, hence these regions underwent atrophy and disruption of connectivity in individuals with depression [286,297]. Taken together, exercise could produce multiple benefits (**Figure 4**) to neural plasticity including neurogenesis and synaptogenesis and cause antidepressant effects [298]. A meta-analysis concludes that exercise has a large and significant antidepressant effect in people with depression, even when publication bias was accounted for. The authors underlines that these results strongly support the claim that exercise is an evidence-based treatment for depression [299]. Most studies of antidepressant effects of exercise have been performed with moderate intensities, but it is not known whether this intensity is superior to HIIT when it comes to antidepressant effects.

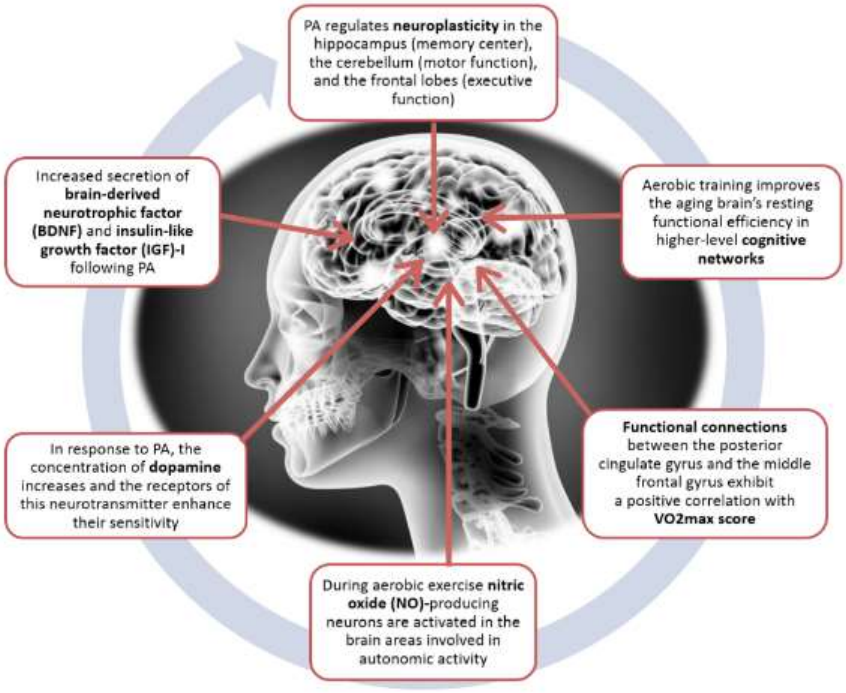

Figure 4. The main pathway effect of aerobic exercise on the central nervous system [300] reprinted with permission.

Aims of the thesis

The primary aim of this thesis is to elucidate the potential role of HCA_1-receptor activation in exercise-induced neurogenesis and depressive behavior, as well as the levels of some soluble neurotrophic factors which regulate neurogenesis in stroke. The study is conducted in mice and humans.

Specific aims are:

1. To determine the effects of HCA_1-dependent neurogenesis in the two main neurogenic niches in the brain *in vivo* (Paper I).

2. To determine whether different interval exercise intensities (MIIT; HIIT) or lactate treatment can contribute to reduce depressive-like behavior in mice *in vivo*, and whether the effect is mediated via activation of HCA_1 (Paper II).

3. To determine whether plasma levels of some key neurotrophic factors differ between patients with acute ischemic stroke and age and gender matched controls (Paper III).

Summary of papers

Paper I:

L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1.

Lambertus, M.*, Øverberg, L.T.*, Andersson, K.A., Hjelden, M.S., Hadzic, A., Haugen, Ø.P., Storm-Mathisen, J., Bergersen, L.H., Geiseler, S. and Morland, C.

* These authors contributed equally

Acta Physiologica (2021)

The underlying mechanisms for the positive effects of exercise are not yet fully understood, but lactate, which is secreted from the muscles during strenuous exercise, has been suggested as an important contributor. Previously, our group has shown that exercise induces angiogenesis through activation of the lactate receptor HCA_1, but whether activation of HCA_1 by lactate also induces neurogenesis in the SGZ and V-SVZ was unknown. To investigate if HCA_1-activation induced neurogenesis in these two neurogenic niches, WT and HCA_1 KO mice received treatment with either high-intensity interval exercise (HIIT), or subcutaneous injection with lactate or saline, 5 days per week for 7 weeks. Markers for proliferating cells (Ki-67) and immature neurons (doublecortin; DCX) were used in the immunostaining to identify neurogenesis in the two neurogenic niches.

We found that HIIT stimulates neurogenesis in both genotypes in the SVZ. However, only reaching statistical significance for DCX in WT mice ($p = 0.039$). Furthermore, WT mice treated with lactate showed an even greater increase in neurogenesis than what was found in mice exposed to exercise ($p = 0.0004$); this effect of lactate was not seen in the HCA_1 KO mice. These data combined demonstrate that HCA_1-dependent mechanism(s) is involved in the regulation of neurogenesis in the SVZ. In contrast, in the hippocampal SGZ, HIIT significantly induced neurogenesis in both genotypes (WT: $p = 0.003$; KO: $p = 0.038$, respectively). The HCA_1-dependency of SVZ neurogenesis was confirmed by quantification of Ki-67-positive cells. Contrary to what was sees in SVZ, in the SGZ, lactate treatment had no effect on neurogenesis in neither genotype, while exercise induced neurogenesis in both genotypes. Altogether, the results of this study show that neurogenesis in the two main neurogenic niches is regulated differently, by HIIT or lactate treatment, and that SVZ neurogenesis may be stimulated by HCA_1-activation.

Paper II:

The antidepressant effect of exercise is HCA_1-dependent.

Øverberg, L.T.*, Bjørkeng, E. K, Lambertus, M, Geiseler, S, and Morland C.

(Unpublished manuscript)

Physical exercise can be beneficial to prevent and treat depression and the effect size appears to be comparable to that of antidepressant treatment. The optimal exercise intensity to obtain the antidepressant effect and the mechanisms underlying this effect is unknown. In this study, the aim was to investigate whether HCA_1-dependent mechanisms are involved in the antidepressant effects of exercise. The FST, a behavioral test used to study depression-like behavior in rodents, was used to investigate the effects of MIIT and HIIT in WT and HCA_1 KO mice. The animals used in this study were initially part of two larger independent exercise studies, and the depression-like behavior was measured after two and six weeks of exercise, respectively. At the latter time point, lactate treatment was also included to directly determine whether lactate signaling though HCA_1 was involved. We found that two weeks of exercise, with either MIIT or HIIT, reduced the floating time of the WT mice in the FST indicating a reduction in depressive-like behavior in these animals. In the HCA_1 KO mice, on the other hand, the floating time were unaffected by MIIT or HIIT. Hence, the data suggest the involvement of HCA_1-dependent antidepressant effects. After six weeks of exercise, on the other hand, only MIIT reduced depressive-like behavior of the WT mice. The same tendency was seen in the HCA_1 KO mice but to a much lesser degree (not reaching statistical significance). Furthermore, we revealed that lactate, through HCA_1-activation contributed to the positive effects of exercise in depressive-like-behavior, as WT mice treated with lactate displayed shorter floating time than their saline-treated counterparts. Again, the effect was not present in HCA_1 KO mice. Our results indicate that the antidepressant effect of exercise and lactate treatment is partly HCA_1-dependent.

Paper III:

Plasma levels of BDNF and EGF are reduced in acute stroke patients.

Øverberg, L.T.*, Lugg E. F., Gaarder, M., Langhammer, B., Thommessen, B., Rønning, O.M., Morland, C.

Heliyon 8 (2022)

Intrinsic levels of irisin and GFs may be important contributors to the recovery after stroke. These substances may also act as biomarkers for disability and progression post-stroke. In the present study we used the commercial sandwich enzyme-linked immunosorbent assay (ELISA) to analyze the plasma concentration of BDNF, EGF, bFGF, and irisin. We found significantly lower levels of BDNF ($p < 0.005$) and EGF ($p < 0.005$) in the stroke patients compared to a group of age and gender matched healthy controls. The plasma levels of bFGF and irisin did not differ between the two groups. The results also showed large individual differences in GF levels among the stroke patients. However, whether these can predict long-term prognosis needs to be investigated further. Not unexpected, the healthy controls outperformed the stroke patients in the cognitive tests. However, due to the lack of information about cognitive status before stroke onset, we cannot rule out the possibility that the stroke patients may have had cognitive deficits pre-stroke. Taken together, we suggest that the lower levels of GFs in the acute phase of stroke may indicate that there is a mismatch between the need to induce plasticity mechanisms and repair post-stroke, and the brain`s ability to induce these processes.

Discussion of the results

I. Neurogenesis in the SGZ is induced by exercise but is independent of the HCA_1

In paper 1, we found that exercise resulted in an increased number of cells expressing DCX, a marker for immature neurons, indicating enhanced neurogenesis in the SGZ in both WT and KO mice. This is in line with research reporting that physical exercise (e.g., running) is a potent modulator of hippocampal neurogenesis [301] (**Figure 3**). Since we wanted to investigate whether the HCA_1-receptor had an initiating role in the neurogenic process in the hippocampal formation, as well as whether activation of this receptor could mimic the neurogenic effects of exercise, mice were also subjected to lactate treatment resembling the serum lactate levels obtained during high-intensity exercise. The results, however, showed that lactate treatment did not induce neurogenesis above control levels in the SGZ, and that the level of neurogenesis did not differ between WT and HCA_1 KO mice. We concluded that neurogenesis in the SGZ niche is exercise-induced but independent of HCA_1-activation. Interestingly, unpublished data from our group, investigating the effects of different exercise intensities on neurogenesis, indicate no difference in nestin, a marker of neural progenitor cells (NPCs), expression levels [302], suggesting that exercise-induced neurogenesis in the SGZ engage mainly the proliferation of type III cells and not of the type I cells (Lambertus, manuscript in preparation). This finding is partially supported by Lev-Vachnish and colleagues who used the HCA_1-specific agonist 3,5-dihydroxybenzoic acid to demonstrate that adult hippocampal neuronal proliferation is independent of HCA_1. The latter study, however, reported that lactate injections increased proliferation of neurons, and that this response was dependent on intracellular lactate, as suppression of MCTs was found to counteract it.

The enterpretation of the results of Lev-Vachnish and colleagues are not conclusive, however, as the inhibitor used, alpha-cyano-4-hydroxycinnamic acid (4-CIN), would likely affect lactate transport through MCT2s on peripheral as well av central cells [303]. Hence, whether the reported effects of 4-CIN on neurogenesis are directly related to metabolic effects of lactate in the hippocampus or indirectly through systemic effects, which alter brain physiology, is difficult to anticipate. Indirectly linking lactate metabolism to hippocampal neurogenesis, though, another study reported that knockdown of MCT2 lead to impaired long-term memory and learning [304]. The latter study showed that the memory impairment resulting from the

knockdown of MCT1 and MCT4 could be rescued by administration of pyruvate or the ketone body beta-hydroxybutyrate, while the memory impairment seen in response to MCT2 knockdown could not be rescued with such energy substrates. Further adding to the complexity, chronically elevated levels of lactate in the hippocampus has been found to cause reduced neurogenesis in the mouse SGZ [280].

Adult hippocampal neurogenesis and angiogenesis has been suggested to be co-regulated [305] and angiogenesis appears to stimulate neurogenesis [38,71]. For example, in stroke, neuroblasts migrate from the SVZ region to the penumbra of the stroke in order to contribute to brain repair, and the migration appears to occur along or close to cerebral vessels [71]. The dentate gyrus of the hippocampus is a highly vascularized environment [306,307], and newly generated capillaries express BDNF, which again promotes the recruitment and migration of newborn neurons [308,309]. In addition, exercise has been shown to induce angiogenesis as well as neurogenesis in the hippocampus by increasing the expression of NGF and BDNF [310,311]. Our results, however, do not support a direct link between angiogenesis and neurogenesis. In paper 1, we did not find HCA_1-dependent neurogenesis in the SGZ. Interestingly, these analyses were performed on the same animals where our group has previously reported HCA_1-dependent angiogenesis in the dentate gyrus [247]. Hence, it appears that angiogenesis and neurogenesis are not causally linked, but rather that they are regulated by some of the same mechanisms and hence, in some conditions, may be co-increased by these mechanisms.

II. Neurogenesis in the SVZ is stimulated by HCA_1-activation

Although for decades it has been acknowledged that exercise, especially running, increases neurogenesis in the hippocampus, there has been a debate whether this process is exercise-dependent also in the SVZ. In paper 1, we report that WT mice treated with either exercise or lactate for seven weeks had increased numbers of DCX and Ki-67-positive cells in the SVZ, and that this effect was not observed in the HCA_1 KO mice. Recently, this result was replicated by our group in a study investigating whether neurogenesis in the two main neurogenic niches (SGZ and SVZ) is affected differently by high or medium exercise intensity in a shorter exercise duration of three weeks. The latter study was performed in the same animals used for the FST in paper 2, and demonstrated that HIIT or MIIT both triggered the neurogenic process in both

niches, but that neurogenesis was HCA_1-dependent only in the SVZ (Lambertus, manuscript in process).

In previous research voluntary wheel running, as opposed to forced running on a treadmill, was the method of choice to observe the effect of running on neurogenesis. In a study by Brown and colleagues [265], adult mice were housed under three conditions: enriched environment, voluntary wheel running and standard housing, before cell proliferation in the SVZ, newborn granule cells in the olfactory bulb and SGZ neurogenesis were analyzed. The brain tissue was stained with BrdU and the astrocytic marker, cytoplasmic calcium-binding protein (S100β) to label dividing cells. The study failed to detect any differences in the number of newly generated cells in the ventricle wall. However, it was found that voluntary running and enrichment produced a doubling in the amount of new hippocampal granule cells. With no control over exercise intensity between the individual animal, in voluntary wheel running, the variance may therefore have masked smaller effects of exercise. In another study more than a decade later, DCX as well as Ki-67, nestin and other cell cycle stage markers were used to demonstrate that voluntary exercise increased neurogenesis also in the SVZ [267]. In the latter study, Ki-67 produced similar effect as those reported with BrdU by Brown and colleagues mentioned above. However, an increase in DCX positive cells was found in mice who had free access to a running wheel. This is in line with the findings of paper 1, where we showed exercise-induced neurogenesis in both the SVZ and the SGZ. Our data further confirms that neurogenesis of the two niches is regulated differently, as HCA_1-activation seems to be involved in the regulation of neurogenesis in the SVZ only.

In sum, our data confirms that exercise increases neurogenesis in the SVZ, preferably when performed at lactate producing intensities (see next section), and suggest that HCA_1-activation is a regulator of neurogenesis in this niche.

III. The effect of high-intensity exercise and stress on neurogenesis

There is mounting evidence to indicate that physical activity in the form of endurance exercise is beneficial for improving the neuronal plasticity of the hippocampus in both animals and humans [253,263], as well as enhancing synaptic plasticity, learning and memory, neurogenesis, dendritic complexity and spine density [312]. However, the optimum exercise intensity is still disputed, for instance low-intensity exercise has been shown to be beneficial to hippocampal

neuronal plasticity through elevating the expression of BDNF [46,313]. While one study suggested that acute exercise with higher intensity and lactate production, promotes hippocampal plasticity by increasing the content of AMP-response element binding protein, synapsin-I, and insulin growth factor [314]. In contrast, a study reported that chronically elevated hippocampal lactate levels, in vivo, reduces neurogenesis in the SGZ [280], another study reported that acute mild exercise is better than intense exercise at enhancing neuronal activity and increasing the synthesis of neurotrophic factors, such as BDNF [315].

Different types of HIIT have shown to induce superior improvements in cardiorespiratory fitness, and molecular and vascular function, as well as positive effects on the brain in response to intermittent increases in lactate. During exercise, lactate accumulates depending on the intensity and the duration of the exercise. However, randomized controlled trials (RCTs) investigating the impact of HIIT on the brain are surprisingly limited [213]. The HIIT protocol used in the present study was designed for optimal gain in cardiovascular function and to reach about 90% of $V0_2max$, mimicking the HIIT protocol often used in human research, such as 4 x 4 minute intervals [316].

Another factor that is negatively correlated to neurogenesis is the exposure to stress. It is well known that stress, especially chronic stress, negatively affect neurogenesis [35,317]. In experimental animal studies exercise can either be controlled, where the experimenter decides the intensity and duration the animals should run on a treadmill, or voluntary, where the animals freely get on and off the running wheel. Research has shown that voluntary exercise may reduce the negative effect of stress on neurogenesis, however, controlled (forced) exercise has previously been shown to have a better effect despite anticipated higher levels of stress [318]. For instance, one study by Inoune and colleagues reported that high-intensity exercise (classified as faster than 20 m/min. running speed in rats) gave significantly increased corticosterone levels, while exercise with lower than 20 m/min. produced no significantly elevated levels of corticosterone [315]. In both paper 1 and 2, the mice were subjected to HIIT, which was defined as 80-90% of the maximum running capacity (high-intensity: 16-20 m/min.). In paper 2, some mice were subjected to MIIT, performed at 50% of the maximum running capacity (medium-intensity:9-12 m/min.). These running speeds cannot be compared directly between species, but research has reported that rats in general obtain lower running speeds compared to mice at similar maximal oxygen uptake (VO_2max) levels [319]. It is therefore most likely that the rats in Inoune`s study were running at a higher intensity than in our studies.

IV. The antidepressant effects of exercise are HCA_1-dependent

In paper 2, we found that the two weeks exercise regimes with HIIT or MIIT reduced the floating time in the FST equally (although only reaching statistical significance for MIIT), indicating that both exercise intensities reduced depression-like behavior. After six weeks of exercise however, only MIIT, and not HIIT, resulted in reduced depression-like behavior. This antidepressant-like-effect was present in the WT mice, but not in the HCA_1 KO mice, indicating that activation of the HCA_1-receptor contributes to this anti-depressive effect. Further, we saw reduced floating time in mice treated with lactate, but this was not statistically significant. A meta-analysis from 2016 concluded that publication bias is evident in exercise RCTs, and that this has largely resulted in an underestimation of the effect size of exercise. The authors claimed that exercise has a large antidepressant effect, hence, support the use of exercise as an evidence-based treatment for depression [299]. Nevertheless, the fact that lactate contributes to the antidepressant effect, and that this occurs through receptor actions, has not previously been reported.

Hyperactivity of the HPA-axis, resulting in increased cortisol levels, which enhances the likelihood for MDD [320] and hypercortisolemia or other HPA-axis disturbances have been found in approximately 40 to 60% of the persons with MDD [321]. Increased cortisol levels in plasma, urine, and cerebrospinal fluid (CSF) [322] and decreased levels of neurotrophins, such as BDNF [323,324] and VEGF are also observed in depression [325,326]. Interestingly, in paper 2, we found no reduction in floating time in the mice treated with HIIT for six weeks compared to sedentary WT mice, despite observing a reduction in floating time after two weeks of HIIT. This suggest that six weeks of HIIT five times a week induces stress and increases glucocorticoids which counteracts the beneficial effects of exercise. Adult hippocampal neurogenesis has been suggested as a mediator of the response to antidepressant treatments [158,327] and may explain the delay in response of these drugs [317]. Looking collectively at the result from paper 1 and 2 as well as the unpublished neurogenesis data from the animals used in the FST (Lambertus et al., unpublished), it does not seem like the HCA_1-dependent effect on depressive symptoms is directly related to neurogenesis in the hippocampus. Hippocampal neurogenesis was induced by 7 weeks of HIIT (Paper 1) and by 3 weeks of MIIT or HIIT (Lambertus et al., unpublished), equally in HCA_1 KO and WT mice. The antidepressant effect of HIIT or MIIT in these animals (Paper 2), however, was HCA_1-dependent. Nevertheless, we

did find HCA_1-dependent neurogenesis in the other niche, the SVZ, after seven weeks of HIIT (Paper 1) and three weeks of MIIT or HIIT (Lambertus et al., unpublished), but the literature does not suggest that SVZ neurogenesis is involved in the regulation of depression.

Another mechanism suggested to be involved in depression is cerebral hypoperfusion, which has been reported in human patients with depression [328,329] as well as in animals [330]. In this respect, it should be noted that we have previously shown that seven weeks of HIIT induced angiogenesis in a HCA_1-dependent manner [247]. The effect of HCA_1-activation on angiogenesis appears to be mediated via an increase in hippocampal VEGF levels [247]. Decreased VEGF in the brain has been detected in depression [325,326], hence, this HCA_1-dependent increase in VEGF levels may counteract depressive symptoms by enhancing cerebral perfusion or through other effects of VEGF. As we have not investigated direct connections between neurogenesis, angiogenesis, or VEGF-levels and depressive symptoms, this is purely speculations.

V. The potential role of HCA_1 and key neurotrophins in stroke and post-stroke recovery

In the aftermath of an acute stroke, several cascades contributing to the reshaping of the stroke core and penumbra are activated. A delayed effect of stroke is the increase in adult neurogenesis, in both the SVZ and the SGZ niches, which augments the chances for a regenerative strategy to counteract neurological deficits in post-stroke survivors [27]. Neurogenesis is, however, not a stand alone mechanism in post-stroke recovery. For instance, angiogenesis has been found to improve the functional recovery after stroke [71]. Both processes, along with synaptic and cellular plasticity, are regulated by GFs and other mediators. Ischemic stroke promotes neurogenesis by increasing the levels of different GFs such as FGF-2, IGF-1, BDNF, VEGF, and chemokines. Angiogenesis is also induced in stroke and is regulated by VEGF, endothelial nitric oxide synthase, and angiopoietin-1/TIE receptor tyrosine kinase (ANG1/Tie2) to name a few.

Research by Buscemi and colleagues demonstrated that intravenous injections with lactate at one hour and three hours after permanent middle cerebral artery occlusion (MCAO) reduced the lesion size and improved neurological outcome. However, three hours after ischemia onset they saw a weaker effect. Additionally, they report that lactate administrated after treatment with recombinant tPA had a positive effect on functional outcome, and attenuates the

deleterious effects of recombinant tPA, although not as strongly as lactate treatment alone [331]. Preliminary data from our group suggest that in a mouse model of cortical stroke distal MCAO, L-lactate injections administrated at 24 hours and 48 hours after stroke decreased the stroke lesion (Geiseler and colleagues, data not published). The effect was, however, only evident three weeks after stroke (and not at one week), and only in WT mice, suggesting that the effect evolved over time and was dependent on the HCA_1-receptor. In our preliminary data, the saline-treated HCA_1 KO mice showed smaller lesions compared to the saline-treated WT mice, suggesting that compensatory mechanisms make up for the lack of HCA_1. In a recently published study from the Rinholm group [332], however, untreated neonatal HCA_1 KO mice were shown to develop larger lesions than the untreated WT littermates. Hence, whether the endogenously increased lactate levels in the brain during a stroke contribute to the recovery after stroke is not known; neither is it known what the net effect of HCA_1-dependent mechanisms in stroke would be in the human situation.

In paper 3, we analyzed plasma levels of several key GFs, irisin, and inflammation markers in stroke patients and a group of healthy age and gender matched controls. We reported significantly lower plasma levels of BDNF and EGF in the stroke patients the day after admission compared to the controls, suggesting a lower potential for neuroplasticity and repair. Although BDNF levels were lower in the stroke patients, there was no correlation between BDNF levels and stroke size, or between BDNF levels and Barthel activities of daily living (Barthel ADL) scores among the stroke patients. This can be explained by a small sample size, and the fact that we had a low number of medium and large stroke size in our study. However, our findings are in line with a systematic review and meta-analysis by Karantali and colleagues investigating 26 articles which found significant lower levels of BDNF in the stoke patients than in the controls. Additionally, a significant negative correlation between the National institutes of health stroke scale (NIHSS) and BDNF serum levels during the acute phase of stroke was seen [333]. There are, however, reported opposite findings: In a study where serial samples of venous blood (from admission to four days post-admission) was collected from 10 stroke patients, no significant change in plasma BDNF was reported [334]. In support, two other studies also report no reduction in BDNF levels between day one and day seven in the stroke patients [291,335]. In contrast, one study reported increased BDNF levels in stroke patients compared to a control group [336]. Nevertheless, most studies show reduced BDNF levels in stroke patients compared to healthy controls. While we found a 2.5-fold lower BDNF plasma levels in the stroke patients, the research field reports a huge variation from 3.8-fold [337] and

a 2-fold [338] lower serum BDNF in stroke patients compared to healthy controls. The differences in BDNF levels might, however, be diverse because the meta-analysis mention above investigated serum blood at the day of admission, while in the present study (Paper 3), we had plasma blood drawn the day after admission.

To the best of our knowledge only very few studies have analyzed plasma BDNF levels after ischemic stroke. Di Lazzaro and colleagues measured plasma BDNF and S100β, a marker for brain damage and BBB disruption, reporting no change in BDNF levels. They did, however, find a significant increase in S100β levels on day two and day three after stroke onset [334]. In study 3, we also attempted to measured S100β, however, the levels were below the detection limit for most of the samples, and therefore were not included in paper 3. Another difference between the latter study by Di Lazzaro and our study is the timing of the blood samples after stroke onset. In paper 3, the blood samples were drawn the day after admission, while in Di Lazzaro`s study they reported that an increase was detected from day two after admission. In another study, Mourão and colleagues compared plasma levels of BDNF at three timepoints; at hospital admission (within 24 hours of onset), 72 hours after hospitalization and at hospital discharge, finding no significant differences during hospitalization. However, they did find that patients with lower BDNF levels had worse neurological deficits in NIHSS and overall functional performance on the modified Rankin scale (mRS) (measuring the degree of disability or dependence in the daily activities of people who have suffered a stroke or other causes of neurological disability) [128].

Even though BDNF is reported to decrease in the normal brain with age, we did not find a correlation between age and plasma BNDF levels in paper 3, neither in the control group nor in the stroke patients. This may be because of a small sample size, bringing uncertainty to the conclusion. Along with the lower BDNF levels, we also demonstrated that plasma EGF levels were nearly 6-fold lower in stroke patients compared to the controls. To the best of our knowledge, circulating EGF levels in acute stroke patients, have not previously been investigated. However, experimental research by Garcia del Barco-Herrera has reported some interesting results regarding co-treatment with EGF and GHRP-6 affecting both the clinical and the pathological outcomes in a global brain ischemia model. It was found that the EGF and GHRP-6-treated group exhibited a better clinical evolution and survival, and 96% of the animals from the vehicle-treated group exhibited ischemic damage [131]. EGF has protective effects on ischemic brain injury via activation of EGFR, which reduces infarct volume and neurological deficits in brain tissue [339]. However, EGF does not support the long-term

survival of newly generated cells, nor does it enhance the generation of new neurons [340]. The effects of giving EGF after stroke is uncertain. Based on our findings in paper 3, we suggest that therapies to increase EGF levels during the acute phase of stroke should be further explored as a potential target for neuroprotection.

Further, in paper 3, we found that the stroke patients were outperformed on all the cognitive tests (MMSE and TMT A-B) by the healthy controls, as expected. The same was observed in the self-sufficiency tests (mRS and Barthel ADL). In the MMSE, the stroke patients had a significantly lower score than the controls, however, it must be emphasised that the mean score for the stroke patients was above 25, which represents a normal cognition. It has been reported that 10% of stroke patients have pre-stroke dementia; and that an additional 10% will develop incident dementia going through a first stroke [341]. Therefore, we cannot with certainty conclude that the differences in cognitive abilities between the two groups are caused by the stroke, as there is a chance that the stroke patients had an affected cognition even before stroke onset.

In recent years, growth factor therapy has emerged as a potential treatment for ischemic brain injury in animals as well as in humans. While exogenous growth factor therapy has shown promising effects for treating ischemic brain injury, more research are necessary to reveal optimal timing, dosing, and mode of administration, as well as possible combinations of growth factors and their effects when mixed with current management strategies. Clinical studies have shown promise in pilot trials, but there is a need for more pre-clinical testing to optimize combination therapy [342].

Methodological considerations

In the present thesis, both animal and human research was conducted, which involves numerous practical, theoretical, and ethical considerations. The phrase from 'bench to bedside' describes the process of bringing knowledge from the laboratories into the clinics so that it can directly benefit patients. Although it seems like a simple task, it is often a long and complex process, and it is a well-known fact that the majority of animal research gets 'lost in translation' and therefore do not deliver public health gains [343]. Nevertheless, animal models may provide important mechanistical data not otherwise avaialble. In the following section some of the topics mentioned above will be elaborated on starting with the relevance of conducting animal experiments to understand human physiology or pathophysiology, as well as some methodological and ethical challenges we encountered in our human research.

I. Using animal models to explain human physiology and pathophysiology

In most cases, it would be preferable to use human subjects in research regarding human conditions. However, this is not always possible or feasible. In this thesis, even though the long-term goal is to increase the understanding of the effects of exercice, GFs and HCA_1-activation on the human brain, conducting such research would be both impossible and unethical due to the invasive character of the experiments, which requires post vivo processing of the brain. Consequently, the experiments in paper 1 and 2 were conducted in laboratory mice (Mus musculus) of the C57BL/6N strain.

The pros and cons of using animal models

There are many advantages in using mice in animal research to mimic human conditions; mouse models have been used for decades and are well tested; humans and mice are both mammals and mice have DNA coding sequences estimated to be 70 to 85% similar to humans, as well as being biologically very similar. In fact, nearly all the genes in mice share functions with the genes in humans. For instance we develop from egg and sperm, we have the same types of body parts (heart, brain, lungs, kidneys, etc.), similar circulatory, hormonal, digestive, reproductive, and nervous systems. All this makes it somewhat easier for scientists to investigate mice physiology to acquire knowledge regarding human growth, aging and diseases developments [344-346]. Further, it is most likely that the molecular pathway in the mouse resembles the one

in the human brain. Additionally, mice are easy to handle due to their small size, they have a short generation time and an accelerated lifespan (one mouse year equals about 30 human years). Mice are relatively cost-effective to maintain, and the time required to perform research is often manageable. Using mouse models provides better experimental control, as the housing, feeding, day/night cycle, and temperature can be controlled. Furthermore, experiments can be performed on littermates to reduce the genetic variation. All in all, this makes mice an ideal laboratory animal model [347]. In addition, new technological advances in gene manipulation have led to refinements and improvements in the generation of more precise mouse models, which have overcome some of the limitations that earlier mouse models had by adding specificity, reproducibility and efficiency to the generation of alleles (a variant form of a gene) that can expand our knowledge of disease pathogenesis [348,349]. This has specifically been beneficial in our group, having used the knockout mouse line for HCA_1 [350] to investigate how the absence or activation of HCA_1 affects adult neurogenesis.

However, there are some downsides to using animals as research subjects. First and foremost, there are major ethical concerns which must be addressed, such as the fact that animals are bred for the purpose of being subjected to diseases and various medical or behavioral tests, which may be both stressful and painful, and then euthanizing them which lessens the value of life. In study 1 and 2, no disease was induced on the animals, however, the treadmill running exercise and the FST may imply some degree of stress (to be discussed later), as well as the lactate injections which also may induce a short-time pain. Working in animal research, requires an insight and understanding of these downsides, as well as knowledge of the three R`s (Replacement, Reduction and Refinement) providing a framework for performing more humane animal research, improving the welfare of animals used in research [351,352], as well complying with other ethical guidelines.

Approvals and ethics

The animal studies included in the present project was approved *a priori* by the Norwegian Animal Research Authority (FOTS-6292/6505/6590/6720/6758/8243/14204/12521) and the domestic animal use and care committee at the University of Oslo. All experiments have been performed in strict accordance with the national and regional ethical guidelines, and all animal handling and experiments were performed in strict accordance with national and international regulations by Federation of European Laboratory Animal Science Associations (FELASA) certificated personnel. To ensure a comprehensive and transparent description of our in vivo

experiments, we have, in standardized manners performed and documented our experiments in accordance with the Animal Research: Reporting *in Vivo* Experiments (ARRIVE) guidelines, version 2.0 [353].

The debate about the value of model organisms will probably never stop, and when working with animal models, one must keep in mind that a model is just a model and will never be a human being. That's to say a model will never replicate the whole complexity of a human disease or human physiology and psychology. However, information generated from model organisms has had a profound and durable impact on human health and will probably keep on doing so. Promoting reproducibility and proper reporting is important to ensure that the use of preclinical model organisms advances translational research in the most efficient and effective way [354].

Voluntary wheel running vs. forced treadmill running

Animal research, either voluntary using av running wheel or forced using a treadmill, reports huge positive impact on physical health [225,355]. Mice run spontaneously when given access to a running wheel, and distances up to 20 km and up to 7 hours a day have been measured [356]. Voluntary running presents several advantages such as: it is more similar to natural running behavior of mice, no-stress condition is involved, they can run long distances in their active hours (at night); strengthening their skeletal and cardiac muscles, there is no interference from the researcher, and it can be used in long-term studies. However, there is one major disadvantage. The researcher has no, or little, experimental control over the running intensity or duration which is important to avoid variations within, as well as between experiments [356].

The positive aspects of voluntary running, mentioned above, are not contradictory in relation to the forced treadmill running exercise regime conducted in our group, which also provides advantages and disadvantages. Forced exercise, also called controlled exercise, is defined as physically placing the mice on the treadmill which 'forces' them to run. The term 'forcing' can be misleading and create negative associations. In fact, most of the time the mice run voluntarily, and the training protocol includes both fixed breaks between the intervals where the mice can choose whether to run or sit still. In addition, they can take small breaks if they need to during the intervals (based on the operators assessment). Actually, in our study most of the animals, chose to run rather than sitting still, during the two minute rest periods between the intervals. This may indicate that the stress did not reach aversion. Forced running is the preferred modality to ensure experimental control and that highly similar exercise is performed

by all animals, a criterion essential for the reproducibility [357]. In animal research, lack of transparency is a problem and ARRIVE calls for an improved and more detailed reporting of the methods in experimental designs [353,357].

The forced exercise regime enables the researcher to ensure that the mice perform exercise at the desired intensity and duration. In the present thesis, since we wanted to investigate the exercise-induced effects of HCA_1 it was important to ensure that the mice ran above their aerobic threshold, elevating the blood lactate levels. Regarding stress afflicted on the mice, the experimenters had the opportunity to give a mild electrical foot shock to the mice if they refused to run. This was, however, very rarely used in our experiments. Some of the mice were also injected with lactate intraperitoneally, which may have caused pain to the mice. However, only trained personnel conducted the procedure. Despite this, the animals showed no signs of distress such as fur alteration, loss of weight and stereotype behavior.

Lactate treatment

The exercise regime used in study 1 and 2 has been validated extensively [316], and blood lactate levels at ~10 mmol/L have been reported in mice during treadmill exercise close to VO_2max [358]. In the experiment, in paper 1 and 2, the mice were randomized into three groups: treadmill running, sodium L-lactate injections or saline injections (control). The mice treated with lactate received a subcutaneous injection of sodium L-lactate (2 g/kg bodyweight). Injection of lactate in high concentration has shown to mimic HIIT, leading to an increase in blood lactate levels similar to exercise. However, a side effect reported after intravenous infusion of large doses of lactate in humans is panic attacks. Therefore to rule out the possibility that the results were confounded by anxiety, our group has previously tested mice in a rodent test for anxiety, the 'elevated zero maze test', immediately before and 15 minutes after the injection of L-lactate (see Methods in [247]). The test showed no increase in anxiety in response to lactate, compared to saline-treatment.

To ensure that the mice reached a sufficient exercise intensity, a maximal exercise capacity test (MECT) of all the mice was performed every second week throughout the exercise period. MECT results have shown to correlate with VO_2max of the mice and may serve as a proxy for the lactate level. The mice ran with a 25° incline at a speed set at 70% of the MECT for the HIIT protocol which is equal to 90% VO_2max [319]. Morland and colleagues showed that this exercise regime seems to have huge effects on brain function [247]. In addition, in another study

in our group, the running speed required for the MIIT animals was performed at 50% of the maximum running speed, representing 60% $V0_2max$ [319].

Immunofluorescence

Immunofluorescence (IF) is a method that depends on the use of antibodies chemically coupled to a fluorophore which can be visualized under a fluorescence microscope. For an IF labeling to be efficacious it is imperative to have a high-quality antibody that will specifically identify the antigen(s) within the molecule (usually a protein) of interest. Furthermore, when the localization of protein is used to identify specific cell types or tissue components, the protein of interest must be expressed selectively in this structure [359].

In the present study, the IF method was utilized to be able to visualize NPCs and quantify the neurogenic effect of exercise or lactate treatment. Two well-established markers for proliferating cells (Ki-67) [360] and immature neurons (DCX) [361] were used to investigate neurogenesis in the SGZ and the SVZ, respectively. Ki-67 is widely used as a cell proliferation marker to grade tumors. However, since most neurons in the brain are post-mitotic Ki-67 labeling is commonly used to detect proliferating cells in neurogenesis. In later developmental stages, NPCs express a specific type of microtubule-associated protein called doublecortin (DCX). It is only expressed by immature neurons at a later developmental stage [361]. These two proteins are therefore mainly expressed by the specific cell types we aim to detect, and not by most other cells in the brain. In addition, since neurogenesis appears in distinct niches, we also validated the labeling by looking at localization of the Ki-67 or DCX-positive cells as well as the morphology of these cells within the region of interest.

The antibodies in this study have been utilized in immunostaining in other previous studies [362,363] and our labeling resembles the pattern seen in those. Hence, the cells detected in paper 1 are identified with well established markers and detected by highly selective antibodies, making the neurogenesis data highly reliable.

Confocal Microscopy

Currently, confocal microscopy is one of the best imaging methods to picture the outcomes of IF staining of biological section. In paper 1, Zeiss LSM 880 with Airyscan detector was used. The advantages of using Airyscan detector is that it has a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT) area detector collecting a pinhole-plane image

at every scan position instead of single-point detector found in a conventional confocal microscopy detector. This results in better resolution and signal to noise.

In paper 1, the images obtained from the confocal microscopy were manually analyzed counting the DCX and Ki-67 positive cells by an observer who was blinded to the genotypes and treatments. First, the length of the SVZ and SGZ was measured using the Fiji distribution of ImageJ (version 2.0.0-rc-69/1.52p; Java 1.8.0_172) or in ZenBlue 2.3. The density of newborn neurons was calculated as the number of DCX of Ki-67 positive cells divided by the measured length of the corresponding SVZ or SGZ for all the animals.

II. Forced swim test – a test for depression-like behavior

FST was design in the 1970`s as a primary screening test for new antidepressant drugs [364,365]. In the FST mice are placed in cylinders filled with water without the possibility to escape for six minutes. The animals then alternate between actively swimming or trying to escape (referred to as being mobile) and passively floating (referred to as being immobile). Animals which spend more time immobile is thought to be in despair, which is interpreted as a sign of depression-like-behavior. The main advantages of FST are that it is low cost, fast, relatively easy to perform, and the results are easily and quickly analyzed. In addition, it is a valid tool to qualitatively screen antidepressant effects [366], and a reliable test for potential antidepressant treatment with a strong predictive validity [367]. In fact, the FST has shown to have a great sensitivity with all the antidepressant classes [367]. Furthermore, for a long time, FST has been the main test for analying depressive behavior in rodents, and hence comparison with other data is possible although strain-to-strain variations are well known to influence FST results [365].

In recent decades, the FST has also emerged as a valid model to understand stress coping [368]. Stress is known to contribute to depression but also many other conditions. Using FST to understand the biology of stress can be of valuable help in the treatment of stress-related conditions. However, this test also has disadvantages, and it has been criticized over the years for several reasons. Firstly, it has shown to be sensitive to acute treatment with an antidepressant drug in rodents, whereas in humans several weeks or months of antidepressant treatment is required before symptoms are relieved and a clinical response is reported [150,369]. Furthermore, due to the aversiveness of the FST, it is important to be aware of possible influences it might have on brain structure and function, if brain analysis is to be carried out following this

procedure [365]. Critics also claim that the experimenters have been using this procedure to make false and uninformed determinations about an animals' mood and to use these determinations to infer potentially false conclusions about biology related to human health [370,371]. Despite this criticism, FST is still one of the most commonly used animal tests to investigate depression-like-behavior in rats and mice, and as a tool for screening antidepressants [365]. In the present study the mice were acclimatized before the test was conducted in order to reduce stress affecting the results. Since the mice used for FST in paper 2 had already been exercising or receiving injections for two or six weeks, they were already familiarized to being handled by experimenters. During the FST the mice were handled at the minimum required to conduct the test. To further reduce the level of stress, the mice were dried and put in a heated cage immediately after finishing the test, and they could choose to stay in the heated or non-heated part of the cage.

Additional tests exists to measure depresive-like behavior in mice, but non of them are ideal. The tail suspension test (TST) is a test of antidepressant activity in mice that shares its experimental rationale with the FST and also has many of the same advantages and disadvantages. In this test, the mouse is suspended from its tail for a fixed time period, using a piece of adhesive tape in such a position that it cannot escape or hold on to nearby surfaces. Its movements are recorded and the resulting escape oriented behaviors are quantified [372,373]. In sum, TST and FST roughly measure the same behavior, despair. Both are to a certain extent stressful for the animals, and both are usually scored manually, with the sources of error that may limit the accuracy of the experimental results. Consequently, in theory both tests could be combined with our exercise experiments however, the FST was chosen because it is the most commonly used test. Another test, often recommended before these two presented tests, is the SPT. This is a test measuring anhedonia in mice based on a two-bottle choice paradigm [374]. If given a choice between sucrose solution or regular water, rodents will naturally consume sweet food and selectively drink sweet solution. However, when exposed to stress-based models of depression, rodents fail to drink sweetened water. This has shown to be a useful parameter to test for anhedonic behavior in rodents [375]. However, the downside is that it takes eight days to conduct this test. Since the mice in the present study were participating in larger exercise experiments (exercising 5 x per week), and the FST had to be done on a day with no exercise, it was impossible to use the SPT.

Analyzing the forced swim test

The present experiment was video recorded for six minutes, the entire duration of the swimming test. The first two minutes were ignored as recommended in the protocol, as the behavior of the mice in the early phase is assumed to represent anxiety more than depressive-like behavior. For the remaining four minutes the behavior of the animal was categorized into 'mobile' or 'immobile' (behaviors where the animal did not swim or make movements other than those necessary to maintain balance and/or keep their nose above water). There are two viable analyzing methods to measure the swimming behavior in FST: An automatic software system or manual scoring of the behaviors. An automated measurement is, in theory, time efficient and unbiased. However, since rodents may show different swimming behaviors (swimming horizontally or more vertically, or trying to climb on the glass walls) designs of a universal algorithm to analyze the swimming behavior has not been straight forward [376]. Manual analysis of the FST is therefore still the most wildly used method. It has a relatively high variability in the scores of the duration of floating or swimming between individual observers [377], and hence carefully establishing the criteria for each behavior and training the observers to reach a sufficient level of reliability is essential if several observers are to score animals within the same experiment. In the present study we used the latter method, were two independent operators, blinded to the genotype and treatment of the mice, used a stopwatch to measure the floating time. Both observants scored all the animals presented in figure 2 of paper 2, and to further ensure valid measurements, both operators analyzed the FST twice, with several days apart. All the results were then sent to a third member of the group, who held the code key and the data presented in paper 2 are based on an average of the two measurements made by one of the observants. Although reaching slightly different absolute values for immobility times, the two observants reached very similar conclusions regarding the effects of HIIT or MIIT on depression-like behavior. Hence, the data presented in figure 3 of paper 2 is based on two measurements from one observer only (the same observer as in figure 2).

III. Human subjects in research

Ultimately, research on human subjects is necessary to improve human health and welfare. The Declaration of Helsinki from 1964 and the Belmont report from 1978 [378,379] were created to prevent human subjects from being mistreated and harmed in research, and provide guidance for researchers working with identifiable human material and data. In short, they state that the priority is to adhere to the ethical guidelines and laws, as well as to obtain an

approved research protocol. This is to protect the welfare and interests of those enrolled in the study and to maximize the benefits and minimize the harms. Vulnerable persons with weakened autonomy are entitled to be protected, such as the terminally ill, mentally handicapped, imprisoned and people with dementia. Further, it is important to avoid discrimination of special groups such as welfare patients, institutionalized persons, ethnic and racial minorities (with the principle: 'justice for all'). In Norway all research involving human subjects must be pre-approved by the appropriate regional ethical committee and registered in Norwegian Agency for Shared Services in Education and Research (SIKT) [380]. Projects involving human subjects may also be subjected to external or internal (institutional) revision at any time to ensure adherence to all necessary rules and protocols.

Ethics and approvals

The present study was approved by the Regional Committee for Medical and Health Research Ethics (REC; ID 2018/2555; see Appendix A) and the Norwegian Center for Research Data (NSD; ID 539270; see Appendix B), and it was conducted in accordance with the Declaration of Helsinki of 1964 [379]. In addition, the research was reported according to the Biospecimen Reporting for Improved Study Quality (BRISQ) recommendation [381]. In paper 3, we used plasma samples from a group of stroke patients included from the health registry and research biobank for neurological diseases at Akershus University Hospital (Ahus) (REC; ID 2011/1015). In addition, we recruited a group of age and gender matched healthy elderly individuals (controls) from which we drew peripheral blood samples. These individuals also performed a set of cognitive and motor test such as MMSE, CDT, TMT A-B, and hospital anxiety and depression scale (HADS) measuring symptoms of depression and anxiety. In brief, the participants were verbally informed about the project, their right to withdraw from the study and that no individual results would be provided. All signed a written informed consent (Appendix C) prior to participation in the study. In addition, all the data gathered were deidentified with a participant number, blinding the researchers to the identity of the participants and safeguarding their anonymity.

In January 2020, the Norwegian Board of Health Supervision (Statens Helsetilsyn) initiated a paper based supervision of consent based health research projects at several Norwegian institutions, including the University of Oslo (UiO) and Oslo Metropolitan University (OsloMet). In both institutions, we were asked to fill inn a self-evaluation form. Following this, the current project was randomly selected for a full internal revision by external consultants on

behalf of the UiO. No deviations were found during the revision confirming that the project was conducted in compliance with regulations and protocols.

IV. Plasma as a medium

The most ideal medium to investigate the effects of neurotrophic factors in the brain would be CSF, as its direct contact with the CNS would provide a more immediate picture of the CNS milieu. However, spinal puncture is not part of the standard procedure duringa an acute stroke, and was regarded too invasive to perform for this study alone. Consequently, for both research groups, it was considered unethical. Therefore, in paper 3, we collected peripheral blood samples from the healthy controls, and we also got venous blood of stroke patients from Ahus. With the understanding that the GFs we were going to analyze, were able to cross the BBB and that possible changes in their concentration levels would also be reflected in the blood, plasma was a satisfactory substitute.

In short, plasma is whole blood minus erythrocytes, leukocytes, and thrombocytes (platelets). Plasma is extracted from whole blood by centrifugation where blood cells end up at the bottom, and the yellowish liquid that remains is the plasma containing more than 90% water [382]. In the present study, plasma samples were analyzed. This involved several considerations, and there are advantages and disadvantages related to using plasma as a medium instead of for instance serum. Plasma is easier and more time efficient to separate, as there is no need for the blood to clot to make plasma. Furthermore, the yield volum percentage of plasma is higher compared to serum, generating more volume to be used in the analysis, than using serum which provides lesser volume. However, when measuring BDNF, serum BDNF has shown to be beneficial due to the fact that it is about 100-fold higher than plasma levels [383]. In the literature both plasma and serum are used wildly in the analyzing of soluble mediators in many different conditions [174,335,384] making it difficult to compare studies and findings.

In the present study, we used dipotassium ethylenediamine tetraacetic acid (K_2EDTA) tubes in the blood sampling. 'K_2EDTA' is an anticoagulant that is sprayed as a coating in the tubes. The presensce of K_2EDTA may be a disadvantage when measuring BDNF levels, as it may activate platelets and release BDNF [385]. This may be problematic because the BDNF released from the platelets *in vitro* may camouflage differences in BDNF released from the brain in response to exercise and/or stroke [333,386].

In paper 3, we did analyze VEGF, and other GFs and inflammation markers such as IGF-1, IL-6, IL-10, S100β, TNF-α. However, the results were for most participants under detectable levels, and the result were left unpublished. We may only speculate if having used serum we might have got levels over the detection limits. More importantly, the plasma samples were treated identically and analyzed using sandwich ELISA assay. Therefore, using plasma, serum, or CNF is unlikely to cause the huge differences in some of the concentration between the acute stroke patients and the healthy control group.

Sandwich ELISA – a valid immunoassay method

In paper 3, plasma concentrations of BDNF, EGF, bFGF, and Irisin were determined using sandwich ELISA, a validated technique to quantitatively detect an antigen within a sample. The measuring was conducted in strict accordance with the protocols and instructions of the manufacturer (R&D Systems Inc. Minneapolis, USA). The advantage of using sandwich ELISA compared to other types of ELISAs, is the utilization of two antibodies which recognize non-overlapping epitopes of the protein of interest, enhancing the overall selectivity of the detection. In addition, sandwich ELISA tends to be more sensitive, robust and is a validated immunoassay to measure target analytes such as antibodies, hormones, and protein biomarkers in different samples including serum, plasma, full blood, urine, and salvia to name some [387,388]. As discussed for IF labelling above, ELISA detections are also relying on the specificity of antibodies. In addition, the antigen must be large enough to allow two antibodies to bind simultaneously. It is not always easy or possible to find compatible capture and detection antibody pairs to function effectively were cross-reactivity can be problematic. The antibodies used in the present study were bought as parts of validated ELISA kits, containing all the solutions and antibodies needed to do an analysis. We used DuoSets containing matched and paired antibodies, optimized and validated by the manufacturer, thus lowering the possibility for cross-reactivity. Further, these kits were also validated for serum and plasma samples, the latter was the medium used in the present study. Such kits in general involves many steps, introducing greater potential for error (**Figure 4**).

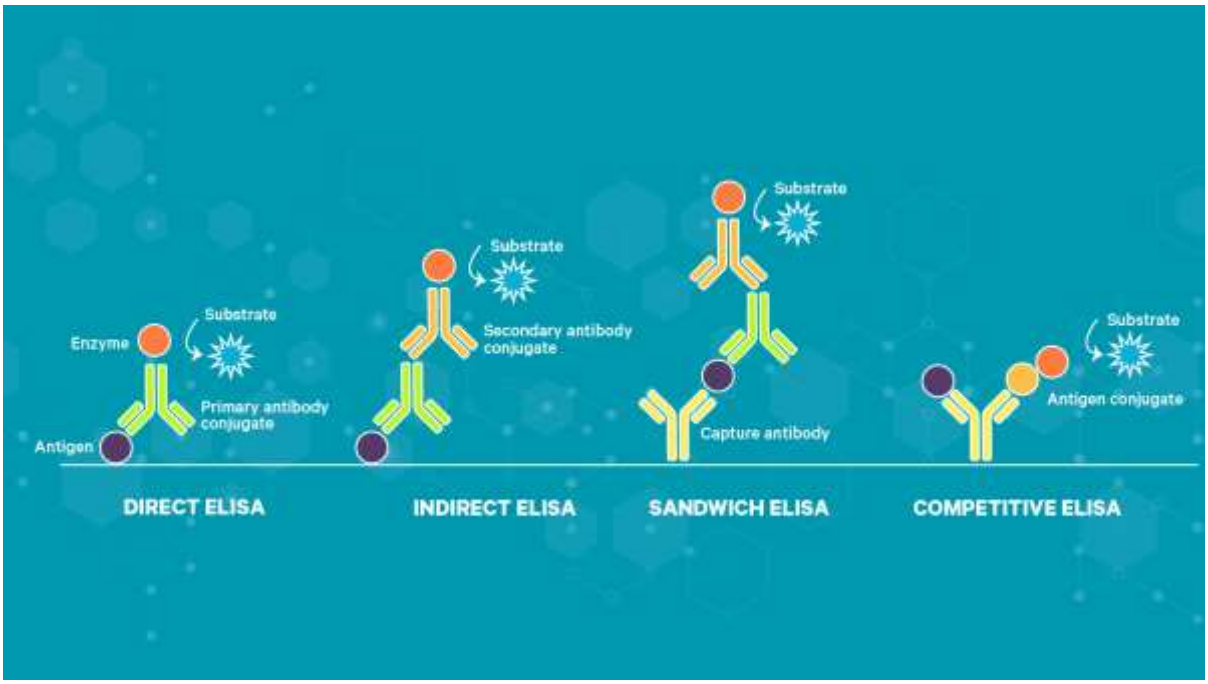

Figure 4: Types of ELISA. In the Direct ELISA, antigens or antibodies in a sample are adsorbed directly to the plate in a non-specific manner; Indirect ELISA works in a very similar way to the direct ELISA except for the addition of a secondary antibody step; **Sandwich ELISA**, the 'capture' antibody (yellow), specific for the antigen, is attached to a solid surface. Then the antigen is added (purple dot), followed by addition of a second 'detection' antibody (green) which binds the antigen at a different epitope than the capture antibody. The sandwich ELISA 'sandwiches' the antigen between the two antibodies [389]. Finally, competitive ELISA, the sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen. Figure from [388] reprinted with permission.

In the present study we also encountered a specific cross-reactivity situation. When measuring plasma BDNF, there was a chance to detect both proBDNF and mBDNF with the BDNF antibodies. Since the epitopes used in the human/mouse BDNF DuoSet ELISA-kit are nonunique for mBDNF, there is a risk that the antibody binds to the proBDNF too. To minimize any impact of platelet-associated BDNF and thus obtain a more valid measure of circulating BDNF, some manufacturers recommend centrifuging a second time within 30 minutes of collection at ~10,000 g for 10 minutes. Other manufacturers do not recommend using plasma for BDNF measurement nor offer any information on how to prepare the blood samples for plasma analyses [390]. All the kits used in this study were, however, verified for both plasma and serum samples as mentioned above. In addition, for the kit used to analyze BDNF there was no available information from the manufacturer about the topics mentioned, nor the affinity for mBDNF contra for the proBDNF. BDNF from platelet, however, is reported to be low compared to the plasma levels, and hence, BDNF measured in plasma is likely to mostly represent the concentration of circulating BDNF. However, normal plasma contains platelets, which could potentially affect the amount of BDNF measured. Despite obvious

differences between serum and plasma BDNF, these measures are still used interchangeably in human literature (51). Consequently, this may give false positive results which must be taken into consideration when interoperating the results. However, the ratio between mature and proBDNF is normally rather high [391]. Hence, BDNF found in this study can be expected to mainly represent mBDNF.

Main conclusions

Whilst most researchers largely agree upon the positive benefits of PA and exercise in cognition, brain plasticity and depression, they still represent underutilized interventions. While some studies imply that the effects of exercise on psychological outcome largely depends on placebo or non-specific psychosocial effects (e.g., attention by staff) [201,203], others claim that most physicians do not regularly assess or prescribe PA and exercise to their patients due to time constraints, complex comorbidities and the lack of training or education on PA counselling and prescribing [392]. In contrast, researchers have for years, promoted recommendations for exercise prescriptions for the general public, partly to prevent stroke or cognitive decline, as well as for people suffering of MDD. The optimal treatment regime however, regarding the relation between dose (frequency, intensity, duration) and therapeutic response is still unknown.

The overall aim of this thesis was to investigate whether HIIT or MIIT, to different degrees, induced neurogenesis and affected depression-like symptoms in mice. Furthermore, to investigate whether lactate signaling was involved in the effects of exercise, the experiments were conducted in HCA_1 KO mice in addition to in WT mice, and in some experiments a lactate-treated group was added.

In paper 1, we report that neurogenesis in the SVZ was enhanced by HCA_1-activation, as treatment with exercise or lactate resulted in increased neurogenesis in the WT, but not in HCA_1 KO mice. In the SGZ of the hippocampus, on the other hand, neurogenesis was induced by exercise in both genotypes, but unaffected by lactate treatment. In paper 2, we found that two weeks of HIIT or MIIT were equally effective at reducing depression-like behavior in mice, and the effect was seen in WT mice but absent in HCA_1 KO mice. After six weeks of exercise, however, MIIT, but not HIIT, reduced depression-like behavior of WT mice but not HCA_1 KO mice. The findings, thus, indicate that HCA_1-dependent mechanism(s) are pivotal for exercise-induced antidepressant effects, perhaps opening for HCA_1 as a novel target for new antidepressant therapies which mimic the antidepressant effects of exercise. Finally, in paper 3, we studied the levels of some key neurotrophic factors in another condition where brain lactate levels are increased, namely acute ischemic stroke. By comparing the plasma levels of these neurotrophins in stroke patients with age and gender matched controls, we were able to detect reduced levels of BDNF and EGF, but unaltered levels of bFGF and irisin, during the acute phase of stroke. These alterations suggest that a mismatch is created in the acute phase of

stroke, between the need for neuroplasticity and the brain's ability to induce such mechanisms. Large individual differences in GF levels were seen among the stroke patients, but further studies are needed to investigate whether these levels can be used as predictors of long-term prognosis. Future studies are also needed to clarify whether HIIT leads to increased levels of neurotrophic factors in ageing individuals and can be used as an efficient means of stroke prevention.

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Paper I

Lambertus, M.*, Øverberg, L.T.*, Andersson, K.A., Hjelden, M.S., Hadzic, A., Haugen, Ø.P., Storm-Mathisen, J., Bergersen, L.H., Geiseler, S. and Morland, C., (2021).

L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1.

Acta Physiologica, 231(3), https://doi.org/10.1111/apha.13587

Received: 8 June 2020 | Revised: 21 November 2020 | Accepted: 23 November 2020

DOI: 10.1111/apha.13587

REGULAR PAPER

ACTA PHYSIOLOGICA

L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1

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Funding information
Norges Forskningsråd, Grant/Award Number: 214458 and 262647

Abstract

Aim: Adult neurogenesis occurs in two major niches in the brain: the subgranular zone of the hippocampal formation and the ventricular-subventricular zone. Neurogenesis in both niches is reduced in ageing and neurological disease involving dementia. Exercise can rescue memory by enhancing hippocampal neurogenesis, but whether exercise affects adult neurogenesis in the ventricular-subventricular zone remains unresolved. Previously, we reported that exercise induces angiogenesis through activation of the lactate receptor HCA1. The aim of the present study is to investigate HCA_1-dependent effects on neurogenesis in the two main neurogenic niches.

Methods: Wild-type and HCA_1 knock-out mice received high intensity interval exercise, subcutaneous injections of L-lactate, or saline injections, five days per week for seven weeks. Well-established markers for proliferating cells (Ki-67) and immature neurons (doublecortin), were used to investigate neurogenesis in the subgranular zone and the ventricular-subventricular zone.

Results: We demonstrated that neurogenesis in the ventricular-subventricular zone is enhanced by HCA_1 activation: Treatment with exercise or lactate resulted in increased neurogenesis in wild-type, but not in HCA_1 knock-out mice. In the subgranular zone, neurogenesis was induced by exercise in both genotypes, but unaffected by lactate treatment.

Conclusion: Our study demonstrates that neurogenesis in the two main neurogenic niches in the brain is regulated differently: Neurogenesis in both niches was induced by exercise, but only in the ventricular-subventricular zone was neurogenesis induced by lactate through HCA_1 activation. This opens for a role of HCA_1 in the physiological control of neurogenesis, and potentially in counteracting age-related cognitive decline.

KEYWORDS
adult neurogenesis, exercise, GPR81, HCA_1, HCAR1, lactate

Marvin Lambertus and Linda Thøring Øverberg contributed equally to this work.

1 | INTRODUCTION

The prevalence of age-related memory decline and age-related neurodegenerative diseases is likely to escalate in the years to come, in pace with the rapid ageing of the population. In humans, evidence from both long-term prospective studies[1,2] and cross-sectional studies[3-5] demonstrate that exercise may reduce cognitive decline and the risk to suffer from dementia.[6] Physical exercise has shown beneficial effects in young people as well as in elderly people with mild cognitive decline.[7] The mechanisms underlying these exercise-induced effects are not completely understood. Exercise may improve executive function and attention through direct actions on the brain: for instance exercise may counteract the decline in cerebral blood flow seen in ageing and age-related neurodegenerative diseases.[8-10] In addition to direct effect of the brain, exercise may affect brain function indirectly though peripheral mechanisms, including regulation of glucose metabolism, and reduction of obesity and type 2 diabetes; all of which have secondary effect on cognitive functions (for review, see Ref. 11). Physical exercise, especially running, also enhances hippocampal adult neurogenesis in rodents.[12-14] Neurogenesis, the generation of new neurons from neuronal stem cells, and their integration into the neuronal circuits may enhance memory function. Most studies find exercise to be as efficient as pharmacological antidepressant therapy at treating mild to moderate depression.[15-24] The antidepressant effect of exercise, as well as that of antidepressants, appears to involve neurogenesis.[22,25-30] For this reason, adult neurogenesis has gained interest as a potential target for manipulation in the combat against depressive symptoms, as well as against neurodegeneration and memory loss associated with ageing and age-related diseases.

In the adult brain, neurogenesis occurs primarily in two niches: One niche is the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. Cells from this niche migrate throughout the dentate granular layer of the hippocampal formation, serving a role in memory function.[31] The second neurogenic niche is the ventricular-subventricular zone (V-SVZ), which is located alongside the ependymal cells that line the lateral ventricles.[32] In rodents, progenitor cells originating from this niche migrate predominantly anteriorly to the olfactory bulb where they differentiate into interneurons.[33] In this process, activated type B neuronal stem cells divide and give rise to transit amplifying type C cells, which again give rise to type A neuroblasts (type A cells) (Figure 1). Eventually, the type A cells migrate along the rostral migratory stream to the olfactory bulb, where they are incorporated into the existing circuitry and mature into GABAergic or dopaminergic neurons.[34-37]

To what degree hippocampal neurogenesis occurs in the adult human hippocampus has been a hot topic for debate during the last years, because of opposing findings.[38-40] There is a consensus that neurogenesis in the SGZ is reduced over the lifespan: some researchers find this decrease to take place rapidly after birth, resulting in undetectable neurogenesis during adulthood and ageing.[39] Other researchers find that neurogenesis persists throughout life,[41] but at a progressively slower rate as we age.[42] Neurogenesis in rodents and non-human primates is important for memory and learning as well as in the prevention of mood disorders like depression. Ageing of the neurogenic niches, resulting in declining neurogenesis is not fully understood. Exhaustion of the neural stem cell pool and age-related changes in the circulatory milieu are probably important[43,44] but a broader 'niche ageing' mechanism may also depend on other factors, for instance vascular ageing.[45]

Neurogenesis in the V-SVZ of adult humans seems to be less controversial than in the SGZ, but the role of this stem cell niche in memory and learning is less obvious. In the ageing V-SVZ, a reduction of the stem cell pool has been demonstrated to be paralleled by a loss of the characteristic cytoarchitectural hallmark: the pinwheel structure where the apical cilium of radial glia is surrounded by ependymal cells.[46] Neurogenesis in the V-SVZ and the migration, maturation, and integration of the newborn neurons into the olfactory bulb circuitry is of importance for odour-recognition/olfactory memory.[47,48] Olfactory memory is distinct from the hippocampus-dependent spatio-visual memory but may provide contextual clues to the hippocampal neurons. As discussed by Eichenbaum and Robitsek,[49] odour-recognition may be essential for higher-order cognitive processing and reduced olfactory memory may therefore correlate with cognitive decline in ageing. In humans [50] and non-human primates,[51-53] V-SVZ-derived neurons appear to migrate to the striatum, where they may serve to protect against Parkinson's disease.[54,55] Similarly, a reduction of adult-born neurons in the striatum has been implemented in Huntington's disease,[50] where cognitive decline is an early symptom.

Physical exercise decelerates the age-related decline in cell proliferation and neuroplasticity. In fact, exercise affects hippocampal adult neurogenesis at multiple levels: Exercise increases the proliferation and differentiation of neurons[12-14] but also increases the integration of newly formed neurons into the functional hippocampal network.[56-59] Hippocampal neurogenesis is accompanied by plasticity-related changes in neuronal morphology, such as increased spine density and arborization of the dendritic tree, as well as long-term potentiation.[60,61] In line with this, exercise efficiently delays some of the functional declines associated with brain ageing. At the functional level, exercise enhances learning, memory, and problem solving, and may thus be particularly conducive to prolonging autonomy during ageing. Exercise counteracts the pathology of Alzheimer's disease (AD) in several ways: In mouse models where mutated human genes

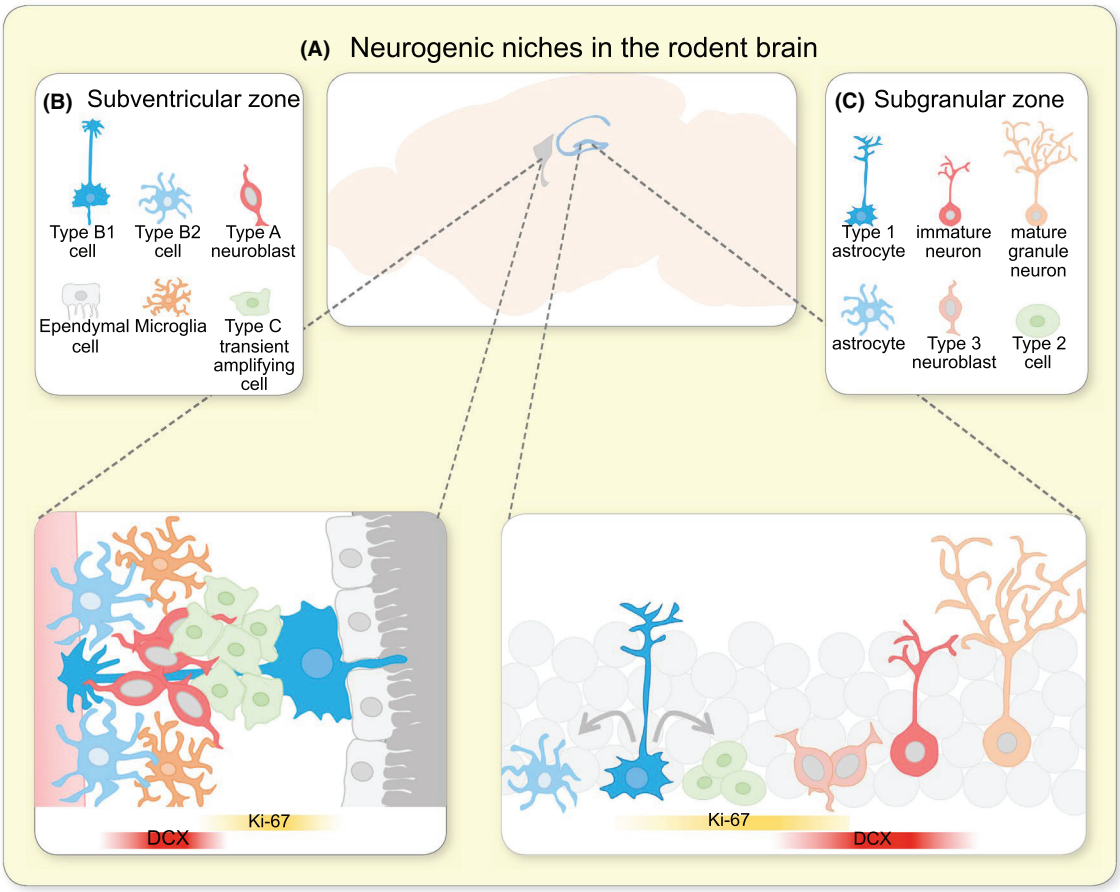

FIGURE 1 A, The location of the two major neurogenic niches -the V-SVZ and the SGZ- in a sagittal section of a mouse brain. B, Schematic drawing of the neurogenic process of the V-SVZ niche. Quiescent type B neural stem cells (NSCs; blue) send an apical process through a layer of ependymal cells (light grey) that line the ventricle (dark grey). A basal process of the type B cell makes contact with the blood vessels of the vascular plexus. Upon activation, the type B cells divide to give rise to type C transit amplifying cells (green). Type C cells rapidly divide and differentiate to type A neuroblasts (red), which migrate to the olfactory bulb. Resident mature astrocytes (blue) and microglia (orange) also inhabit the V-SVZ neurogenic niche. C, Schematic drawing of the neurogenic process of the SGZ niche and the migration into the granular cell layer (light grey cells). Type 1 neuronal stem cells (radial glia; blue) may give rise to neurons (right-pointing arrow) or astrocytes (left-pointing arrow). In neurogenesis, type 1 cells give rise to non-radial type 2 cells (green), which further give rise to intermediate progenitor cells (not shown) that proliferate to give rise to type 3 neuroblasts (pink). As the neuroblasts differentiate to form immature neurons (red) and later fully mature granule neurons (orange), they migrate into the granule cell layer. The stages where Ki-67 and doublecortin (DCX) are expressed are indicated

that increase the accumulations of β-amyloid and/or tau are inserted, running has been reported to reduce the hyper-phosphorylation of β-amyloid and tau,[62,63] which triggers protein aggregation. Interestingly, high intensity exercise was shown to more efficiently reduce β-amyloid levels than exercise at lower intensities, presumably because of an upregulation of several proteases involved in β-amyloid clearance.[64] Whether lactate, which is enhanced in blood and brain in response to high intensity exercise, is involved in the regulation of β-amyloid degradation, is not known. In humans, β-amyloid aggregation in the brain cannot be measured directly (except post mortem), but one study reported an inverse correlation between physical activity and the level of β-amyloid 42 in the cerebrospinal fluid.[65] Supporting this finding, β-amyloid burden was lower and hippocampal volume was greater in old people who were physically active compared with an age matched inactive group.[66] Mouse models for AD consistently show an association between physical activity or exercise and preserved cognitive functions.[67-70] In line with this, exercise increases dendritic arborization,[71] maturation of dendritic spines,[72,73] hippocampal neurogenesis, and reduces the AD-related loss of spatial memory.[74]

Whether exercise also induces neurogenesis in the V-SVZ remains controversial. While some studies report exercise-induced neurogenesis in the V-SVZ, others fail to detect so. Recent data suggest that exercise induces neurogenesis in additional smaller niches, including the hypothalamus and ependymal lining of the third ventricle, and this has been linked to the recovery of homeostatic functions after brain injury.[75] Furthermore, the number of V-SVZ-derived neurospheres has been shown to increase in response to running exercise in aged mice.[76] Lee and colleagues[77] found

that corticosterone-induced reduction in V-SVZ neurogenesis could be rescued by running. Along the same lines, Mastrorilli and colleagues[78] showed that 12 days of voluntary running wheel exercise restored the impaired neurogenesis in the V-SVZ in mice lacking the antiproliferative gene Btg1. In the Btg1 knock-out (KO) mice, an exercise-induced shortening of the cell cycle caused type B cells to accumulate, and hence led to a re-establishment of the physiological progression of neurogenesis the SVZ. This effect was not observed in the wild-type (WT) mice.[78] Nevertheless, the idea that exercise may regulate cell cycle kinetics is in line with previous studies from the hippocampus.[56,79]

Although exercise is known to induce neurogenesis in the hippocampus and is likely to do so in the SVZ as well, the initial molecular signal involved in this process remains unresolved. L-lactate (hereafter referred to as lactate) increases in the blood in response to strenuous exercise and may activate the lactate receptor hydroxycarboxylic acid receptor 1 (HCA_1, also known as HCAR1 or GPR81).[80] HCA_1 is a G_i-protein coupled receptor expressed at the plasma membrane, with the lactate binding site facing extracellulary. Hence, activation of this receptor is distinct from the metabolic effects of lactate and does not require entry of lactate into cells via the monocarboxylate transporters (MCTs).[81] A recent study showed that lactate induced neurogenesis in the hippocampus.[82] This effect was presumably through a metabolic action, as blocking the lactate transporter MCT2 prevented lactate-induced neurogenesis. In line with this, lactate administration prior to learning has repeatedly been shown to enhance memory.[83,84] Lactate signalling through HCA_1, however, also directly affects the excitability and firing properties of neurons independently of metabolic action.[85,86] Recently, a biphasic action of lactate in memory was suggested, where metabolic lactate enhanced memory when administrated before or during the learning process, while HCA_1 signalling mainly enhanced memory consolidation.[87] Hence, evidence for a role of HCA_1 activation on memory is emerging. Furthermore, lactate has been suggested to mediate some of the beneficial effects on neurobioenergetics, and hence to act as an exercise mimetic.[88] We have previously presented that seven weeks of exercise or lactate injections, up-regulates cerebral vascular endothelial growth factor A (VEGFA) levels and angiogenesis in the hippocampus and neocortex through activation of HCA_1.[89] Recent progress in the field suggests that increased angiogenesis correlates with an increase in hippocampal neurogenesis in humans and rodents.[38,90] In these studies, neurogenesis occurs predominantly in neuro-angiogenic foci where neuronal, glial, and endothelial precursors divide into tight clusters. The formation of these clusters near newly formed capillaries also ensures a steady supply of nutrients, oxygen and growth factors to maintain the proliferation of the stem and precursor cells. Taken together, these data suggest that angiogenesis and neurogenesis might be mechanistically linked.

Based on our previous demonstration of HCA_1-dependent angiogenesis and the known stimulation of neurogenesis by exercise, we here investigate whether lactate induces HCA_1-dependent neurogenesis in the SGZ of the hippocampus and/or in the V-SVZ.

2 | RESULTS

2.1 | HCA_1 activation, by exercise or exogenous lactate, induces neurogenesis in the V-SVZ

To investigate whether activation of HCA_1 could be an initial event leading to neurogenesis, we exposed WT mice and HCA_1 KO mice to high intensity interval exercise (HIIT), five consecutive days per week for seven weeks.[89] In the V-SVZ of WT animals, the exercise regime resulted in an increased density of DCX positive neuroblasts compared to what was found in sedentary controls (Figure 2B vs c; quantified in 2 hours). A similar trend was seen in the HCA_1 KO animals exposed to exercise but did not reach statistical significance (Figure 2E vs 2f; quantified in 2 hours). The observed control density of DCX positive cells was higher in KO than in WT, perhaps reflecting compensatory changes that may blunt the effects of interventions in the KO. This did not reach statistical significance ($P = .23$). Interestingly, injections of L-lactate (2 g kg^{-1} body weight; 200 mg ml^{-1}; 18 mmol kg^{-1}; pH 7.4) five days a week for seven weeks, increased neurogenesis in WT mice, to a larger degree than what was found in mice exposed to exercise (Figure 2B vs d; quantified in 2 hours). In HCA_1 KO mice, on the other hand, lactate injections did not increase the density of DCX-positive cells above control levels (Figure 2E vs g; quantified in 2 hours). The lactate treatment used in the present study increases serum lactate to about 10 mM.[89] The dependency of enhanced neurogenesis on HCA_1-activation in the V-SVZ, as determined by the quantification of DCX positive cells after lactate injections in WT and KO mice, was confirmed by quantification of Ki-67 positive cells (Figure 1I), and of cells co-expressing DCX and Ki-67 (Figure 2J). DCX and Ki-67 represent cells at different stages of neurogenesis; DCX identifies neuroblasts (type A cells) while Ki-67 identifies type C cells.

2.2 | Hippocampal SGZ neurogenesis is induced by high-intensity interval exercise but is independent of HCA_1

Since neurogenesis in the V-SVZ was shown to be HCA_1-dependent, we investigate whether activation of HCA_1 could initiate neurogenesis also in the SGZ of the hippocampus. In the SGZ (Figure 3A), exercise resulted in an increased

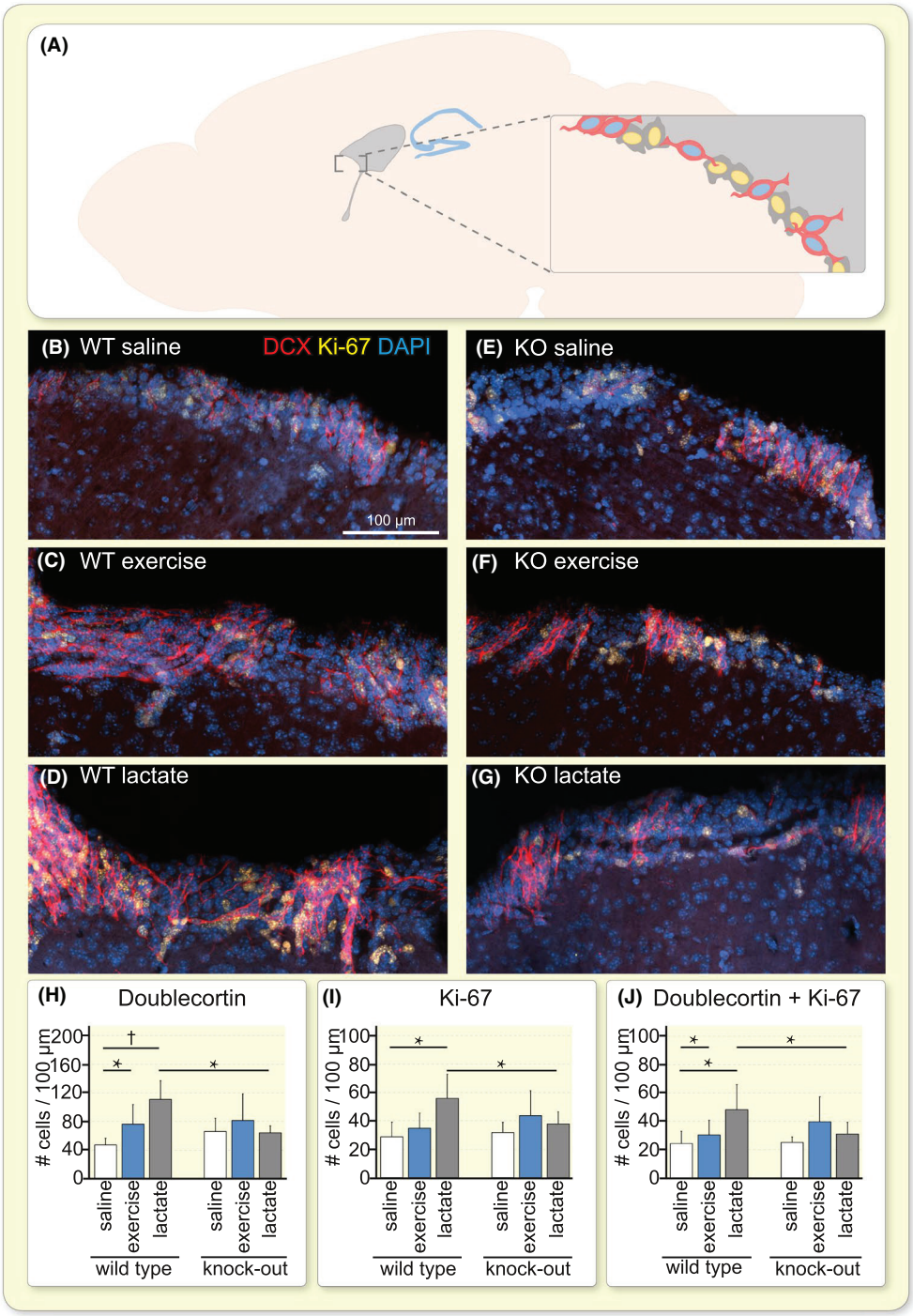

FIGURE 2 The effect of HCA_1 on SVZ neurogenesis on the dorsal part of the lateral ventricle. A, Schematic view of the SVZ. B-G, Confocal micrographs of WT (b-d) and HCA_1 KO (e-g) showing labelling for DCX (red), Ki-67 (yellow), and DAPI (blue). H, The density of DCX-labelled cells increased with high intensity exercise (blue bars) in both genotypes (compared to their respective untreated control groups; white bars), but this only reached significance in the WT ($P = .039$). In the WT mice, lactate treatment (grey bar) lead to an even larger increase in DCX-positive cells ($P = .0004$). The effect of lactate on the density of DCX positive cells was not present in the KO mice (grey bar vs white bar). I, The HCA_1-dependent neurogenic effect of lactate was reproduced by quantification of Ki-67-positive cells. J, Cells which are positive for both Ki-67 and DCX. As for DCX, the number of Ki-67-positive cells increased in response to lactate treatment (grey bar vs white bar) in the WT mice ($P = .003$), but not in the HCA_1 KO mice. $^*P < .05$, $^\dagger P < .01$; one way ANOVA; LSD post-hoc test, WT n = 5,7,4; KO n = 4,4,6). Scale bar = 100 µm

density of DCX positive neuroblasts in both genotypes compared to sedentary controls, ie, independently of HCA_1 activation (Figure 3B vs c; and Figure 3E vs f; quantified in 3 hours). In this region, lactate injections did not induce neurogenesis above control levels (Figure 3B vs d; and Figure 3E vs g quantified in 3 hours).

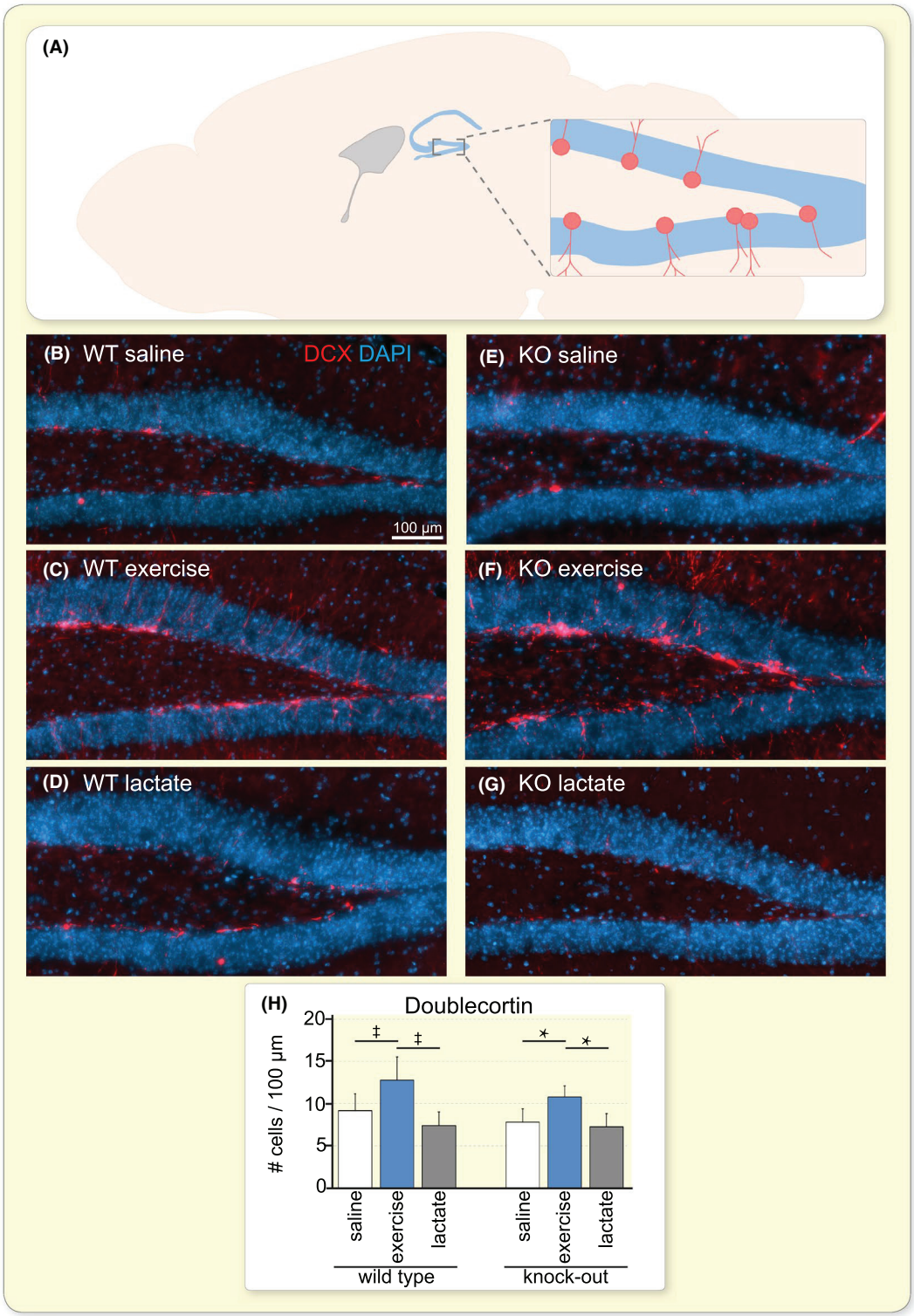

FIGURE 3 The effect of HCA_1 on SGZ neurogenesis on the granule cell layer of the hippocampal formation. A, Schematic view of the SGZ neurogenic niche. (b-g) Confocal micrographs of WT (b-d) and HCA_1 KO (e-g) showing labelling for DCX (red), and DAPI (blue). H, The density of DCX-labelled cells (cells per mm SGZ length) increased in response to high intensity exercise (blue bars) in both genotypes (compared to their respective untreated control groups, white bars); WT ($P = .003$); KO ($P = .038$). Contrary to what was seen in the SVZ, lactate treatment (grey bars) did not lead to increased density of DCX-positive cells neither in the WT mice nor in the KO mice. Data are average ± SD; *$P < .05$, ‡$P < .005$; one way ANOVA; LSD post-hoc test, n = 7,7,6; KO n = 5,4,6). Scale bar = 100 µm

2.3 | HCA_1 activation leads to activation of the Akt/PKB pathway

Data from the HCA_1-mRFP reporter mouse line show that HCA_1 is localized in leptomeningeal fibroblasts.[89] In a recent study,[91] we show that HCA_1 is highly enriched also in fibroblast-like cells in the dorsal part of the wall of the third ventricle, in parts of the choroid plexus and in the tela choroidea. We do not find high levels of HCA_1-mRFP in cells of the SGZ or the SVZ, suggesting that HCA_1-containing leptomeningeal fibroblasts represent the primary site of action for lactate via HCA_1. Release of growth factors from these cells in response to HCA_1 activation may underlie the selective effect in the V-SVZ. In line with this, we demonstrate that primary cultures of leptomeningeal fibroblasts isolated from the HCA_1 (WT) mouse line responded to lactate by an increased phosphorylation of Akt/PKB at 5 minutes (Figure 4A). This effect was not seen after 15 minutes (Figure 4A). After longer exposure times to lactate, p-Akt levels were reduced compared to control (Figure 4B). The latter finding likely represents internalization of the HCA_1 receptor,[92] as the effect on p-Akt is nullified by co-incubation with the selective β-arrestin/β2-adaptin interaction (endocytosis) inhibitor, barbadin (Figure 4B). Furthermore, the exogenous HCA_1 agonist 3-chloro-5-hydroxy benzoic acid (3-Cl-5-OH)[93] produced a similar effect as did lactate, highlighting that the observed effect results from receptor activation, distinct from metabolic effects of lactate.

Despite three independent attempts (3-6 mice in individual wells per attempt) to culture leptomeningeal fibroblasts from the HCA_1 KO mouse line in parallel with the WT fibroblast cultures, we did not succeed in obtaining KO fibroblast cultures. The KO fibroblasts stopped proliferating and/or died prior to reaching confluency (data not shown), suggesting that HCA_1 is important for viability in these cells.

3 | DISCUSSION

The present study demonstrates that in the V-SVZ, injections of lactate, five days a week for seven weeks, induced neurogenesis in WT mice, as evidenced by increased densities of cells expressing markers of newly formed neuroblasts (DCX) and/or earlier developmental stages (Ki-67) (Figure 2). This effect

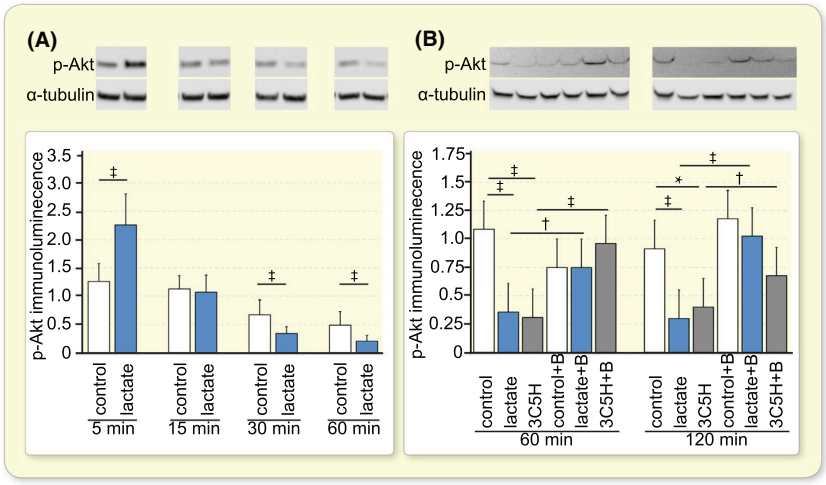

FIGURE 4 The effect of HCA_1 activation on the level of phosphorylated Akt (p-Akt) in leptomeningeal fibroblasts. A, Representative Western blots for p-Akt (band at 60 kDa) and α-tubulin (band at 50 kDa) after exposure of leptomeningeal fibroblasts to PBS (control) or PBS with sodium L- lactate (20 mM) for 5, 15, 30 or 60 minutes. Below: Quantitatve assessment of the immunoluminescence intensity of the p-Akt band, relative to the loading control. To be able to combine results from different experiments (n = 7), the results were normalized to the average of all the control samples on the same blot. The results are presented as mean ± SD. Exposure to lactate (blue bars) resulted in an increased level of p-Akt compared to control (white bars) after five minutes ($P = .00002$; paired Student´s *t*-test). This effect was not seen after 15 minutes. After longer exposure times, 30 and 60 minutes, p-Akt levels were reduced in response to lactate treatment (blue bars) compared to control (white bars; $P < .005$ for both comparisons; paired Student´s *t*-test for each time point). B, Representative Western blots for p-Akt after exposure of leptomeningeal fibroblasts to PBS (control), PBS with sodium L-lactate (20 mM) or 3-Cl-5-OH (3C5H; 40 μM) in PBS in the absence or presence of the endocytosis blocker barbadin for 60 or 120 minutes. Quantitative results shown below are immunoluminescence intensity of the p-Akt band, relative to the loading control. To be able to combine results from different experiments (n = 4), the results were normalized to the average of all the control samples on the same blot. As expected, lactate (blue bars) produced a reduction in the p-Akt levels after 60 or 120 minutes compared to the respective controls (white bars; $P < .005$; one-way ANOVA for each time point; LSD posthoc test). These findings were reproduced by exposure to the exogenous HCA_1 agonist, 3-Cl-5-OH (grey bars; $P < .05$; one-way ANOVA; LSD posthoc test) compared to the respective controls. Barbadin (B) completely inhibited the effects of the HCA_1 agonists, suggesting that the reduction of p-Akt produced by lactate and 3-Cl-5-OH after 60 or 120 minutes were because of internalization of the HCA_1 receptor. *$P < .05$; †$P < .01$; ‡$P < .005$

was abolished in HCA_1 KO mice, indicating that the lactate receptor regulates V-SVZ neurogenesis (Figure 2). The lactate treatment regime increases plasma lactate to about 10 mM in 5-15 minutes, returning to basic levels within 45-60 minutes.[89] High intensity interval exercise, which increases lactate to a similar, but somewhat lesser extent (see Ref. 89), tended to induce neurogenesis in both genotypes, reaching statistical significance only for DCX in WT, but not for Ki-67 or double labelled DCX + Ki-67 cells in either genotype. While exercise did not increase the density of DCX labelled cells in KO, it should be noted that KO mice had a control level of DCX labelling that was slightly higher than in the WT. This difference did not reach statistical significance, but still, it opens for the possibility that the lack of HCA_1 could have caused compensatory changes, masking an exercise induced increase of DCX density in KO mice. Based on these results we cannot conclude whether the effect of lactate injections in HCA_1 WT animals is an additional effect on top of a smaller exercise-induced neurogenesis (occurring through HCA_1-independent mechanisms), or whether the effect is induced by HCA_1-activation alone. Nevertheless, our data demonstrate that HCA_1 is a pivotal regulator of neurogenesis in the V-SVZ.

The HCA_1 dependency of neurogenesis in this niche was not caused by differences in running speed or exercise intensity, as the knock-out and wild-type mice performed equally well in maximal exercise-capacity tests, which were performed every second week throughout the exercise period.[89] As reported previously, the mice rapidly increased their maximal exercise capacity during the first weeks of exercise, reaching a plateau at about 140% after 5 weeks, as predicted for HIIT.[89]

In the other main neurogenic niche of the brain, the SGZ of the hippocampus, exercise induce neurogenesis in both genotypes, but this effect could not be replicated by lactate injections (Figure 3). This is in line with previous studies demonstrating that even mild exercise is enough to induce hippocampal neurogenesis, through several mechanisms.[94]

Neurogenesis has been suggested to occur in close proximity to newly formed capillaries.[45] We have previously shown that angiogenesis in the dentate gyrus is increased in response to exercise or lactate injections in an HCA_1-dependent manner.[89] Since these analyses were performed in the same animals as used in the present study, we can conclude that the regulation of hippocampal neurogenesis, being independent of HCA_1, is not secondary to an HCA_1-dependent angiogenesis. Hence, in the present study, neurogenesis occurred independently of angiogenesis.

Which HCA_1-expressing cells may be the primary sites for the action of circulating lactate resulting in HCA_1-induced increase in V-SVZ neurogenesis? Using a HCA_1-mRFP reporter mouse line, we have previously demonstrated that HCA_1 is expressed at high levels in the pia mater, where it localizes on fibroblast-like cells accumulated along pial blood vessels.[89] The pial localization of HCA_1 was confirmed by qPCR analysis, and is consistent with data presented in the Allen Brain Atlas (http://mouse.brain-map.org/gene/show/89056; http://mouse.brain-map.org/experiment/siv?id=77464856&imageId=77469798&initImage=expression&contrast=0.5,0.5,0,255,4). The HCA_1-mRFP labelling was stronger along arteries than veins, and did also line some of the larger blood vessels penetrating into the brain parenchyma.[89] This labelling could be consistent with a layer of pial fibroblasts reported to continue along the glia limitans along the branching arteries that penetrate into the brain parenchyma.[95,96] Growth factors like VEGF[97] and IGF-1, all of which can be released from fibroblasts, are known to regulate neurogenesis: The proper regulation of the neurogenesis process depends on VEGFA,[97] or the coordinated action of fibroblast-growth factor 2 (FGF2) and insulin-like growth factor 1 (IGF1).[98] Fibroblasts are known to release all of these growth factors, opening for the possibility that pial fibroblasts are the main site of action for lactate via HCA_1. In fact, we have previously shown that hippocampal VEGFA levels increased in response to HCA_1 activation in vivo. Interestingly, we recently showed that pial fibroblast-like cells, expressing HCA_1, are also found along the dorsal region of the wall of the third ventricle, and the adjacent parts of the choroid plexus and the tela choroidea.[91] In the present study, we demonstrate that primary leptomeningeal fibroblasts respond to HCA_1 activation by enhanced phosphorylation of Akt/PKB. A possible result of this increase in p-Akt is an increase in CREB, leading to enhanced expression of growth factors.[99] Various growth factors are also known to regulate the Akt-pathway though activations of their respective plasma membrane receptors, but given the fact that our data were obtained in monocultures of fibroblast and that increased p-Akt is detected within five minutes, an indirect effect via growth factor release is less likely. We therefore believe that the activation of the Akt-pathway occurs downstream of HCA_1 activation, in line with previous evidence from different cell types.[100,101] The results on p-Akt in leptomeningeal fibroblasts expand on the previous observation[92] that HCA_1 activation over time (minutes) leads to downregulation of the response to below basal levels, because of internalization of the receptor. The observations indicate that intermittent stimulation, like by lactate generated in high intensity interval exercise, will act differently from continuous stimulation, such as by administration of an exogenous agonist with a longer half-life. This phenomenon has important implications for using HCA_1 as a target of therapeutic intervention. It may also serve to explain apparently contradictory results of HCA_1 activation. Our data suggest that the HCA_1-containing fibroblasts and ependymal cells have the ability to respond to alterations in brain, blood, and CSF lactate to release their growth factors directly into the CSF where they may gain access to the cells of the subventricular zone. This remains to be investigated but could theoretically represent an explanation of why neurogenesis in the V-SVZ, but not in the SGZ, is sensitive to lactate/HCA_1-regulated neurogenesis. In this context, it should be noted that the walls of the

lateral ventricles, where the V-SVZ is located, do not contain pial fibroblasts, so a local action within the ventricle wall is not likely. In our previous study,[91] we also found HCA_1-mRFP in cells along smaller vessels in the neural tissue. Based on their perivascular localization and their expression of platelet-derived growth factor receptor beta (PDGFR-β), we suggested that these HCA_1-containing cells were immature pericytes, but PDGFR-β is also expressed by immature fibroblasts. Although not detected explicitly in the HCA_1-mRFP reporter mice, the localization of HCA_1 on other cell types in the brain has been suggested. For instance, Jean-Yves Chatton's lab have convincingly shown HCA_1-dependent electrophysiological effects in cultured neurons, indicating that neurons express HCA_1.[85] An astrocytic localization of HCA_1 has also been suggested.[102] Localization of HCA_1 on neural stem cells has not been reported but cannot be excluded. Consequently, we cannot conclude as to which cell type is responsible for the HCA_1-mediated influence on neurogenesis in the V-SVZ, but lactate-sensing fibroblasts is a likely option.

Taken together, our results demonstrate that neurogenesis in the two main neurogenic niches of the brain are regulated differently: while physical exercise is important for SGZ neurogenesis, it appears to serve a smaller role in V-SVZ neurogenesis. In contrast, HCA_1-dependent lactate-induced neurogenesis was only detected in the V-SVZ, and not in the SGZ. HCA_1 may therefore represent a potential target for future drugs aiming to increase neurogenesis in the V-SVZ niche selectively. The functional consequences of V-SVZ neurogenesis remain unresolved. Nevertheless, recent data suggest that V-SVZ neurogenesis and the insertion of newborn neurons into the olfactory bulb circuitry plays a role in odour-recognition/olfactory memory,[47,48] which may be important for higher-order cognitive processing and olfactory memory.[49] Although distinct from the spatio-visual memory, which is dependent on the hippocampus, olfactory memory may affect hippocampal memory by providing contextual clues. While hippocampal neurogenesis is important in memory recollection and spatio-visual memory, some researchers claim that olfactory memory deficit may be the rodent equivalent of human age-related cognitive decline.[49] Furthermore, in humans[50] and non-human primates,[51-53] V-SVZ-derived neurons appear to migrate to the striatum where they may serve to protect against Parkinson's disease[54,55] and Huntington's disease.[50] Therefore, it is not unlikely that an HCA_1-regulated increase in neurogenesis may decelerate age-related cognitive decline.

4 | MATERIALS AND METHODS

4.1 | Animals and animal treatment

The study was performed in strict accordance with the national and regional ethical guidelines, including the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All animal handling and experiments were performed by Federation of European Laboratory Animal Science Associations (FELASA) certified personnel and approved by the Animal Use and Care Committee of the Institute of Basic Medical Sciences, The Faculty of Medicine, University of Oslo, and by the Norwegian Animal Research Authority (FOTS6292, FOTS6505, FOTS6590, FOTS6720, FOTS6758 and FOTS8243). The experiments are reported in accordance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines[103] and in agreement with the recommendations for good publishing practice in physiology.[104]

The HCA_1 KO mouse line was generated as described.[105] At seven weeks of age, HCA_1 KO or WT mice were semi-randomized into three groups: high-intensity interval training (HIIT), sodium L-lactate injections (LAC), or saline injections (control), ensuring an equal distribution of males and females in each group. The HIIT regime used in the present study was designed for optimal gain in cardiovascular function and to reach about 90% of VO_{2max}.[106,107] It consisted of interval exercise as described,[89] five consecutive days per week for seven weeks. Each session consisted of 10 min warm-up at 5 m min^{-1}, followed by 10 × 4 minutes of high-intensity intervals on a treadmill (Columbus Instruments, USA) at a 25 degrees incline. Between the intervals there were two minutes of active rest at 8.6 m min^{-1} 25 degrees incline. Every second week throughout the exercise period, a maximal exercise capacity test (MECT) was performed for each individual mouse, to adjust the running speed of the intervals. The MECT was performed as follows: After a 15 minute warm up-period at 9.6 m min^{-1}, the band speed was increased by 1.8 m min^{-1} every 2 minutes until exhaustion, ie, the mice refused to run further, despite being manually pushed on to the band or receiving electrical stimuli (<1.5 mA, maximally 1-2 per day by the intrinsic device of the treadmill).

The mice that were treated with lactate, received a subcutaneous injection of sodium L-lactate (2 g kg^{-1} body weight; 200 mg ml^{-1} dissolved in 0.9 % saline; pH adjusted to 7.4; ie, 18 mmol kg^{-1}). The control mice received the same volume (per kg body weight) of 0.9 % saline. The lactate or saline injections were administered subcutaneously five days a week for seven weeks. The mice were weighed every week for dosage-adjustment.

At 6 hours after the end of the exercise or 6 hours after the last dose of lactate or saline, the mice were anaesthetized with zolazepam 3.3 mg ml^{-1}, tiletamine 3.3 mg ml^{-1}, xylazine 0.5 mg ml^{-1}, fentanyl 2.6 μg ml^{-1}; 0.01 ml g^{-1} body weight, intraperitoneally (i.p.) and perfused transcardially with 4 % formaldehyde in 0.1 M sodium phosphate buffer pH 7.4 (NaPi) for 8 minutes. After perfusion with the fixative, the brains were gently removed from the skull, and stored in

4 % formaldehyde at 4°C overnight, before the brains were transferred to a 1:10 dilution of the fixative. During the experiment, one KO mouse exposed to exercise was excluded after 5 weeks, as it suddenly performed worse than expected (based on the previous performance of the same mouse) during two consecutive exercise section. It was therefore considered injured or sick. Two mice were excluded because of stereotypical behaviour (one KO saline, and one KO exercise). Two mice were excluded because of suboptimal perfusion fixation (1 WT saline; 1 KO exercise). The remaining animals were WT: 7, 7, 6 (saline, exercise, lactate) and KO: 5, 4, 6 (saline, exercise, lactate).

4.2 | Immunofluorescence

Before sectioning, the brains were transferred to 30 % sucrose in 0.1 M NaPi and allowed to sink in this solution overnight. Sagittal sections (20 µm thickness) were cut on a sliding microtome. Free-floating sections obtained 0.75-0.80 mm laterally from the midline were used. Immunofluorescence was performed largely as described.[89] Free-floating brain sections were rinsed three times in PBS (10 mM NaPi, 0.9 % NaCl) and antigen retrieval was performed by incubation of the section in citrate buffer (0.01 M, pH 8.7) for 30 minutes at 80°C. The sections were then rinsed in PBS and unspecific antibody binding sites were blocked by incubating with 10 % newborn calf serum and 0.5 % Triton X-100 in PBS for 2 hours. The sections were then incubated with primary antibodies (rabbit anti-Ki-67, Abcam AB15580; final dilution 1:500) and guinea pig anti-doublecortin (DCX, Merck AB2253, final dilution 1:2000), overnight at room temperature under careful shaking. Then, the sections were rinsed in PBS and incubated with secondary antibodies: Alexa Fluor 594 Anti Guinea Pig (Invitrogen A11076) and Alexa Fluor 647 Anti Rabbit (Invitrogen A21241), both at a dilution of 1:500, for 2 hours at room temperature. From this step on, the sections were covered from light. After incubation with the secondary antibodies, the sections were rinsed, stained with DAPI (Thermo Fisher, UK) for 15 minutes and rinsed again. Finally, the sections were mounted with Prolong Gold Antifade Mounting Reagent (Thermo Fisher, UK) and coverslipped (Assistant, Germany).

Confocal microscopy was performed with a Zeiss LSM880 microscope with Airyscan detector at 20× magnification with each Z-stack slice 0.5 µm, combined with a maximum intensity projection option. The cells were counted manually by an observer who was blinded to the genotypes and treatments, and the length of the V-SVZ or SGZ was measured in the Fiji distribution of ImageJ[108] (version 2.0.0-rc-69/1.52p; Java 1.8.0_172, ImageJ, RRID:SCR_003070) or in ZenBlue 2.3. The density of newborn neurons was calculated as the number of DCX or Ki-67 positive cells divided by the measured length of the corresponding V-SVZ or SGZ for each animal. For the V-SVZ, six sections were excluded from the analysis, either because they had artefacts in the region of interest, or because they were not in the narrow anatomical region included in this study. The excluded sections were two WT saline, two WT lactate and one KO saline.

4.3 | Isolation of primary leptomeningeal fibroblast

Leptomeningeal fibroblasts were isolated[109] from WT (n = 5) and HCA_1 KO (n = 6) mice. The mice were deeply anaesthetized with isoflurane and euthanized by decapitation. The brain was gently removed from the scull and put in ice cold PBS. A thin layer of the neocortex was dissected loose, transferred to sterile culture medium and cut into smaller pieces. The pieces of cortical tissue from each animal were distributed into three of the wells of a six-well plate (VWR, United Kingdom). Growth media (DMEM/F12 GlutaMAX (Thermo Fisher Scientific), with 10 % Foetal Bovine Serum (FBS) (Thermo Fisher Scientific, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, USA)) was added at a volume that prevented the tissue from floating yet kept them moist (about 850 µL in each well). The tissue pieces were grown at 37°C with 5 % CO_2. The media was gently replaced twice a week. When a confluent layer of leptomeningeal fibroblasts covered the entire well, after approximately four weeks, the remaining tissue pieces and the medium was removed. During this 4-weeks period, all the KO cells stopped growing and/or died. The WT cells were washed with trypsin-EDTA (0.25 %; Sigma-Aldrich, USA) in PBS and the detached cells were pipetted into 15 mL falcon type. To inactivate the trypsin, growth media containing 10 % FBS was added, and the cells were centrifuged (Heraeus Megafuge 1.0 R, Thermo-Fisher Scientific, USA) at 1000 *g* for about 5 minutes. The resulting cell pellets from all animals were pooled and grown in the T75 flask until confluency. For experiments, the cells were split to several six-well Tissue Culture plates, with 2 mL growth medium in each well. The cells used in the present study were generation 7-11 ex vivo.

4.4 | Exposure of cells to HCA_1 agonists

At 24 hours before the experiment, the medium was replaced by either growth medium containing 10 % FBS or medium without FBS. The following day, the cells were exposed to either sodium L-lactate (Sigma-Aldrich, USA) 20 mM in PBS or vehicle, for five, 15, 30 or 60 minutes. To study whether HCA_1 was internalized in response to receptor activation, other cells were exposed to sodium

L-lactate (20 mM), the HCA_1 agonist 3-chloro-5-hydroxybenzoic acid (3-Cl-5-OH: Sigma-Aldrich, USA; 40 μM), or PBS (control), in the absence or presence of the β-arrestin/AP2-dependent G-protein-coupled (GPCR) endocytosis inhibitor, barbadin.[110] In the cultures treated with barbadin, barbadin was added at 30 minutes prior to the HCA_1 agonists. Since 3-Cl-5-OH was dissolved in DMSO, all wells were supplemented with DMSO 1 μL per 2 mL PBS. The exposure times for the latter experiment were 60 and 120 minutes. The cells were harvested in 100 μL ice-cold RIPA buffer with protease- and phosphatase inhibitor (Sigma-Aldrich, USA), transferred to Eppendorf tubes and snap frozen in liquid nitrogen before they were stored in −80°C.

4.5 | Western blotting

The protein content of the harvested fibroblast samples was measured with the Bicinchoninic acid (BCA) assay (Thermo-Fisher Sientific, USA). The samples were heated at 95°C for 2 minutes, and diluted in 25% 4X Laemmli buffer (94 mM Tris HCl pH 6.8, 10% (w/v) SDS, 25% (v/v) glycerol, 0005% (w/v) bromophenol blue, in milli-Q water) with 5% 2-Mercaptoethanol (Sigma-Aldrich, USA) to a final concentration of 1 μg protein μL^{-1}. The samples (10 μg protein/lane) was applied to each well of a Bio-Rad precast gradient gel (4 %-15 % or 4 %-20 %; Bio-Rad Laboratories, Irvine, CA, USA). The electrophoresis was run on 150 volts for approximately one hour. The proteins were then blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, USA) at 2.5 A and 25 V in the Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories, USA) for 10 minutes. The quality of the transfer of the proteins, was confirmed by Ponceau S staining.

The membrane was incubated with 5 % dry nonfatty milk for one hour to block unspecific protein-interaction sites. Then the membrane was incubated with primary antibody (Phospho-Akt (Ser473) (D9E) XP® Rabbit IgG, catalogue number: 4060; Cell Signaling Technology, USA; final dilution 1:2000) overnight. The next day, the membrane was washed 3 × 10 minutes in TBST and exposed to species-specific secondary antibodies (Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey), catalogue number: NA934 (GE Healthcare Life Sciences, United Kingdom); final dilution 1:5000) for one hour. The membrane was washed 3 x 10 minutes and then exposed to an HRP-substrate (Luminata™ Classico Western HRP Substrate; Merck Millipore, USA) for 5 minutes. The protein bands were visualized and imaged (Syngene Chemi Genius 2 Bio Imaging System, USA). Finally, the membrane was rinsed (3 × 10 minutes), blocked for 1 hour with 5% dry milk and incubated with an HRP-coupled α-tubulin antibody for 3 hours. Then the blots were exposed to the HRP-substrate again and imaged as described.

The p-Akt antibody produced one predominant band at the correct molecular mass (60 kDa) with no other visual bands. Quantitative analysis of the chemiluminescence signals of the protein bands, representing p-Akt (60 kDa), was performed using the Image Studio Lite Version 5.2.5 (LI-COR Biosciences, USA) program. Measured chemiluminescence signal for protein binding of the above proteins was normalized against measured chemiluminescence signal for protein-binding of the loading control, α-tubulin (50 kDa) from the same membrane. For the shorter exposure times (Figure 4A), seven separate cell experiments and Western blots, each with two replicates, were performed. For the longer exposure times, four independent cell experiments and Western blots, each with two replicates, were performed.

4.6 | Statistical analysis

Data were analysed with pairwise student´s *t*-test for the Western blot data presented in Figure 4A, or by one-way ANOVA; LSD post-hoc test in SPSS (SPSS, RRID:SCR_002865) for all other data sets.

ACKNOWLEDGEMENTS

This work was supported by grants from the Research Council of Norway: 262647/F20 (CM, SG, MSH, and ML) and grant number 214458/F20 (CM, KAA, LHB, JS-M.), the MLS^{UiO} (CM), The Faculty of Health Sciences, Oslo Metropolitan University (LTØ), and the Faculty of Dentistry, University of Oslo (ØPH). The authors thank Stefan Offermanns for providing the HCA_1 KO mice. Some sets of images used in the quantification of hippocampal neurogenesis were acquired at the Norbrain Slidescanning Facility at the Institute of Basic Medical Sciences, University of Oslo, a resource funded by the Research Council of Norway.

CONFLICT OF INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

LHB, JS-M and CM designed the study. CM, KAA, ØPH and AH performed the in vivo studies. CM and KAA planned these studies and harvested organs. LTØ, ML, and CM performed immunohistochemistry. LTØ performed the analysis of neurogenesis in the SGZ (supervised by CM). ML performed confocal imaging of neurogenesis in V-SVZ and performed the analysis (supervised by SG and CM). MSH performed the cell culture and Western blot experiments. CM wrote the draft manuscript. LTØ, ML and CM prepared the figures. ML, LTØ. and JS-M revised the draft

manuscript. All authors discussed the results, critically revised the manuscript and approved the final version of the manuscript. ML and LTØ contributed equally to the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Lambertus M, Øverberg LT, Andersson KA, et al. L-lactate induces neurogenesis in the mouse ventricular-subventricular zone via the lactate receptor HCA_1. *Acta Physiol*. 2021;231:e13587. https://doi.org/10.1111/apha.13587

Paper II

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The antidepressant effect of exercise is HCA_1-dependent.

(Unpublished manuscript)

The antidepressant effect of exercise is HCA_1-dependent

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Running title: The antidepressant effect of exercise is HCA_1-dependent

Abstract:

The prevalence of depression is increasing, especially in the younger generations. Therapy resistance, low compliance, and remission are major concerns with the current antidepressant drugs, and hence there is an urgent need for new therapies. Exercise has shown to be equally effective as antidepressant drugs in the treatment of mild to moderate depression, but no consensus has been reached regarding the optimal exercise intensity. The mechanisms underlying the exercise-induced antidepressant effect are only partly understood. Here we report that two weeks of high-intensity interval training (HIIT) or moderate-intensity interval training (MIIT) reduced depression-like behavior in mice, with equal effectiveness between the exercise intensities. In contrast, after six weeks of exercise MIIT, but not HIIT, reduced depression-like behavior. At both time points the antidepression-like effect of exercise was present in WT mice, but not in mice lacking the HCA_1 L-lactate receptor, indicating that HCA_1-dependent mechanism(s) are pivotal in exercise-induced antidepressant effects. The results suggest that HCA_1 can be a target for new antidepressant therapies, to mimic the antidepressant effects of exercise.

Keywords: depression; forced swim test; exercise, HCA_1, L-lactate, animal model.

Introduction

Major depressive disorder (MDD) is one of the most common and serious mood disorder and is characterized by a persistent feeling of loss of interest or pleasure accompanied by cognitive and behavioral changes such as irritability, fatigue, poor concentration, sleep disturbances, weight gain or loss, a feeling of worthlessness or guilt, reduced energy and activity level, and sometimes suicidal tendencies [1]. In clinical populations, depression is often accompanied by anxiety disorders, with an estimated comorbidity as high as 80% [2]. In fact, people with MDD experience substantially poorer physical health in general, significantly affecting the individual, family and society [3]. The cause of depression includes a complex interaction between social, psychological, and biological factors and may be triggered by loss, childhood trauma, or other aversive experiences [1]. Globally, 280 million people -equivalent to 3,8% of the world's population- are estimated to suffer from depression, and this proportions is increasing [4]. Alarmingly, depression is increasing rapidly in adolescence [5]. The high prevalence, combined with the poor health and productivity of people with depression, makes depression one of the largest causes of disability [6].

MDD is a complex and multifactorial disorder and the etiology is not completely clarified [7]. Low levels of the neurotransmitters serotonin and norepinephrine in some brain regions, is one important finding however, it is not known whether this is a consequence -or a cause- of the disease [8]. It is however, acknowledged that mood and attention are regulated by these transmitters, and the "monoaminergic hypothesis" for depression has formed the basis for treatment for decades. This hypothesis states that a functional reduction in serotonergic and noradrenergic signaling underly the depressive symptoms [9-11]. In line with the monoamine hypothesis, depression is often treated with antidepressant drugs affecting monoaminergic signaling, such as selective serotonin reuptake inhibitors (SSRI) or serotonin and noradrenaline reuptake inhibitors (SNRI), which blocks the serotonin transporter (SERT) or both SERT and the noradrenalin transporter (NET), respectively, hence increasing the levels of these transmitters in the synaptic cleft. Other commonly used antidepressant drugs like the tricyclic antidepressants (TCA) and inhibitors of monoamine oxidase A (MAO-A), also affect monoaminergic signaling, although less selectively [12]. The combination of antidepressant drugs and non-pharmacological treatments like psychotherapy are the first-line intervention for depression, but their efficacy is variable. Often, these treatments have no more than moderate efficacy for the short-term treatment of acute MDD [13,14].

To this day, there are many challenges in the antidepressant treatment therapy. For unknown reasons, about 30% of the people taking antidepressant drugs are characterized as therapy resistant [15,16] and do not achieve satisfactory relieve of the clinical symptoms [10,13,17-19]. Furthermore, antidepressant drugs often cause unpleasant side-effects such as weight gain, headache, dry mouth, and sexual dysfunction amongst others [20]. While these side effects often are most pronounced during the first 1-2 weeks of the treatment, the clinical effect is often delayed by 1-4 weeks or more. Together, this may lead to a lack of adherence to the pharmacotherapy. For instance, Van Tiem and colleagues reported that approximately one third of the patients discontinue antidepressants within the first four weeks of treatment and 44% discontinue them by the 12th week of treatment. Patients reported no benefits of the medications and the high cost for the medication as reasons for low adherence [21]. While the primary effects of antidepressant drugs on monoaminergic signaling appears instantly, this does not lead to an instant relieve of the symptoms. The mechanism(s) behind this inconsistency in drug actions is unknown however, in recent years it has become evident that MDD results from a complex combination of pathophysiological mechanisms in addition to the decrease in monoamines, for review, see; [22,23]. MDD has been linked to reduced neuroplasticity, including inhibition of adult hippocampal neurogenesis, which can be restored by antidepressant drugs [24-26]. As the development of mature neurons from neuronal stem cells may take several weeks, for review see; [27], adult neurogenesis may be one mechanism that explain the delayed effects of antidepressant drugs.

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is another mechanism which has been linked to the development of MDD in adulthood [28]. Evidence from cross-sectional studies has shown higher levels of cortisol in plasma [15], urine, and cerebrospinal fluid (CSF) in MDD patients compared to healthy controls [29,30]. In fact, 40-60% of the persons with MDD have hypercortisolemia or other HPA-axis disturbances [31,32]. Decreased levels of neurotrophins, such as brain derived neurotrophic factor (BDNF) [33-35] and vascular-endothelial growth factor (VEGF) can also contribute to progression of depression [36,37]. There are however, considerable discrepancies across studies, with some indicating higher VEGF, some unaltered levels, and some lower concentrations in MDD patients compared to nonaffected subjects [38]. Together with the increase in cortisol levels, reduced levels of neurotrophins negatively effects neuroplasticity, including synaptic plasticity, neuronal survival, and neurogenesis [22,39]. Increased neuroinflammation is also suggested to contribute to the pathology of MDD, as increased levels of pro-inflammatory cytokines and markers of

oxidative stress [23,40] have been reported in animal models of the disorder. Interestingly, cerebral hypoperfusion has been reported in human patients with depression [41,42]. To which degree all these mechanisms are linked is a matter of debate, and the contribution of each of these mechanisms may differ between patients, as all of these changes are not detected in every patient. Genetic factors are estimated to account for 42%–50% of the differences in the response to, and adverse effects of, antidepressant drugs [16].

To date, there are no treatment that is universally effective, and the antidepressant response rates are low due to great heterogeneity of MDD [15]. Hence, there is an urgent need for alternative treatment strategies, preferably with less side-effects. Exercise is one such alternative treatment [43] which alleviate depressive symptoms and prevent the risk of developing depression [44-46]. Exercise has been suggested to improve brain serotonin synthesis, increase the levels of antioxidant enzymes, increase the serum and brain BDNF and VEGF levels, and reduce serum and brain cortisol levels, for review see; [9,23]. Through these -and perhaps other- mechanisms, exercise decreases neuroinflammation, increases brain plasticity, including hippocampal neurogenesis, and increases blood flow, in part by increasing angiogenesis. Most of the studies on antidepressant effects of exercise are performed at low to medium exercise intensities. Hence, it is unknown whether different exercise intensities affect depressive symptoms to the same degree.

High-intensity exercise (HIT) leads to an increased production of L-lactate in the muscles. L-lactate released from the muscles is transported in the blood stream and crosses the blood-brain barrier (BBB) via specific transporters (monocarboxylate transporters (MCTs) to access the brain [47,48]. The brain can use L-lactate as an energy source, but L-lactate signaling through the hydroxycarboxylic acid receptor 1 (HCA_1, also known as HCAR1 or GPR81) [49], on which L-lactate is the main endogenous agonist, also contributes to cerebral effects of L-lactate in response to exercise. We have previously demonstrated that HCA_1-activation leads to enhanced VEGF levels in the hippocampus and mediates exercise-induced angiogenesis [50], which in theory may contribute to the antidepressant effects of exercise. Furthermore, HCA_1 is involved in the regulation of neurogenesis in the subventricular neurogenic zone, but not in the hippocampal, subgranular, neurogenic zone [51]. The HCA_1-receptor is located on cells along the blood vessels in the Pia mater, as well as alongside some larger arteries and veins in the brain parenchyma [50], and in some parts of the brain's ventricular system, at the brain/CSF interface [52]. Whether the antidepressant effects of exercise are mediated via HCA_1 is

unknown, but a study in mice reported that 10 days of L-lactate treatment resulted in less vulnerability to stress, social avoidance and anxiety [53], without testing for HCA_1-dependency.

In this study we directly compared the effects of HIIT and MIIT at reducing depression-like behavior in mice. To investigate whether HCA_1-dependent mechanisms are involved in the antidepressant effects of exercise, the effects of exercise in wild-type (WT) mice was compared to the effects in HCA_1 knockout (KO) mice. We measured depression-like behavior after two or six weeks of exercise, and at the latter time point we also included L-lactate treatment to pinpoint whether L-lactate signaling was involved.

Materials and Methods

Animals: All in vivo experiments were formally approved a priori by the Norwegian Animal Research Authority (FOTS ID: 12521, approval date June 16th, 2017 and FOTS ID: 14204, approval date April 19th, 2018). The in vivo experiments were carried out by Federation of Laboratory Animal Science Associations (FELASA) certified personnel in strict compliance with the 2010/63/EU directive of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and reported in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines 2.0 [54]. The HCA_1 knockout line was a gift from Prof. Stefan Offermanns (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany). The generation of this knockout line has previously been described [50,55]; the exon encoding murine HCA_1 was replaced by a cassette encoding β-galactosidase (LacZ) and neomycin resistance by homologous recombination in embryonic stem cells. The line was backcrossed nine times into a C57Bl/6N background after arrival in our lab. The animals were stalled in groups up to 8 mice per GreenLine cages (Sealsafe Plus GM500 or GM900, Buguggiate, Italy) in a 12:12 light/dark cycle at the Department of comparative medicine, Institute of basic medical sciences, Faculty of medicine, University of Oslo. The mice had access to food and water *ad libitum.* In addition to standard bedding, the cages were enriched with paper for nest-building, a wooden stick, and a toilet roll core or a small plastic house. As this study examined the effects of exercise, running wheels were not installed as enrichments in the cages. The mice were earmarked for identification at 4 weeks of age. The biopsy taken during earmarking was used for genotyping.

Treatments: The experiments presented in the present study were conducted as part of a larger study where mice were exercised for three or seven weeks. Depressive-like behavior was

measured by the forced swim test (FST) one week before the end of the experiments (after two or six weeks of exercise intervention, respectively), on a day where the animals did not exercise. Mice treated for two weeks prior to the FST: WT and HCA_1 KO mice (8.5-18.5 weeks old) were randomized to three treatment groups: HIIT, MIIT or a sedentary control group. Distribution of mice to the different intervention groups was: WT control: n = 8 (3 males, 5 females), WT MIIT: n = 8 (2 males, 6 females), WT HIIT: n = 6 (4 males, 2 females) and KO control: n = 7 (2 males, 5 females), KO MIIT: n = 8 (2 males, 6 females) and KO HIIT: n = 8 (3 males, 5 females). Mice treated for six weeks prior to the FST: WT and HCA_1 KO mice (six weeks of age at the start of the experiment) were randomized to either MIIT, HIIT, intraperitoneal (i.p.) L-lactate injections (2g/kg body weight; 200mg/ml dissolved in physiological saline; pH = 7.4. This is equivalent to 18 mmol L-lactate per kg body weight), or to a sedentary control group who received injections of physiological saline (0.9 %, the same volume per kg body weight as the L-lactate injections). Distribution of mice to the different intervention groups was: WT saline (control): n = 16 (6 males, 10 females), WT MIIT: n = 15 (7 males, 8 females), WT HIIT: n = 15 (6 male, 9 females), WT L-lactate: n = 17 (11 males, 6 females), KO saline (control): n = 16 (9 males, 7 females), KO MIIT: n = 15 (9 males, 6 females), KO HIIT: n = 17 (7 males, 10 females) and KO L-lactate: n = 16 (7 males, 9 females). For the mice who received exercise interventions for either three or seven weeks, a test for Maximum Exercise Capacity Test (MECT) was performed on the second day of the training regime and then again, every second week (**Figure 1**).

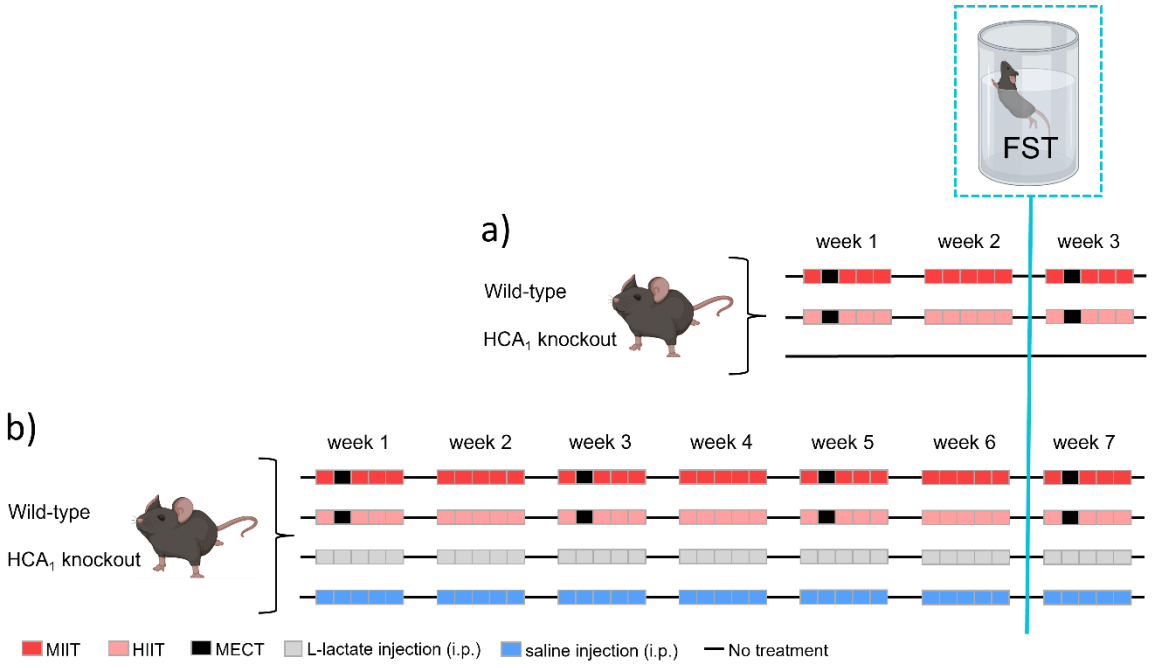

Figure 1: Timeline of the project. **a)** One group of wild-type mice or HCA_1 knockout mice received three weeks of either MIIT (red) or HIIT (pink) for five consecutive days per week, with two days of rest (black line) between; control mice of both genotypes received no treatment (black line). A MECT (black) was performed on the second exercise session of week one and week three. An FST was performed after two weeks of exercise (turquoise), on a day where the mice did not exercise. **b)** Another group of wild-type mice or HCA_1 knockout mice received seven weeks of either MIIT (red), HIIT (pink), or i.p. injections of L-lactate for five consecutive days per week, with two days of rest (black line) between; control mice of both genotypes received i.p. injections of saline injections five days a week for seven weeks. A MECT (black) was performed on the second exercise session of week one and then every second week throughout the exercise intervention. An FST was performed after six weeks of exercise (turquoise), on a day where the mice did not exercise.

Exercise regimes and MECT test: The mice exercised by running on a treadmill, five consecutive days a week for either three or seven weeks. The MIIT, HIIT and MECT protocols were performed on an Exer-3/6 treadmill (Columbus Instruments, USA) at 25% incline. The MECT sessions consisted of warming-up for 15 minutes at a speed of 9.6 m/min. The speed was increased by 1.8 m/min every two minutes until exhaustion, and the test was completed without breaks. Exhaustion was defined as when the mice refused to run further despite being pushed gently or lifted back on the band or given an electric foot-shock (intensity of <1.5 mA). The foot-shock was administered by individual switches for each mouse. It was decided a priory that a maximum of two electric shocks could be given to one mouse per MECT session, but in practice electric shocks were rarely used. The results of the MECT were used to adjust the speed of the next MIIT and HIIT sessions. Both training protocols (MIIT and HIIT) started with a 10-minute warm up at a speed of 8 m/min followed by 10 intervals of 4 minutes each with 2 minutes of rest between. During the rest period, the treadmill was set to 8.6 m/min and the animals could choose to run at this speed (active rest) or sit still (total rest). For HIIT, the speed was set at approximately 80% of the running speed obtained in the MECT but was increased by 0.2 - 0.6 m/min per training session depending on the performance of the mice. For MIIT, the running speed was approximately 60% of their maximum capacity and the speed was kept constant until the next MECT [50]. Exclusion criteria: Throughout the experimental period, animal welfare was evaluated using a standardized scoring scheme, which addressed weight development, fur quality, and stereotypical behavior. If any of the animals showed signs of illness or other discomfort, they were closely monitored by the experimenters and/or a veterinarian, to assess whether the animal was able to continue the experiment. If an animal performed worse than expected (based on the previous performance of the same mouse) in one HIIT or MIIT session, the animal was given additional breaks as needed. If the same animal performed worse than expected for two consecutive days of exercise, the animal was presumed to be injured or sick and was withdrawn from the experiment. Among the animals assigned to three weeks of

exercise, three mice from the WT HIIT group were excluded due to this criterium. Among the animals assigned to seven weeks of exercise two animals, one WT MIIT, one KO MIIT were withdrawn from the study due to the exclusion criteria. All these mice were excluded because they refused to run and/or performed worse than expected for two consecutive days.

Forced swim test: FST was performed in the light part of the day; between 9.00 a.m. and 2.00 p.m. one day after the last exercise session of week two or week six of the exercise intervention, respectively. The mice were habituated to the behavior lab for 60 minutes before the experiment started. The FST experimental setup consisted of three transparent glass cylinders (height 40 cm; diameter 17 cm), separated by walls to ensure that the animals could not see each other during the tests. The cylinders were filled with fresh water (23.8 ± 0.1°C) to a height of 20 cm, hindering the mice from resting with their tails on the bottom of the cylinder during the experiment. The FST room was illuminated with indirect light and the brightness just above the water level was 60-70 lux, measured at the start and again at the end of each experimental session day. The water in the cylinders were replaced between each animal to avoid odors from the previous animals affecting the performance of the next animal. Prior to the experiment, the mice were weighed, tail-marked and randomized to one of the three cylinders. Two rooms were used during the FST, and the rooms were separated by a curtain. The FST experiment was performed in one room while the other room was used to stall the mice before and after the tests. Immediately after placing all three mice in their respective cylinders, the experimenter left the FST room. The experiment lasted for 6 minutes, and at the end of the experiment, the mice were immediately picked up, carefully dried with paper, and placed in a cage under a heating lamp for 10 minutes. The lamp was placed so that only half of the cage was heated, allowing the mice to decide whether to stay on the heated or the non-heated side of the cage. All trials were recorded by a video camera connected to EthoVision XT (Noldus Information Technology, Netherlands) and the videos were used for analysis.

Analysis of FST: The mice were videotaped for 6 minutes in the FST. The first 120 seconds were ignored as recommended, as the behavior of the mice in this phase is assumed to primarily represent anxiety/despair [56]. The behavior of the mice during the remaining 240 seconds of the test were categorized into “mobile” or “immobile” and measured manually with a stopwatch. The analyses were performed by two independent operators who were blinded to the genotype and treatment of the animals. The operational definition for “immobility” was behaviors where the animal did not swim or making movements other than those necessary to

maintain balance and/or to keep the nose above water. All other active movements were recorded as “mobile”.

Data presentation and statistics: Data from the FST are presented as the number of seconds the mice spent immobile (out of a total of 240 seconds) ± standard error of the mean (SEM). Statistical analyses were performed using a Kruskal-Wallis test followed by Dunn‘s multiple comparisons test (GraphPad Prism, version 9.2.0), and the significance level was set at 5% ($p<0.05$).

Results

To investigate whether exercise at different intensities affected depression-like behavior in mice and, if so, whether the L-lactate receptor HCA_1 was involved in the effect, we exposed WT mice and HCA_1 KO mice to FST after two weeks or six weeks of exercise intervention in the form of either MIIT or HIIT. After two weeks of exercise, the WT mice exposed to MIIT showed significantly shorter floating time (49.8 ± 9.04 seconds; mean ± SEM) compared to the sedentary WT (control) mice (97.0 ± 11.1 seconds; $p = 0.030$¸ Kruskal-Wallis test; Dunn’s multiple comparisons test), suggesting a lower degree of depression-like behavior. The WT mice exposed to HIIT (WT HIIT) tended to show shorter floating time (51.0 ± 15.5 seconds) than the WT control mice, but this did not reach statistical significance ($p = 0.094$). Floating time for the WT mice exposed to MIIT did not differ from the floating time of the WT mice exposed to HIIT ($p > 0.999$). In KO mice, the floating time was unaffected both by MIIT (85.0 ± 4.74 seconds; $p = 0.492$) and HIIT (65.9 ± 8.12 seconds; $p > 0.999$) compared to the sedentary KO control group, and there was no difference in floating time between KO mice exposed to MIIT and KO mice exposed to HIIT ($p = 0.461$; Kruskal-Wallis test; Dunn’s multiple comparisons test) (**Figure 2**).

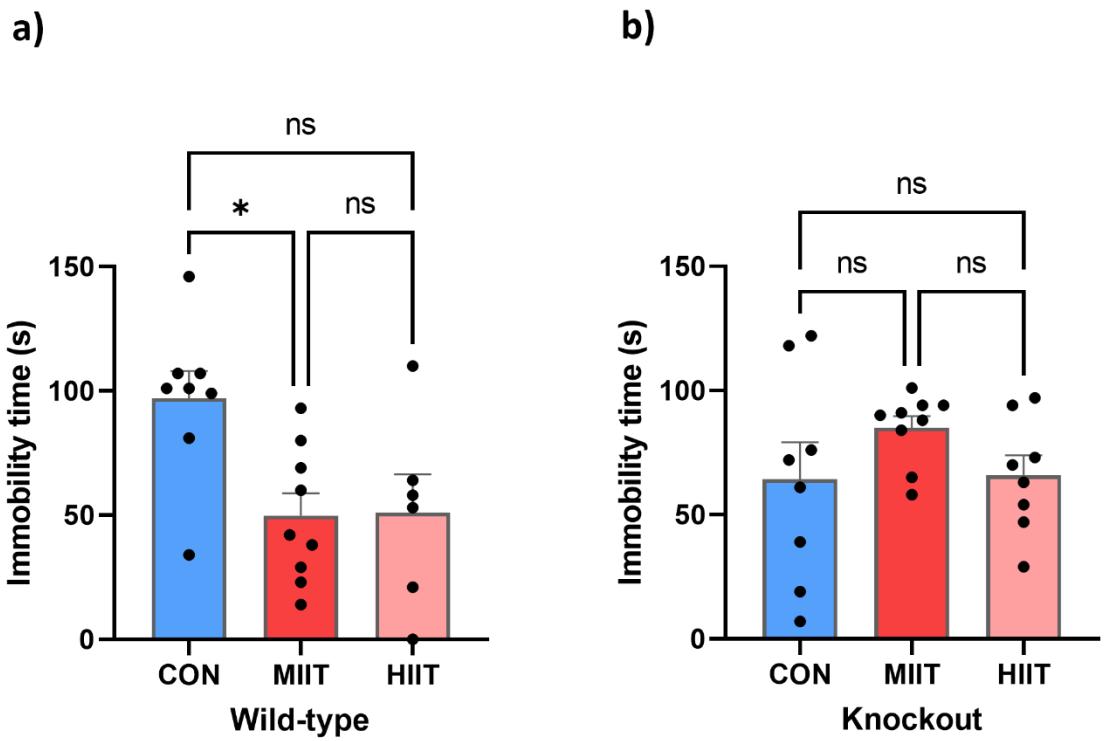

Figure 2. Results from the FST performed in sedentary control mice (CON; blue), or after two weeks of exercise at different intensities: MIIT (red), HIIT (pink). The exercise interventions were administered on five consecutive days per week, and the FST was performed on a day where the animals did not exercise. The bars show average immobility time ± SEM; the immobility time for each animal is indicated by the black dots. **a**) Immobility time in WT animals. **b**) Immobility time in HCA_1 KO animals. *: $p<0.05$; ns: not statistically significant ($p>0.05$); Kruskal-Wallis test; Dunn's multiple comparisons test.

As reported for the mice treated for two weeks, WT mice exposed to MIIT for six weeks spent less time passively floating (17.3 ± 4.43 seconds) compared to the WT control mice (49.9 ± 6.15 seconds; $p = 0.0004$). Contrary to what was observed after two weeks, there was no difference between WT mice exposed to HIIT (40.1 ± 7.92 seconds) and WT control mice ($p = 0.8299$). In WT animals, treatment with L-lactate injections resulted in a tendency towards lower floating time (29.6 ± 4.08 seconds) compared to the WT control ($p = 0.1152$). In the KO mice, neither MIIT (22.9 ± 4.01 seconds), HIIT (32.7 ± 7.26 seconds) nor L-lactate treatment (44.7 ± 5.20 seconds) resulted in lower floating time compared to the sedentary KO control mice ($p = 0.4680$ for KO MIIT vs. KO control, $p \geq 0.9999$ for the remaining comparisons; Kruskal-Wallis test; Dunn's multiple comparisons test) (**Figure 3**).

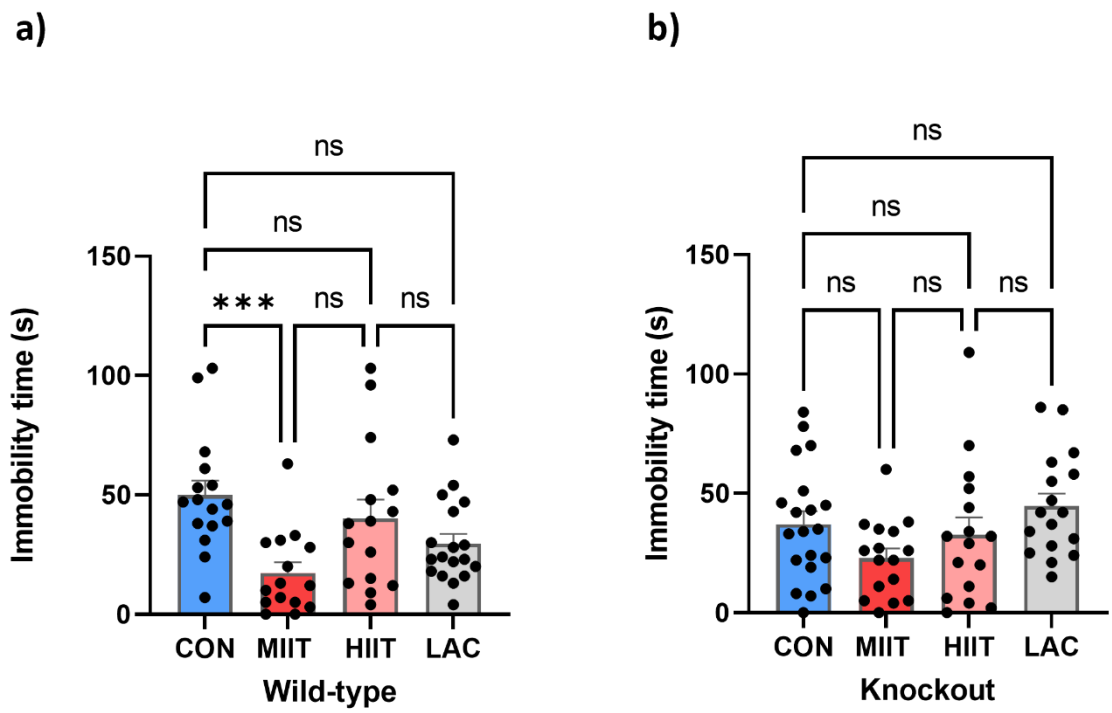

Figure 3. Results from the FST performed after six weeks of i.p. saline treatment (CON; blue), exercise at different intensities: MIIT (red), HIIT (pink), or after six weeks of i.p. L-lactate (LAC; grey) treatment. All interventions, both the exercise and the i.p. injections were administered on five consecutive days per week, and the FST was performed on a day where the animals did not exercise. The bars show average immobility time ± SEM; the immobility time for each animal is indicated by the black dots. **a**) Immobility time in WT animals. **b**) Immobility time in HCA_1 KO animals. *: $p<0.05$; ns: not statistically significant ($p>0.05$); Kruskal-Wallis test; Dunn's multiple comparisons test.

Discussion

In the current study we found that activation of the L-lactate receptor HCA_1 by exercise for two or six weeks resulted in reduced floating time in the FTS, which can be interpreted as a reduced depression-like behavior. This antidepressant effect of exercise was seen in WT mice but not in HCA_1 KO mice, suggesting that HCA_1-dependent mechanisms contribute to this effect. Further supporting that the antidepressant effect of exercise was regulated by HCA_1-activation, L-lactate injections tended to reduce depression-like behavior in the WT mice ($p = 0.115$), but not in the KO mice. The HCA_1-dependency of the antidepressant effect of exercise could not be explained by a difference in running speed or exercise intensity, as the MECT results did not differ between KO and WT mice, and mice of both genotypes ran together on the same treadmill. Using the same HIIT regime and L-lactate treatment as in the present study, we have previously shown that HCA_1-activation in response to exercise or L-lactate treatment increased hippocampal VEGFA levels and enhanced the cerebral capillary density of mice [50]. As cerebral hypoperfusion is a suggested mechanism underlying depression, this exercise-induced, HCA_1-dependent, angiogenesis may contribute to the antidepressant effects seen in the present

study. The HCA_1-dependent antidepressant effect seen in the present study is however, not likely to result from HCA_1-dependent increase in hippocampal neurogenesis, as we have previously reported that an identical HIIT regime for seven weeks resulted in increased hippocampal neurogenesis which was independent of HCA_1-activation [51]. Supporting this conclusion, the same study did not find that injections with L-lactate for seven weeks induced neurogenesis [51]. Furthermore, the animals exposed to three weeks of exercise in the present study (FST performed after two weeks) were also analysed for hippocampal neurogenesis and no role of HCA_1 in exercise-induced hippocampal neurogenesis was found (Lambertus et al., manuscript in preparation). In both studies however, we reported HCA_1-dependent neurogenesis in the sub-ventricular zone. Neurogenesis in the hippocampus, but not in the subventricular zone, has been implicated in depression [57]. Hence, it is not clear whether the HCA_1-dependent subventricular neurogenesis plays a role in the antidepressant effects of exercise detected in the present study.

The present study shows that two weeks of exercise with either MIIT or HIIT effectively reduced depression-like behavior. The effects of HIIT are hard to interpret, as immobility after HIIT was similar to that seen after MIIT, yet it did not reach statistical significance when compared to the control mice. In contrast, after six weeks of exercise, we observed that MIIT - but not HIIT- reduced depression-like behavior. One possible explanation is that six weeks of HIIT (5 days per week) may result in a mild increase in the stress level of the animals. The primary effector of stress responses is the HPA-axis which regulates the circulating levels of cortisol and other stress mediators [58]. The HPA-axis also plays an important role in the regulation of mood, and a dysfunction of the HPA-axis occurs in mood disorders. Cortisol reduces neurogenesis, which presumably is an important mechanism in depression [29,30]. When comparing the exercise-induced neurogenesis between the animals exposed to HIIT for three weeks (Lambertus et al., manuscript in preparation) and the animals exposed to HIIT for seven weeks [51] however, we found that the number of newborn neurons, measured as doublecortin-positive cells, were increased by 100% after three weeks of HIIT, while the increase in neurogenesis after seven weeks was only 25%. These findings are in line with the hypothesis that the longer exercise regime induces a mild degree of stress, which may also underly the lack of antidepressant effect of prolonged HIIT reported in the present study. To investigate the effects of L-lactate signaling, control of the exercise intensity is essential. Therefore, the mice in the present study were subjected to forced, rather than voluntary exercise, which may in theory be more stressful for the animals. During the experiments however, the

animals were closely observed, and any signs of exhaustion the mice were allowed a small additional break before gently being put back on the treadmill. Furthermore, none of the animals showed signs of distress, such as alterations in their fur, weight loss or stereotype, suggesting that they were not subjected to a high degree of stress. It should be noted that as soon as the animals were placed on the treadmill, and as well during the two-minutes of rest between the intervals, most of the animals chose to run at a moderate speed rather than sitting still. This indicates that the exercise was not stressful to a level where it led to aversion.

In this experiment we used the FST, a widely used test for depression-like behavior [59]. The test is based on the observation that escape behavior will occur in animals subjected to aversive, stressful stimuli and that such behavior will decrease with depression [60,61]. Behavioral immobility, as opposed to active swimming or attempts to escape, reflects a state of despair in mice, consistent with what is observed in human patients with depression. Despair is a key symptom of human MDD, but MDD is characterized by other symptoms as well, which are not measured in the FST. Hence, the FST has been criticized for not targeting depression *per se* [59]. In the present study, we used HCA_1 KO and WT mice where no depression has been induced, and we therefore used the FST to measure whether exercise can reduce the normal level of despair, which we refer to as depression-like behavior. Even if not testing all aspect of depression, we compare the level of despair between groups of animals, making us able to conclude that this type of depression-like behavior is affected by the treatment in an HCA_1-dependent manner. The design of our study closely resembles the type of studies that the FST was developed for -namely to test the effects of antidepressant drugs. The FST is still the most commonly used test for antidepressant effects of drugs [56] due to low coasts and its reliability [62], and a recent meta-analysis reported that all types of currently used antidepressant drugs reduced immobility in the FST [63], verifying the external validity and reproducibility of the FST, as well as the relevance of the FST in the present study.

Conclusions

The prevalence of depression is increasing, especially in the younger generations. With about 30% of the patients with MDD being resistant to the current antidepressant drugs, there is an urgent need for new therapies. Exercise has been shown to be equally effective as antidepressant drugs, but the loss of interest experienced by these patients, often hinders adherence to exercise therapy. In the current study we found that two weeks of HIIT or MIIT caused a reduction in

depression-like behavior in mice, with no difference in effectiveness between the exercise intensities. In contrast, after six weeks of exercise only MIIT, and not HIIT, induced a reduction in depression-like behavior. At both time points the antidepressant-like effect of exercise was present in WT mice but not in HCA_1 KO mice, indicating that exercise mediates antidepressant effects through HCA_1-dependent mechanism(s). This study opens for the possibility that the L-lactate receptor HCA_1 can be a target for new antidepressant therapies, to mimic the antidepressant effects of exercise.

Author Contributions: L.T.Ø and C.M designed the study. S.J.G and M.L exercised the mice. S.J.G performed the L-lactate injections. L.T.Ø, E.B and C.M conducted the FST. L.T.Ø and E.B analyzed the data. L.T.Ø made figures and drafted the manuscript. C.M and L.T.Ø wrote manuscript. All authors critically revised the manuscript.

Acknowledgments: The authors would like to thank master students Camilla Brox; Kimberly Dungdung Phan and Teresa Nguyen for their excellent work in training the mice together with S.J.G.

Conflicts of Interest: The authors declare no conflict of interest. The funders had nor role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Paper III

Øverberg, L.T.*, Lugg E. F., Gaarder, M., Langhammer, B., Thommessen, B., Rønning, O.M., Morland, C., (2022).

Plasma levels of BDNF and EGF are reduced in acute stroke patients.

Heliyon 8, https://doi.org/10.1016/j.heliyon.2022.e09661

Heliyon 8 (2022) e09661

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Plasma levels of BDNF and EGF are reduced in acute stroke patients

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ARTICLE INFO

Keywords:
Biomarkers
Disability
Growth factors
Irisin
Ischemic stroke
Plasticity

ABSTRACT

Stroke affects almost 14 million people worldwide each year. It is the second leading cause of death and a major cause of acquired disability. The degree of initial impairment in cognitive and motor functions greatly affects the recovery, but idiosyncratic factors also contribute. These are largely unidentified, which contributes to making accurate prediction of recovery challenging. Release of soluble regulators of neurotoxicity, neuroprotection and repair are presumably essential. Here we measured plasma levels of known regulators of neuroprotection and repair in patients with mild acute ischemic stroke and compared them to the plasma levels in healthy age and gender matched controls. We found that the levels of BDNF and EGF were substantially lower in stroke patients than in healthy controls, while the levels of bFGF and irisin did not differ between the groups. The lower levels of growth factors highlight that during the acute phase of stroke, there is a mismatch between the need for neuroprotection and repair, and the brain's ability to induce these processes. Large individual differences in growth factor levels were seen among the stroke patients, but whether these can be used as predictors of long-term prognosis remains to be investigated.

1. Introduction

Stroke is the second leading cause of death and a major contributor to acquired disability in the world today [1]. The degree of long-term functional loss after stroke can vary from nearly none to different degrees of impaired motor function, speech loss and reduced cognitive function which may greatly affect the autonomy of the patient. More than 80 million stroke survivors worldwide are currently living with such consequences [2, 3]. Ischemic stroke is caused by an occlusion of a cerebral blood vessel which causes interruption of blood supply to the affected brain area. This rapidly results in tissue necrosis at the stroke core, which may be followed by loss of brain functions, disabilities, or even death. According to the annual report from the World Stroke Organization (WSO), stroke has reached epidemic proportions [2]. Although the incidence and mortality rate of stroke is currently decreasing in high-income countries, the prevalence and the public health burden of stroke in developing countries are expected to rise in the years to come [4, 5]. Given the high mortality rate and probability for long-term disabilities, stroke imposes a considerable economic and social burden on the society, as well as on the stroke patients and their families [6, 7].

Functional outcome after stroke depends on the size of the affected area in the brain and the location, how long the blood supply is reduced or absent for, and whether the stroke is ischemic or hemorrhagic. The degree of initial impairment greatly affects the recovery, but variability between stroke patients with the same lesion type and size sometimes makes an accurate prediction challenging. The individual differences are probably due to a combination of factors, whereof some still are unknown. Hence, the degree of improvement can be hard to predict in the early phase of a stroke.

Brain injury induced by stroke results from complex series of pathophysiological events, including increased release of excitatory transmitters, oxidative stress, inflammation, and cell death. At the same time, cytokines and growth factors that increase the survival of brain cells are released. Growth factors are a group of peptides that stimulates cellular processes, including cell survival, growth, proliferation and differentiation

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https://doi.org/10.1016/j.heliyon.2022.e09661
Received 26 January 2022; Received in revised form 22 March 2022; Accepted 31 May 2022

[8]. Growth factors are key regulators of neural plasticity, which is an important part of the repair and regeneration after stroke. Neural plasticity includes processes such as angiogenesis, neurogenesis [9, 10], and rewiring of the brain. All these processes are stimulated by growth factors and are important contributors to the functional recovery after stroke [11, 12, 13]. Irisin, a hormone-like myokine with neurotrophic effects, has also been reported to induce beneficial effects after stroke, including inhibited post-ischemic inflammation, reduced oxidative stress and improved mitochondrial function [14]. In fact, decreased concentrations of irisin are associated with poor functional outcome in ischemic stroke [15].

Growth factors are ubiquitously expressed in the adult brain, and many of them are upregulated in response to ischemia [16]. Irisin is mainly expressed in skeletal muscle but is also produced by brain cells, primarily neurons [17, 18]. Some growth factors remain elevated over several weeks after stroke [19]. Probably the combination of harmful and protective factors released during the acute phase of stroke regulates the balance of neurotoxic and neuroprotective or neuroregenerative processes. The release and effects of growth factors in stroke are subjected to great individual differences and are influenced by a variety of factors including localization of the stroke, genetic factors [20], sex [21] and other—yet unknown—factors. Most growth factors readily pass the blood-brain barrier (BBB), and hence their concentrations in the systemic circulation may be a proxy for the concentration that affects the brain.

Based on the known effects of growth factors and irisin in stroke, treatment strategies to increase regeneration of nerve tissue, for example by supplying larger amounts of growth factors, have received much attention [22]. Although these substances are known to provide positive effects in the healthy brain, the effects of giving them as stroke treatment are less promising. In animal models, post-stroke delivery of brain-derived neurotrophic factor (BDNF) has given promising effects [23]. For various reasons, however, the same effects have not been reached in humans [24]. Similarly, treatments to increase irisin levels are effective in animal models of stroke [14, 25, 26], but have so far not been translated into human stroke patients. The effect of giving basic fibroblast growth factor (bFGF), fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) after stroke is uncertain [27]. Vascular endothelial growth factor A (VEGF A), which regulates the development of blood vessels in the brain, has surprisingly proved to exacerbate the injury of stroke in the acute phase [19], but rescue brain cells in the sub-acute phase [28]. The lack of effect of added growth factors in acute stroke patients may be due to unintentional side effects, low selectivity, low ability to cross the BBB, or difficulty in finding the right dose in transition from animal studies to clinical trials [24]. Intrinsically elevated levels of irisin or growth factors, on the other hand, may be important contributors to—and biomarkers for—recovery after stroke.

In the present study we aimed to determine whether differences in plasma levels of irisin and key growth factors (BDNF, bFGF, and EGF) could be detected in acute stroke patients compared to a group of age and gender matched healthy controls (Figure 1).

2. Materials and methods

2.1. Ethics and approvals

The study was approved by the Regional Committee for Medical and Health Research Ethics, South-Eastern Norway (REC; ID 2018/2555), and the Norwegian center for research data (NSD; ID 539270), and it was registered in the quality assurance system for health and medical research at the University of Oslo, Norway (Helseforsk). The stroke patients were recruited from the health registry and general research biobank for neurological diseases at Akershus University Hospital (Ahus), Norway (REC ID 2011/1015). The study was conducted in accordance with the Declaration of Helsinki of 1964 [29]. The research is reported according to the Biospecimen Reporting for Improved Study Quality (BRISQ) recommendations [30]. The stroke patients were given verbal information about the project and signed a written informed consent the

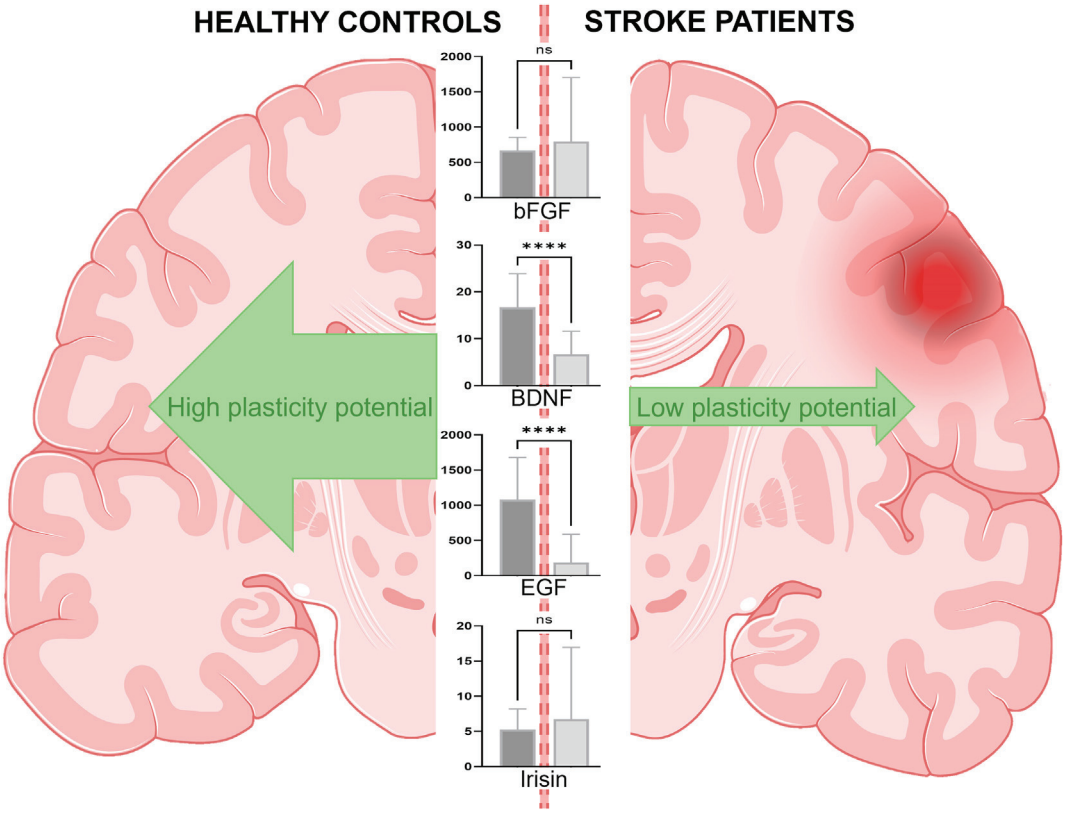

Figure 1. Graphical abstract. Figure partly created with www.BioRender.com.

day after admission to the hospital, prior to inclusion and blood sampling. Participation to this study did not affect the treatment of the stroke patients, except for the extra blood sample taken.

All blood samples, case report forms (CRFs), and test scores were de-identified and labeled with a participant number, hence all analyses were performed by researcher(s) who were blinded to the identity of the stroke patients/healthy controls. The paper file with the participant's identity linked to the study ID was kept secured in a safe, separated from the rest of the study data. The CRFs and test scores were kept in a locked drawer in a room with strict access control (card and code to enter the hallway; key to enter the room; separate key to open the drawer).

2.2. Study groups and design

The study included 94 subjects, 47 stroke patients and 47 age and gender matched controls (17 males; 30 females in each group). A timeline for the study is given in Figure 2. The stroke patients were selected from the established biobank (REC ID 2011/1015) based on the following inclusion criteria: 1) age >50 years, 2) acute ischemic stroke (ICD-10-CM code: I63) was the main cause for hospitalization, 3) cognitive abilities that allowed patients to give an informed consent to participate, and 4) the time between stroke onset and hospitalization had been noted in the patient journal. The stroke diagnosis was verified by a stroke neurologist based on symptoms and brain imaging, either computer tomography (CT) or magnetic resonance imaging (MRI). Patients with hemorrhagic stroke were not included in the study. The stroke etiology was categorized based on whether the stroke was lacunar, cortical/non-lacunar, or transient ischemic attacks (TIA). Patients with TIA were included in the study only if they exhibited unilateral weakness. The non-TIA strokes were further classified into three size categories: large infarctions (cortical/subcortical infarctions with a volume >10 ml), medium infarctions (cortical/subcortical infarctions with a volume of approximately 5–10 ml), and small infarctions (lacunar or non-lacunar infarctions with a volume <5 ml).

The healthy controls were recruited from fitness centers in the south-eastern Norway. The project leaders gave a verbal presentation of the planned study at the end of fitness classes for elderly people. Other controls were recruited independently of the fitness classes through our network of seniors. Those interested to participate wrote their contact information on a list and were then contacted by e-mail with information about the study. All participants signed a written informed consent on the day of participation, prior to inclusion. The inclusion criteria were age >50 years, healthy (defined by self-reported experience of own health), and cognitive ability that allowed for an informed consent. Exclusion criteria: previous stroke or TIA.

2.3. Tests for cognitive and motoric status, and self-sufficiency

Cognitive abilities were tested with mini-mental state examination (MMSE), trail making test A (TMTA) and trail making test B (TMTB). MMSE includes tests of orientation, attention, memory, language, and visual-spatial skills; TMTA and TMTB measure executive functions such as visual attention and psychomotor tempo [31, 32]. For the stroke patients, these tests were performed between day three after admission and the time they were discharged from the hospital (the patients were normally hospitalized for 4–10 days). For the healthy controls, these tests were performed directly prior to the blood sampling. MMSE was performed as described by Folstein and colleagues [33] with the Norwegian revisions by Strobel and Engedal [34]. In the TMTA, the time (in seconds) used by each person to connect 25 numbered circles in an ascending order with a continuous line was measured. In TMTB, the 25 circles were labeled with either a number or a letter, and the time (in seconds) to connect the circles, with a continuous line, alternating between numbers and letters and taking both series in ascending order, from 1-A-2-B etc.,

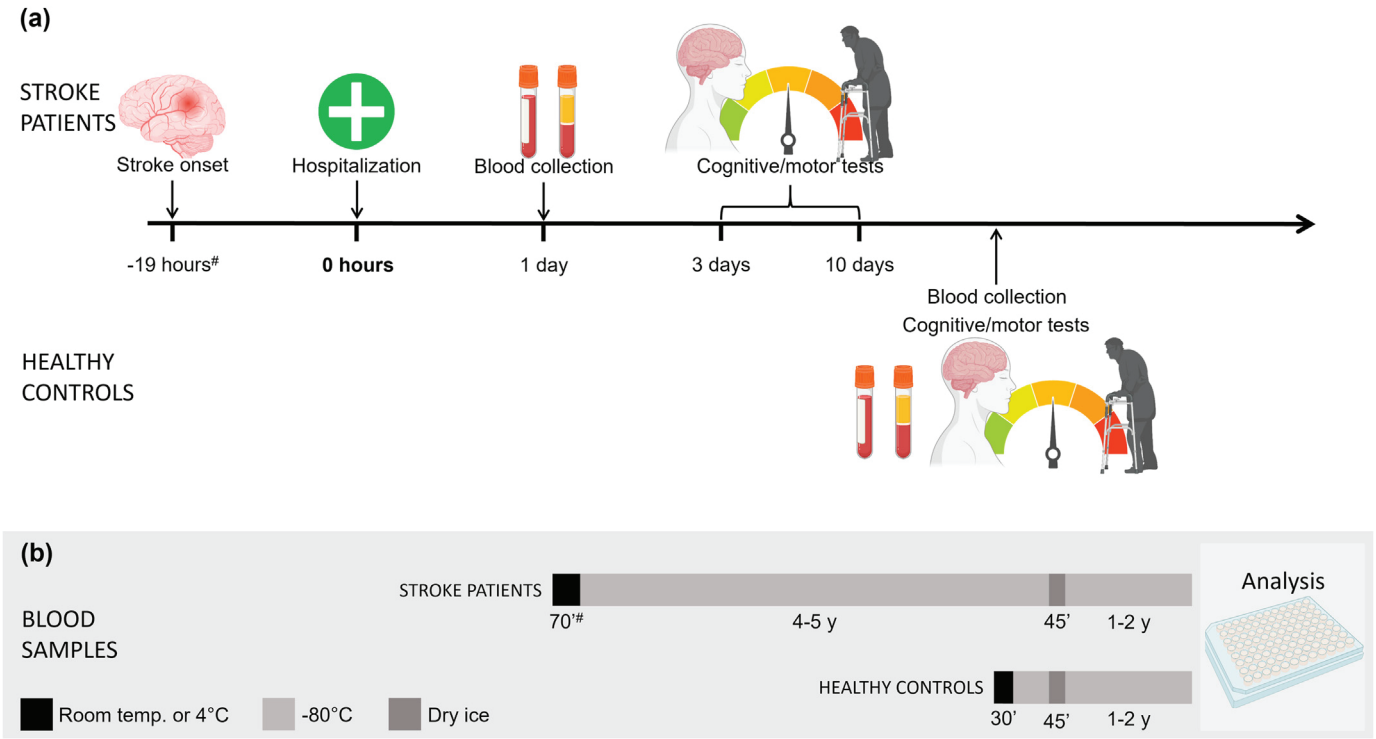

Figure 2. Timeline for the project. (a) Timeline for the stroke patients and the healthy controls. For the stroke patients, the time intervals between stroke onset and hospitalization, and between hospitalization and blood collection and testing are indicated. For the healthy controls, blood sampling and testing were performed on the same day, about 4 years after inclusion of the first stroke patient. (b) Timeline for the plasma samples from stroke patients and healthy controls. The samples were kept at room temperature before centrifugation at +4 °C, and again during pipetting (black). The plasma samples where then kept at -80 °C (grey). During transportation from the biobank to the local lab, the samples were kept on dry ice (dark grey). The samples were then kept at -80 °C (grey) until analysis. #median time; 'minutes. Figure partly created with www.BioRender.com.

was measured. In addition, before discharge from the hospital, the degree of disability was measured according to the Barthel activities of daily living (ADL) Index (0–20) [35, 36] and the mRS [37].

2.4. Blood collections and plasma analyses

The sample of venous blood was drawn from the stroke patients between noon and 1 pm on the day after admission to the hospital. The median time between symptom onset and hospitalization was 19 h but varied from 1 h to >240 h (Figure 2; Table 1). The blood samples were collected in de-identified (labeled with the participant number only) BD vacutainer tubes containing K_2 dipotassium ethylenediaminetetraacetic acid (K_2EDTA) 10.8 mg, ref. 367864). The tubes were gently inverted 10 times and centrifuged within 10–15 min (5810 R, serial number 0036330, Eppendorf Nordic, Hoersholm, Denmark) at 1000 g for 5 min, with temperature of +4 °C. The supernatant (plasma) was aliquoted within 45–140 min (median: 70 min), stored at -80 °C in the biobank for four to five years, and were kept on dry ice for 30 min during shipment before further analysis. From the healthy controls, 6 mL of venous blood was collected from the antecubital area of the arm after at least 30 min of rest. The blood was collected in BD vacutainer tubes and the plasma preparation was performed as described above. The plasma was gently pipetted in 200 μL aliquots in pcr tubes (VWR, kat.nr: 732-0676) and stored at -80 °C until further analysis.

The plasma concentration of BDNF, bFGF, EGF, and irisin were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc. Minneapolis, USA). The optimal dilution of the plasma samples for each analysis was identified in a separate test where four different dilutions of plasma were tested. The following ELISA kits were used, and plasma was diluted as indicated: Human BDNF (DY248), dilution: 1:40; human EGF (DY236-05), dilution: 1:5 for the stroke patients; 1:20 for the healthy controls; human FGF (DY233), dilution: 1:10; human irisin (DY9420-05), dilution: 1:5. The analyses were conducted according to the instructions of the manufacturer. All plasma samples were assayed in duplicates, and samples from stroke patients and healthy controls were evenly distributed on each plate. An intra-assay coefficient of variation (CV) below 20% between the duplicates was considered acceptable, but in most cases, it was well below 10%. Samples where the CV exceeded 20% were reanalyzed. The blood samples were subjected to a maximum of three freeze-thaw cycles, and the time of each thaw was about 1 h (on ice).

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., California, USA). One healthy control was excluded from the analysis as this person had plasma levels several folds above the rest of the healthy controls cross all analyses. To keep the age and sex distribution of both groups equal, the matched stroke patient was also excluded. Some individuals were identified as statistical outliers for single growth factor/irisin analysis. As individual differences are presumably important for stroke recovery, these individuals were included in the analyses and graphs. A separate analysis was conducted to verify that inclusion of these individuals did not affect the conclusion of the statistical comparisons. The normality of each dataset was tested using the Shapiro-Wilk test, and nonparametric tests were found to be applicable. Comparisons between the stroke patients and the healthy controls were analyzed by the Mann-Whitney U test. When comparing more than two groups, a Kruskal-Wallis test was performed. The significance level was set to <0.05.

3. Results

The study group consisted of 47 stroke patients and 47 age and gender matched subjects in the control group. The participants ranged from 53-83 years of age; the majority (85%) were between 60 and 80 years of age (Table 1).

The stroke patients on average had a modified Rankin Scale (mRS) score consistent with slight disability [37] (Table 2). The mean Barthel ADL score reflected a low need for help, but seven of the 47 stroke patients had a Barthel ADL score of 10–19 (reflecting a moderate need for help), and six scored below 9 (reflecting a high need for help) [35] (Table 2). The MMSE score of both the stroke patients (26 ± 4.5 of 30; mean ± SD) and the healthy controls (29 ± 1.0 of 30) were considered normal [33], but the score was significantly lower among the stroke patients than the healthy controls ($p < 0.0001$; Mann-Whitney U test; GraphPad Prism). Among the 39 stroke patients who were tested by MMSE, 27 had a score of 25–30, suggesting no cognitive impairment; 8 had a score of 21–24, consistent with mild cognitive impairment, and 4 patients scored 10–20, consistent with moderate cognitive impairment. None of the patients scored below 10 (severe cognitive impairment). In the healthy controls, all participants scored 27 or above (Table 2).

Time spent to complete TMTA [32, 38] (60.0 ± 35.2 s) for the stroke patients was longer than for the healthy controls (37.6 ± 14.3 s; $p = 0.0006$; Mann-Whitney U test; GraphPad Prism). The time to complete the TMTB [32] varied largely between the individuals but was longer for

Table 1. Summary characteristics for the healthy controls (Control) and stroke patients (Stroke).

Description	Control	Stroke
number of participants (n)	47	47
age, mean ± SD	70.5 ± 7.0	70.3 ± 7.8
females, n (%)	30 (64%)	30 (64%)
stroke type		
lacunar, n (%)		25 (53%)
cortical/non-lacunar, n (%)		15 (32%)
TIA, n (%)		7 (15%)
stroke size		
small, n (%)		32 (80%)
medium, n (%)		4 (10%)
large, n (%)		4 (10%)
Time: stroke onset to hospitalization		
median (hours)		19
min-max (hours)		1- >240

Table 2. Results from cognitive, motoric, and self-sufficiency tests of the healthy controls (Control) and the stroke patients (Stroke).

Description	Control	Stroke
mRS[1]	-	
mean ± SD		1.9 ± 1.4
median (n)		2 (47)
MMSE[2]		
mean ± SD	29.0 ± 0.97	26.1 ± 4.5
median (n)	29 (47)	27 (39)
TMTA		
mean (sec) ± SD	37.6 ± 14.3	60.0 ± 35.1
Median (n)	33.3 (47)	46.5 (26)
TMTB		
mean (sec) ± SD	89.7 ± 47.4	131 ± 64.5
median (n)	75 (47)	114 (25)
Barthel ADL score[3]	-	
mean ± SD		17.5 ± 4.9
median (n)		20 (47)

[1] mRS: 0–5 scale; 0 = no symptoms; 5 = severe disability; constant care needed (6 = dead).
[2] MMSE: maximum score: 30.
[3] Barthel ADL score: maximum score: 20 (= self-sufficient).

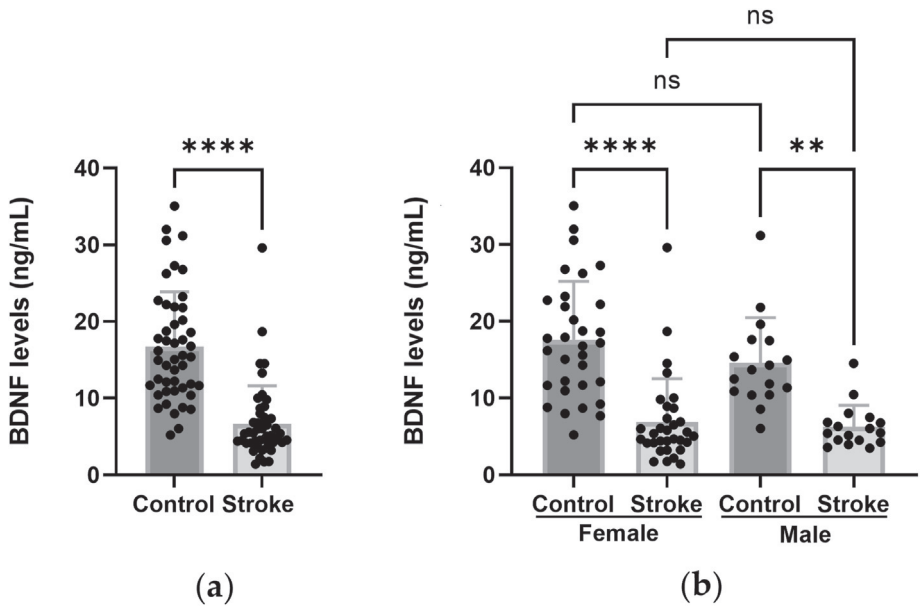

Figure 3. Plasma levels of BDNF is reduced in stroke patients. (a) Plasma levels of BDNF (ng/mL) in healthy controls and stroke patients; (b) Plasma levels of BDNF (ng/mL) in healthy male and female controls and stroke patients. **p < 0.01; ****p < 0.0001; ns: not statistically significant (in (a): Mann-Whitney U test, in (b): Kruskal-Wallis test; GraphPad Prism).

stroke patients (131 ± 64.5 s; mean ± SD) than for the healthy controls (89.7 ± 47.4 s; mean ± SD; p = 0.0015; Mann-Whitney U test; GraphPad Prism) (Table 2).

3.1. BDNF levels were lower in stroke patients than in healthy controls

In the stroke patients, the levels of BDNF ranged from 1.43 to 29.61 ng/mL, and in the healthy controls, the range was 5.23–35.06 ng/mL. The plasma BDNF levels in the stroke patients (6.69 ± 4.92 ng/mL; mean ± SD) were significantly lower than in the age and gender matched control group (16.70 ± 7.20 ng/mL; mean ± SD; p < 0,0001; Mann-Whitney U test, GraphPad Prism) (Figure 3a).

A sub-analysis was performed to establish whether males and females differed in the BDNF levels. The data were segregated based on sex (n = 30 females and 17 males in each group) and reanalyzed (Figure 3b). There was no difference between males and females in any of the groups (p > 0.99 for both comparisons). In females, the BDNF levels were 61% lower in the stroke patients than in the healthy controls, and in males the BDNF levels were 57% lower in the stroke patients than in the healthy controls. Females: 6.88 ± 5.63 ng/mL (mean ± SD) in stroke patients versus 17.58 ± 7.64 ng/mL in healthy controls (p= <0.0001; Kruskal-Wallis test, GraphPad Prism). Males: 6.29 ± 2.78 ng/mL in stroke patients versus 14.59 ± 5.88 ng/mL in healthy controls (p = 0.0013; Kruskal-Wallis test, GraphPad Prism).

3.2. BDNF levels were largely unaffected by age

To study whether the BDNF levels were affected by age, the subjects were categorized into three age groups: <67 years (n = 15; 16 stroke patients and healthy controls, respectively), 68–74 years, (n = 17; 17); >75 years (n = 15; 14). The BDNF levels did not change in relation to age, neither in the stroke patients nor the healthy controls (Figure 4). The

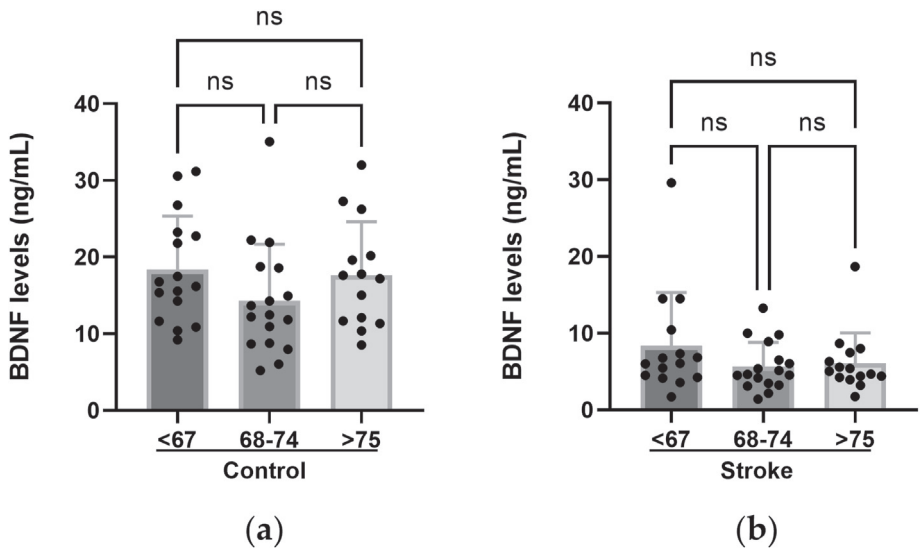

Figure 4. Plasma levels of BDNF are unaffected by age. Plasma levels of BDNF (ng/mL) in different age groups of (a) healthy controls; (b) stroke patients. ns: not statistically significant (Kruskal-Wallis test; GraphPad Prism).

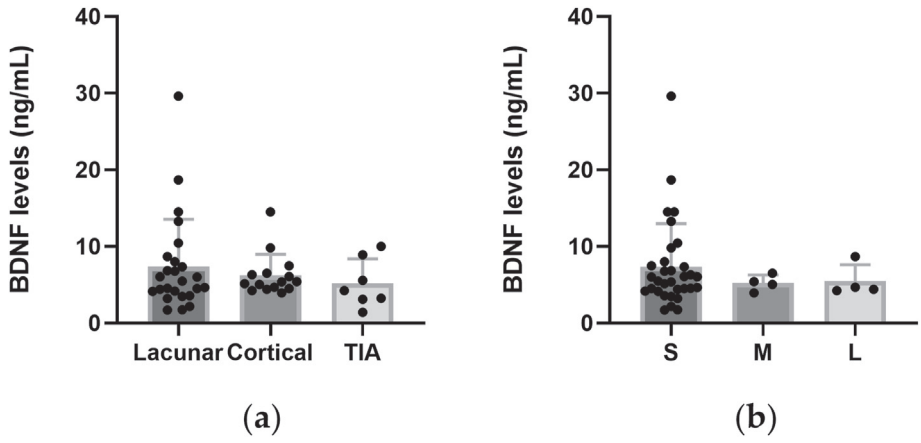

Figure 5. Plasma levels of BDNF are unaffected by stroke type. Plasma levels of BDNF (ng/mL) segregated by (a) stroke type; (b) stroke size ($p = 0.57$ and $p = 0.81$, respectively; Kruskal-Wallis test; GraphPad Prism).

levels of BDNF were lower in the stroke patients than in the healthy controls, irrespectively of age. The difference slightly increased with age: In the youngest age group (<67 years of age) BDNF levels in stroke patients were 45.6% of the levels measured in the healthy controls. In patients who were 68–74 years of age, this percentage was 39.6%, and in patients >75 years of age it was 34.7%.

3.3. BDNF levels were unaffected by stroke size

The stroke patients ($n = 47$) were classified based on whether the lesion was lacunar, cortical/non-lacunar or TIA (Table 1). The levels of BDNF did not differ between the different stroke types (Figure 5a; $p = 0.57$, Kruskal-Wallis test, GraphPad Prism). The non-TIA stroke patients ($n = 40$) were further divided based on the lesion size into small ($n = 32$), medium ($n = 4$), large ($n = 4$). BDNF levels were not dependent on the stroke size (Figure 5b; $p = 0.81$, Kruskal-Wallis test, GraphPad Prism).

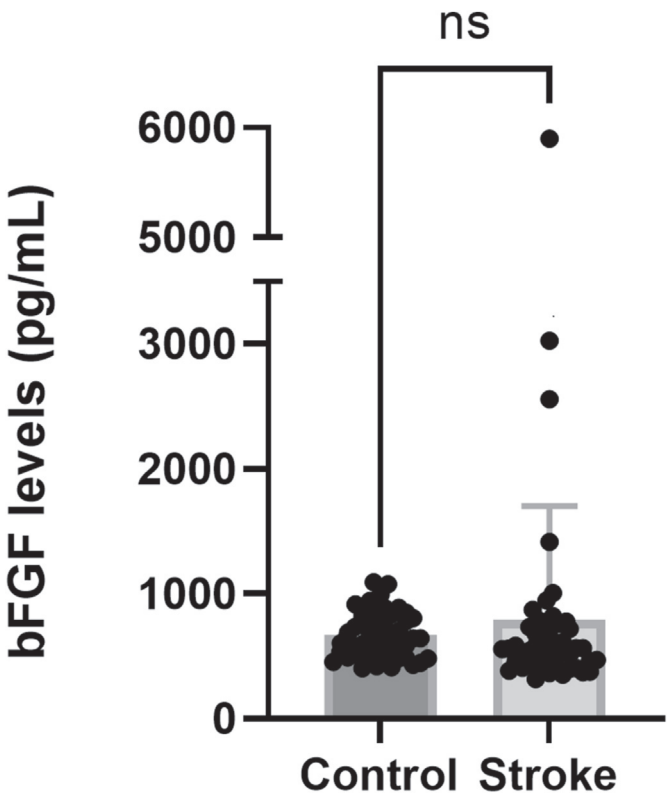

Figure 6. Plasma levels of bFGF. Plasma levels of bFGF (pg/mL) in healthy controls and stroke patients. ns: not statistically significant (Mann-Whitney U test, GraphPad Prism).

3.4. bFGF levels were marginally higher in stroke patients than in healthy controls

The bFGF levels were marginally higher in the stroke patients (795 ± 908 pg/mL; mean ± SD) than in the healthy controls (671 ± 184 pg/mL; $p = 0.068$; Mann-Whitney U test, GraphPad Prism; Figure 6). Four stroke patients were identified as statistical outliers, but these were included in the analysis. A separate analysis confirmed that the inclusion of these patients did not change the conclusion. The bFGF levels did not differ between males and females, neither for the stroke patients nor for the healthy controls ($p > 0.95$; Kruskal-Wallis test; GraphPad Prism; data not shown). Furthermore, the bFGF levels were not affected by the stroke size ($p > 0.65$; Kruskal-Wallis test; GraphPad Prism; data not shown).

3.5. EGF levels were lower in stroke patients than in healthy controls

The EGF levels varied largely between the participants. In stroke patients, plasma EGF varied between 16.5 and 2428 pg/mL, a 15-fold difference. In the healthy controls the EGF levels varied almost 7-fold, ranging from 541 to 3766 pg/mL. The level of EGF was almost 6-fold lower in the stroke patients (184 ± 403 pg/mL (mean ± SD) than in the healthy controls (1080 ± 599 pg/mL; $p < 0.0001$; Mann-Whitney U test; GraphPad Prism) (Figure 7). Some individuals (stroke: $n = 6$; control: $n = 2$) were identified as statistical outliers and had EGF levels several folds higher than the mean levels in the respective groups. These were included in the analysis, but a separate analysis was performed to confirm that the inclusion of these individuals did not change the conclusion.

As reported for BDNF and bFGF, the levels of EGF did not differ between males and females, neither in the healthy controls nor among the stroke patients ($p > 0.99$; Kruskal-Wallis test; GraphPad Prism; data not shown). Furthermore, the EGF levels were unaffected by the stroke size ($p = 0.67$; Kruskal-Wallis test; GraphPad Prism; data not shown).

3.6. Irisin levels were similar in stroke patients and healthy controls

The irisin levels did not differ between the stroke patients (6.74 ± 10.22 ng/mL) and the healthy controls (5.27 ± 2.94 ng/mL; mean ± SD; $p = 0.14$; Mann-Whitney U test; GraphPad Prism) (Figure 8). The irisin levels, however, varied greatly, especially among the stroke patients (ranging from undetectable levels to 50.90 ng/mL). The corresponding range in the healthy controls was 1.85–18.47 ng/mL. The variation in

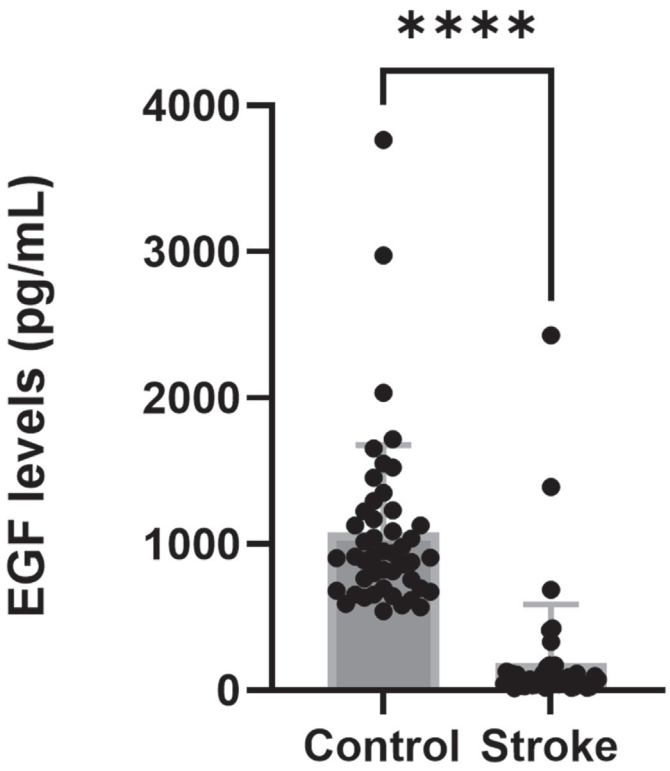

Figure 7. Plasma levels of EGF. Plasma levels of EGF (pg/mL) in healthy controls and stroke patients. ****: $p < 0.0001$; (Mann-Whitney U test, GraphPad Prism).

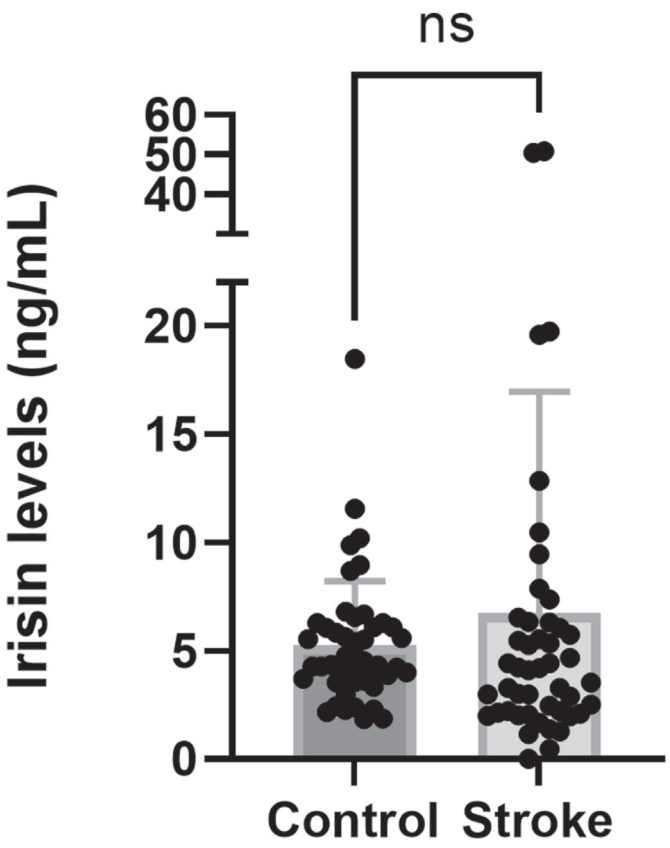

Figure 8. Plasma levels of irisin. Plasma levels of irisin (ng/mL) in healthy controls and stroke patients. ns: not statistically significant (Mann-Whitney U test, GraphPad Prism).

irisin levels in stroke patients was not related to differences in stroke size ($p = 0.23$; Kruskal-Wallis test; data not shown) and was similar in males and females (Kruskal-Wallis test; $p = 0.50$; GraphPad Prism; data not shown).

4. Discussion

The present study demonstrates that plasma levels of the growth factors BDNF and EGF were 2.5- and 5.7-fold lower, respectively, in acute stroke patients than in age and gender matched healthy controls. The levels of bFGF and irisin did not differ between the groups. The levels of growth factors and irisin was unaffected by sex and age. Somewhat surprisingly, we did not find any correlation between the levels of growth factors or irisin and the type of stroke (lacunar, cortical/non-lacunar or TIA) or the lesion size. The low numbers of medium and large infarct sizes, however, calls for caution when drawing a conclusion of no association.

The stroke patients participating in the present study all had cognitive abilities to give an informed consent. The most severe stroke cases, including those who were not capable of approving participation in the study, were excluded. In the cognitive, motoric and self-sufficiency tests, the stroke patients were out-performed by the healthy controls as expected.

Although, stroke is known to be a major contributor to both physical and cognitive decline, we cannot conclude with certainty that the differences in cognitive abilities between the stroke patients and the healthy controls were caused by the stroke *per se*; the possibility that the stroke patients had a lower cognitive score even prior to the stroke must be taken into consideration. In fact, published data indicate that 10% of stroke patients have pre-stroke dementia [39].

The lower levels of BDNF and EGF found in the stroke patients compared to the healthy controls cannot with certainty be ascribed to stroke *per se*; the stroke patients could in theory have lower levels of these growth factors independently of the stroke. The differences are, however, quite large, making it more plausible that they are caused by a major event like stroke than by natural variation. We detected a substantial decrease in plasma BDNF in the stroke patients compared to the healthy controls. Only a few studies have analyzed plasma levels of BDNF after stroke. One reason for this may be that K_2EDTA, which is commonly used as an anticoagulant in the plasma samples—including in the present study—may activate platelets. This can result in the release of BDNF from these platelets, which in turn may mask differences in BDNF released from the brain in response to stroke.

Our findings are, nevertheless, in line with a recent meta-analysis [40] summarizing seven publications where BDNF serum levels in stroke patients during the acute phase were compared to control groups. The magnitude of difference in BDNF levels between stroke patients and healthy controls vary from study to study: Algin and colleagues found that BDNF serum levels in stroke patients at the time of admission to the hospital, were 3.8-fold lower than in a control group who were admitted to the emergency department for non-neurological reasons [41]. Similarly, Chaturvedi and colleagues reported a 2-fold lower serum BDNF in stroke patients compared to in healthy controls [42]. In both studies, the blood was collected at admission, whereas in the present study, blood samples were drawn the day after admission. In another study, the levels of BDNF in stroke patients measured within 10 days after stroke onset (median: 4 days) were found to be reduced compared to the controls [43]. This illustrates the robustness of the BDNF decrease after stroke. Nevertheless, some studies did not find reduced levels of BDNF in stroke patients the first day [44], or seven days after stroke [45]. In fact, even increased BDNF levels have been reported in stroke patients compared to controls [46]. The former study did, however, report that plasma BDNF levels at day one were significantly lower in patients who ended up with a Barthel ADL index score ≤ 80 on day five after stroke onset than in those with a Barthel ADL index score >80 [44]. In the present study a Barthel ADL scale from 0-20 was used, but these scores can easily be converted to

the 1–100 index scale: The average Barthel ADL score in our study was 87.5, and 10 patients had a score >80. The stroke patients participating in the present study were relatively homogenous regarding the Barthel ADL scores which may explain why we did not see a correlation between BDNF levels and Barthel ADL scores. The present study is, however, in line with the majority of studies showing reduced BDNF levels in stroke patients compared to healthy controls.

The robust reduction in BDNF levels found in most studies where acute ischemic stroke patients are compared to controls, combined with the known neuroprotective/neurodegenerative effects of BDNF, suggest that means to increase BDNF levels may be effective therapeutic targets in stroke. Administration of BDNF per se, however, is precluded by a low bioavailability of BDNF in the brain [44]. This reflects a combination of low ability to cross the BBB along with a short distribution time in brain tissue. Means to increase endogenous brain-intrinsic BDNF levels may prove to represent more promising therapeutic approaches. So far no BDNF-increasing therapy has reached clinical use in stroke patients.

In the present study there was no correlation between BDNF levels and stroke size or between BDNF levels and Barthel ADL scores in stroke patients. It should be noted that the sample size, especially for the medium and large stroke sizes, is limited and hence these data are encumbered by uncertainty. Furthermore, in the present study, the stroke size was measured retrospectively from MR images obtained 4–5 days after stroke onset. Despite the fact that BDNF levels decrease with age in the normal brain [45, 47, 48], no correlation between age and BDNF levels were found in the present study. Again, the sample size in each age group were limited, bringing uncertainty to the conclusion. Furthermore, we did not detect different BDNF levels in males and females. This is supported by findings in humans [49] and rodents [50], even though it is known that estrogen increases BDNF expression while testosterone decreases it [51]. Taken together, publications report lower BDNF levels in plasma or serum from stroke patients compared to controls during the early phase of a stroke. Supporting a role of BDNF in early stroke recovery, the val66met single nucleotide polymorphisms (SNP) of the *bdnf* gene is among the top polymorphisms implicated in stroke risk and prognosis [52].

In the present study, bFGF levels were marginally higher in the stroke patients than in the healthy controls ($p = 0.068$). Other studies have shown increased serum bFGF levels when comparing stroke patients to a control group: Guo and colleagues found elevated bFGF levels in serum samples obtained within 48 h after ischemic stroke; the bFGF levels peaked at day three and reminded elevated for 14 days [53]. Elevated levels of bFGF in stroke patients at day three after stroke was confirmed by Golab-Janowska and colleagues even when traditional vascular risk factors were controlled for [54]. Elevated bFGF levels in plasma or serum is consistent with a reported upregulation of bFGF in the brain in response to experimental ischemia in rodents [55, 56] and postmortem in the brain of patients who died 24 h to 43 days after acute ischemic stroke [57]. In the permanent medial cerebral artery occlusion (pMCAO) model in rats, bFGF treatment promote neuroprotection and neurogenesis: Intravenous bFGF injections at 2 h after pMCAO induction resulted in improved functional outcome (rotarod performance) and a substantial reduction of the infarct volume [58]. Intracisternal administration of bFGF at 24 and 48 h after pMCAO led to an increased number of BrdU positive cells in the subgranular zone but did not cause reduced stroke volume [59]. Serum bFGF levels have previously been reported to correlate positively with the infarction size [53]. Such a correlation could not be detected in the present study, perhaps due to the relatively low number of patients with medium or large stroke sizes. A positive correlation between the peak bFGF level and improvement in neurological function between day two and day 20 after stroke has been reported [53], indicating that bFGF may prove to be an early biomarker for progression after stroke.

Through activation of the EGF receptor (EGFR), EGF regulates proliferation and DNA repair. To the best of our knowledge, circulating EGF levels have not previously been investigated in the acute phase of stroke in humans, and effects of EGF in stroke therapy is also missing. In a rat model of stroke, however, intraventricular infusions of EGF and erythropoietin (EPO) together promoted regeneration of the injured neocortex and reversed motor function deficits [60]. Neither EGF nor EPO was able to induce this affect alone. Furthermore, EGFR levels are reported to increase in the penumbra surrounding the stroke core in rodents [61]. In the present study we found that EGF levels in plasma from the stroke patients were nearly 6-fold lower than the levels in the age and gender matched control group. This likely contributes to a reduced capacity for plasticity and tissue regeneration, suggesting that therapies to increase EGF levels during the acute phase of stroke would be beneficial. In order to conclude whether EGF represent a therapeutic target in stroke, the data from the current study need to be confirmed in additional studies, perhaps also including persons with more severe strokes. As described, the evidence for neuroprotective effects of EGF in stroke derives from animals and cell cultures, and the translational value to human patients needs to be determined. We did not detect age or sex dependent changes in EGF levels in the present study, and EGF levels did not depend on the stroke size. The latter may reflect that most of our stroke patients had small lesion sizes, and the limited number of patients with medium or large strokes makes it challenging to detect statistical correlation between EGF levels and stroke size. We therefore cannot conclude whether such a correlation exists, or if the difference in EGF levels between stroke patients and healthy controls would have been even larger if patients with more severe strokes had been included.

Irisin is a myokine released by skeletal muscle during exercise and is known to induce the transformation of white adipocytes to brown adipocytes [62]. During the 10 years since it's discovery, irisin has been implicated in neuroprotection in several neurological diseases, including stroke [63]. Plasma irisin levels have been shown to correlate with levels of irisin in the cerebrospinal fluid (CSF) of healthy humans [64], highlighting the relevance of measuring plasma irisin in stroke patients.

Most studies so far have focused on differences of irisin between groups of stroke patients, and to the best of our knowledge, only one study has reported serum irisin levels in stroke patients compared to a control group of approximately the same age [65]. In their study, Kazimierczak-Kabzińska and colleagues detected significantly lower levels of irisin in stroke patients compared to the control group. However, their control group contained a higher fraction of males, who on average were younger than the stroke patients. Whether or how this bias affects the result is hard to interpret as age has been reported to be negatively correlated with plasma and CSF irisin levels, and irisin have been reported to be higher in males than in females [64]. Further complicating the matter, body mass index—a major risk factor for stroke—is negatively correlated with CSF levels of irisin. Consistent with the results of the present study, Kazimierczak-Kabzińska and colleagues also did not detect differences in irisin levels based on age or sex. This lack of detected correlation may reflect the relatively low number of participants in both studies. Two studies from Chinese populations reported negative correlations between levels of irisin and neurological outcome (measured by the National Institutes of Health Stroke Scale (NIHSS) at admission) and between irisin and stroke volume [66]. Furthermore, serum levels of irisin measured the morning after hospitalization were higher in patients who presented high functional outcomes (measured by mRS) at three months after stroke [66], or six months after stroke [67]. Higher serum irisin levels at the time of admission were also positively correlated to survival for three months [66] or six months [67] post-stroke.

In the present study irisin levels did not differ between the healthy controls and the stroke patients. This finding was somewhat surprising based on the publications presented above. In addition, irisin has been suggested to stimulate the expression of BDNF [18], but in the present study BDNF levels were decreased in stroke patients irrespectively of the unaltered irisin levels. The irisin levels, however, varied between individuals of the same group about 10-fold in the healthy controls, and

from undetectable levels to 50.9 ng/mL in the stroke patients. Based on the correlations of plasma irisin levels with long-term functional and neurological outcomes, the large variation in plasma irisin levels may be of interest in itself, as irisin levels may prove to be a suitable biomarker for long-term stroke outcome. In addition, irisin levels did not differ neither between males and females, nor between different age groups, types of stroke, or stroke sizes. The latter finding is supported by Wu and co-workers [66], who also did not detect correlations between age or sex of the patients, or stroke subtype distribution with irisin levels. In healthy humans, however, plasma levels of irisin were observed to increase with age and to be higher in males than in females [64].

We cannot conclude with certainty that irisin levels were unaffected by the stroke, as differences in the levels between the two groups could in theory have been present before stroke onset, hence masking any changes in the stroke patients in response to the stroke. Low CSF or plasma irisin levels have been associated with risk factors for stroke, like high body mass index, high cholesterol levels or diabetes mellitus type II [67]. These risk factors are expected to be higher among the stroke patients than the healthy controls, so if a baseline difference in irisin levels was present, irisin would likely be lower in stroke patients. Based on the correlation between low irisin levels and stroke severity, an increase in irisin in response to stroke (which would be necessary to compensate for lower baseline irisin) is not likely. Hence, we conclude that in the present study irisin levels did not differ between stroke patients and healthy controls.

5. Conclusion

In the present study we measured plasma levels of known regulators of neuroprotection and repair in acute ischemic stroke patients and compared them to the plasma levels in healthy age- and gender matched controls. We found that the levels of BDNF and EGF were lower in the stroke patients than in the healthy controls, while the levels of bFGF and irisin did not differ. Furthermore, we found large individual differences in irisin and growth factor levels, which may reflect idiosyncratic mechanisms affecting post-stroke recovery. Taken together, the data suggest that during the acute phase of stroke, there is a mismatch between the need for neuroprotection and repair, and the brain's ability to induce these processes. Most of the patients in the current study had mild strokes. Further studies with larger sample sizes should be performed to confirm the current findings -including their applicability for more severe stroke cases- and demonstrate whether variations in these growth factor levels may be a predictor for post-stroke outcome.

Declarations

Author contribution statement

Linda Thøring Øverberg: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Cecilie Morland: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Elise Fritsch Lugg & Mona Gaarder: Performed the experiments; Analyzed and interpreted the data.

Ole Morten Rønning: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Birgitta Langhammer & Bente Thommessen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

The work was supported by Oslo Metropolitan University -OsloMet and by UNIFORFRIMED.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors are grateful to Thor-Øistein Endsjø for valuable help with the recruitment of participants to the healthy control group, and to Head Engineer Hilde Nilsen for help and guidance with the analysis. We would further like to thank the research group: "Male reproductive health" at OsloMet, represented by Prof. Trine B. Haugen and Head Engineer Eldri Undlien Due, for access to lab facilities for blood separation and storage. Finally, we would like to express our appreciation to all the stroke patients and healthy controls who participated in the study.

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Appendix A

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Silje U. Lauvrak	22845520	04.03.2019	2018/2555/REK sør-øst
				D
			Deres dato:	Deres referanse:
			09.02.2019	

Vår referanse må oppgis ved alle henvendelser

Cecilie Morland
Universitetet i Oslo

2018/2555 Blodanalyser som utgangspunkt for individuelt tilpasset oppfølging av slagpasienter

Forskningsansvarlig: Universitetet i Oslo, Akershus universitetssykehus HF, OsloMet - storbyuniversitetet
Prosjektleder: Cecilie Morland

Vi viser til tilbakemelding mottatt 09.02.2019 i forbindelse med ovennevnte søknad. Tilbakemeldingen ble behandlet av komiteens leder på delegert fullmakt.

Prosjektleders prosjektbeskrivelse
Hjerneslag rammer millioner av mennesker årlig. Det er store forskjeller i hvor godt pasientene gjenvinner sine kognitive og motoriske funksjoner etter et slag. Utvikling av depresjon i ettertid kan ikke forutsies utfra objektive mål for funksjonstap. Individuelle forskjeller skyldes sannsynligvis en kombinasjon av faktorer. Disse er lite kartlagt. Vekstfaktorer, kortisol, melkesyre, og markører for oksidativt stress kan frigjøres fra hjernen under slag, og påvirker nervevevets overlevelse. Hypotesen er at balansen mellom disse stoffene er avgjørende for graden av funksjonstap etter hjerneslag. Prosjektet vil identifisere faktorer i pasientenes blod ved innleggelse som, sammenlignet med en kontrollgruppe, kan brukes til å forutsi progresjon, og til å utvikle en individuelt tilpasset behandling for slagpasientene. Blodprøvesvar sammen med kognitive-, motoriske og bildedata vil brukes. Forhold pasientene selv opplever som avgjørende for vellykket rehabilitering, vil også kartlegges.

Saksgang
Søknaden ble første gang behandlet i møtet 16.01.2019, hvor komiteen utsatte å fatte vedtak i saken. Komiteen ba prosjektleder redegjøre for beredskapen i prosjektet, og ba om navn på kontaktperson ved forskningsansvarlige institusjoner.

Prosjektleders tilbakemelding ble mottatt 09.02.2019.

Komiteens vurdering
Komiteens spørsmål er tilfredsstillende besvart, og prosjektleder har kommet med en grundig redegjørelse for beredskapen i prosjektet.

Det skal i prosjektet benyttes prøver fra en tidligere godkjent generell forskningsbiobank (REK-ref.: 2011/1015 «Helseregister og generell forskningsbiobank for nevrologiske sykdommer»). Komiteen anser avgitt samtykke for deltagelse i denne biobanken som dekkende for de analysene som skal gjøres i det omsøkte prosjekt. For hovedstudien som inkluderer 3- månederskontroll med blodprøver, intervju og tester for kognitiv og motorisk funksjon og depresjon, samt for kontrollgruppen, skal det imidlertid innhentes nytt samtykke. Komiteen har ingen innvendinger til samtykkeprosedyren slik den er beskrevet i søknaden.

Besøksadresse:
Gullhaugveien 1-3, 0484 Oslo

Telefon: 22845511
E-post: post@helseforskning.etikkom.no
Web: http://helseforskning.etikkom.no/

All post og e-post som inngår i saksbehandlingen, bes adressert til REK sør-øst og ikke til enkelte personer

Kindly address all mail and e-mails to the Regional Ethics Committee, REK sør-øst, not to individual staff

Etter komiteens syn er prosjektet nyttig, og det er små ulemper forbundet med deltagelse (det er kun 3-månederskontrollen som gjøres utenom normal behandling og oppfølging). På bakgrunn av dette godkjenner komiteen at studien gjennomføres som beskrevet i søknad, protokoll og tilbakemelding fra prosjektleder.

Vedtak
REK har gjort en helhetlig forskningsetisk vurdering av alle prosjektets sider. Prosjektet godkjennes med hjemmel i helseforskningsloven § 10.

Vi gjør samtidig oppmerksom på at etter ny personopplysningslov må det også foreligge et behandlingsgrunnlag etter personvernforordningen. Det må forankres i egen institusjon.

Godkjenningen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknad, protokoll, tilbakemelding fra prosjektleder og de bestemmelser som følger av helseforskningsloven med forskrifter.

Tillatelsen gjelder til 31.01.2030. Av dokumentasjonshensyn skal opplysningene likevel bevares inntil 31.01.2035. Forskningsfilen skal oppbevares atskilt i en nøkkel- og en opplysningsfil. Opplysningene skal deretter slettes eller anonymiseres, senest innen et halvt år fra denne dato.

Forskningsprosjektets data skal oppbevares forsvarlig, se personopplysningsforskriften kapittel 2, og Helsedirektoratets veileder for «Personvern og informasjonssikkerhet i forskningsprosjekter innenfor helse og omsorgssektoren».

Dersom det skal gjøres vesentlige endringer i prosjektet i forhold til de opplysninger som er gitt i søknaden, må prosjektleder sende endringsmelding til REK.

Prosjektet skal sende sluttmelding på eget skjema, senest et halvt år etter prosjektslutt.

Klageadgang
REKs vedtak kan påklages, jf. forvaltningslovens § 28 flg. Klagen sendes til REK sør-øst D. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK sør-øst D, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Vi ber om at alle henvendelser sendes inn på korrekt skjema via vår saksportal: http://helseforskning.etikkom.no. Dersom det ikke finnes passende skjema kan henvendelsen rettes på e-post til: post@helseforskning.etikkom.no.

Vennligst oppgi vårt referansenummer i korrespondansen.

Med vennlig hilsen

Appendix A

Finn Wisløff
Professor em. dr. med.
Leder

Silje U. Lauvrak
Seniorrådgiver

Kopi til: henrik.schultz@farmasi.uio.no; groj@oslomet.no; helge.rosjo@ahus.no
Universitetet i Oslo ved: postmottak@uio.no
Akershus universitetssykehus HF ved øverste administrative ledelse: postmottak@ahus.no
OsloMet ved øverste administrative ledelse: post@oslomet.no

Blodanalyser som utgangspunkt for individuelt tilpasset oppfølging av slagpasienter

Referanse

539270

Status

Vurdert

. N

NSD Personvern

29.05.2019 14:14

Det innsendte meldeskjemaet med referansekode 539270 er nå vurdert av NSD. Følgende vurdering er gitt: Vi viser til endring registrert 14.05.2019 der samarbeidsavtale mellom UiO og OsloMet er ettersendt. Det er vår vurdering at behandlingen av personopplysninger i prosjektet vil være i samsvar med personvernlovgivningen så fremt den gjennomføres i tråd med det som er dokumentert i meldeskjemaet med vedlegg den 29.05.2019. Behandlingen kan fortsette. OPPFØLGING AV PROSJEKTET NSD vil følge opp underveis og ved planlagt avslutning for å avklare om behandlingen av personopplysningene pågår som forventet / er avsluttet. Lykke til videre med prosjektet! Kontaktperson hos NSD: Øyvind Straume Tlf. Personverntjenester: 55 58 21 17 (tast 1)

FORESPØRSEL OM DELTAKELSE **SOM KONTROLLPERSON** I FORSKNINGSPROSJEKTET

BLODANALYSER SOM UTGANGSPUNKT FOR INDIVIDUELT TILPASSET OPPFØLGING AV SLAGPASIENTER

Dette er et spørsmål til deg om å delta i et forskningsprosjekt for å undersøke om faktorer hjernen skiller ut når du sitter i ro, og hvordan disse faktorene påvirkes når du trener, henger sammen med din kognitive funksjon og evnen til å løse spesielle oppgaver. Deltakerne til studien er friske frivillige personer over 50 år som er i stand til å løpe eller gå på tredemølle. I forlengelsen ønsker vi å sammenligne blodprøvene som tas av deg rett etter trening med blodprøver som tas av pasienter med hjerneslag ved Akershus universitetssykehus (Ahus). Siden trening beskytter mot hjerneslag, ønsker vi å se om hjernen frigjør noen av de samme faktorene under trening som under et hjerneslag, og om vi kan bruke denne informasjon til å forutsi hvilke hjerneslagpasienter som vil ha mest nytte av fysisk aktivitet i opptreningsfasen etter et hjerneslag. Prosjektet er et samarbeid mellom Universitet i Oslo, Akershus Universitetssykehus, og OsloMet -Storbyuniversitetet (tidligere Høgskolen i Oslo og Akershus).

HVA INNEBÆRER PROSJEKTET?

Dersom du samtykker til å delta i prosjektet, vil du bli invitert til OsloMet.

Prosjektet innebærer tre blodprøver samt tester for motorisk og kognitiv funksjon. I tillegg vil vi stille deg noen spørsmål om dine trenings vaner, og be deg om å fylle ut et spørreskjema som omhandler angst og depresjon. Det hele vil ta mellom to og tre timer.

Dersom måleresultatene gir mistanke om sykdom eller skade som krever behandling eller vurdering i sammenheng med førerkort el., vil du bli anbefalt å oppsøke fastlegen din for videre medisinsk undersøkelse. Prosjektet videreformidler ikke informasjonen til din fastlege, så det er opp til deg å vurdere om du vil oppsøke legen.

Dersom du tillater det, vil det bli gjort opptak av intervjuet, slik at vi er sikre på at informasjonen du gir blir oppfattet riktig. Dersom du ikke ønsker opptak, vil den som intervjuer deg i stedet ta notater under intervjuet. I tillegg vil vi be deg om å fylle ut et spørreskjema.

<u>Innebærer prosjektet at behandlingstilbudet blir annerledes?</u>
Nei, dette prosjektet er uavhengig av eventuell behandlingen du mottar av fastlege/helsetjenesten. Deltakelse i prosjektet innebærer ikke hyppigere eller annerledes medisinsk behandling og oppfølging enn man vanligvis får.

Prøvetaking:
I blodprøvene dine vil vi analysere forskjellige vekstfaktorer som beskytter hjernevevet. Vi vil også analysere stresshormonet kortisol, og stoffer som skilles ut fra hjernen når den utsettes for skade. Prøvene vi tar av deg i hvile vil brukes som sammenligningsgrunnlag for de prøvene som blir tatt rett etter trening, og 30 minutter etter trening. Noen av analysene vi gjør kan fortelle hvilke gener som ble skrudd av eller på under treningen. Vi vil se etter mulige variasjoner i genet for en melkesyre-sensor som nylig ble oppdage i hjernen, men vi vil ikke gjøre noen systematisk gentesting av deg. Resultatene fra blodprøvene vil kobles opp mot resultatene fra de øvrige testene (kognitive/motoriske tester), men vil ikke lagres i noe offentlig register, og studien vil ikke ha tilgang til opplysninger i din pasientjournal.

Hva skjer når du møter til studien:
Vi vil først måle dine vitale tegn (blodtrykk/puls/vekt) og regne ut din maks puls. Du vil deretter bli bedt om å svare på noen spørsmål om dine treningsvaner. Videre vil vi be deg om å løse noen oppgaver som tester dine kognitive og motoriske egenskaper. Vi vil så be deg besvare et spørreskjema om hvordan du har det, for å avdekke f.eks. depresjon. Ettersom vi ønsker å ta blodprøve av deg på tre tidspunkter, vil vi sette inn en veneflon i armen din, slik at du slipper nye stikk for hver blodprøve. Vi vil ta blodprøve av deg i hvile (før du begynner treningen), rett etter trening og 30 minutter etter at treningen er over.

Treningen:
Treningen vil være oppvarming (10 min.) etterfulgt av intervalltrening (4x4 min.) med høy intensitet på tredemølle, enten løping eller rask gange avhengig av hvor god form du er i.

MULIGE FORDELER OG ULEMPER

Deltakelse i prosjektet påvirker ikke eventuell medisinsk behandling eller oppfølging du får i helsevesenet. Resultatene fra studien vil ikke påvirke din behandlingssituasjon, men kan bidra til økt forståelse av hvordan trening påvirker hjernen, og til bedre oppfølging av slagpasienter senere.

Det forventes ingen bivirkninger eller ubehag utover selve blodprøvetakingen og treningen.

FRIVILLIG DELTAKELSE OG MULIGHET FOR Å TREKKE SITT SAMTYKKE

Det er frivillig å delta i prosjektet. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst, og uten å oppgi noen grunn, trekke ditt samtykke. Dersom du trekker deg fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke deg eller har spørsmål til prosjektet, kan du kontakte prosjektleder, Cecilie Morland, 41547945, cecilie.morland@farmasi.uio.no

HVA SKJER MED OPPLYSNINGENE OM DEG?

Opplysningene som registreres om deg skal kun brukes slik som beskrevet i hensikten med prosjektet. Du har rett til innsyn i hvilke opplysninger som er registrert om deg og rett til å få korrigert eventuelle feil i de opplysningene som er registrert. Du har også rett til å få innsyn i sikkerhetstiltakene ved behandling av opplysningene.

Alle opplysningene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger gjennom en navneliste.

Det er kun prosjektleder, Cecilie Morland, som har tilgang til denne listen.

Opplysningene om deg vil bli slettet senest 5 år etter prosjektslutt.

HVA SKJER MED PRØVER SOM BLIR TATT AV DEG?

Prøvene som tas av deg vil bli anonymisert og oppbevart ved Universitetet i Oslo for analyser. Alle prøvene vil bli destruert så snart alle analyser er ferdige, og senest 10 år etter prosjektavslutning.

GENETISKE UNDERSØKELSER

Vi vil se etter variasjoner i et enkelt gen, nemlig genet for en melkesyre-sensor i hjernen. Denne sensoren er nylig oppdaget i hjernen, og studien ønsker å undersøke om aktivering av denne påvirker skadeomfanget etter hjerneslag. Vi vil derfor gjøre en genanalyse av dette ene spesifikke genet, men ingen systematisk egenanalyse vil bli utført. Funksjonen til melkesyre-sensoren i hjernen er i liten grad kjent, og analyser av variasjoner i dette genet vil dermed foreløpig ikke kunne brukes for å si noe om risiko for senere sykdom. Resultatet vil derfor ikke bli formidlet til deltakerne.

FORSIKRING

Deltakere i denne studien er forsikret gjennom pasientskadeloven.

OPPFØLGINGSPROSJEKT

Det kan hende vi vil kontakte deg igjen senere, dersom det blir aktuelt å forlenge oppfølgingstiden. Du vil da kunne velge om du vil delta eller ikke.

GODKJENNING

Regional komité for medisinsk og helsefaglig forskningsetikk har vurdert prosjektet, og har gitt forhåndsgodkjenning (REK nr. 2018/2555).

Etter ny personopplysningslov har dataansvarlig og prosjektleder Cecilie Morland et selvstendig ansvar for å sikre at behandlingen av dine opplysninger har et lovlig grunnlag. Dette prosjektet har rettslig grunnlag i EUs personvernforordning artikkel6a og artikkel 9 nr. 2 og ditt samtykke.

Du har rett til å klage på behandlingen av dine opplysninger til Datatilsynet.

KONTAKTOPPLYSNINGER

Dersom du har spørsmål til prosjektet kan du ta kontakt med Førsteamanuensis Cecilie Morland, 22844937, cecilie.morland@farmasi.uio.no eller ph.d.-student Linda Thøring Øverberg, loverber@oslomet.no, 67236417.

Du kan også ta kontakt med institusjonens personvernombud dersom du har spørsmål om behandlingen av dine personopplysninger i prosjektet. Personvernombudet ved Universitetet i Oslo er Maren Magnus Voll. Hun kan nås på via e-post: personvernombud@uio.no eller telefon 22859778.

Jeg samtykker til å delta i prosjektet og til at mine personopplysninger og mitt biologiske materiale brukes slik det er beskrevet.

Sted og dato

Deltakers signatur

Deltakers navn med trykte bokstaver

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0130 Oslo

OsloMet Avhandling 2023 nr 19
ISSN 2535-471X
ISBN 978-82-8364-486-9