MUSCLE APELIN MEDIATES THE ANTIDEPRESSANT EFFECTS OF PHYSICAL EXERCISE BY ENHANCING ADULT HIPPOCAMPAL NEUROGENESIS: THE MUSCLE-BRAIN AXIS

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PhD

The Hong Kong Polytechnic University

2025

The Hong Kong Polytechnic University

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Muscle Apelin Mediates the Antidepressant Effects of Physical Exercise by Enhancing Adult Hippocampal Neurogenesis: The Muscle-Brain Axis

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

August 2024

CERTIFICATE OF ORIGINALITY

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Abstract

Physical exercise is well recognized for its potent antidepressant effects, but the underlying molecular mechanisms remain largely unclear. Sarcopenia, an ageing condition with muscle atrophy, is highly associated with a higher risk of developing depression in elderly. This study investigates the role of apelin, a novel myokine linked to sarcopenia, in modulating hippocampal neuroplasticity and its contribution to the antidepressant effects of exercise. Using a mouse model of depression induced by chronic unpredictable stress (CUS), it was found a significant reduction in the protein expression of apelin and its receptor (APJ) in the hippocampus. Remarkably, physical exercise in terms of voluntary wheel running for 4 weeks alleviated depression-like behavior, which is correlated with increased apelin and APJ protein expression and enhanced adult neurogenesis in the hippocampus. Running elevated mRNA expression levels of apelin in skeletal muscles, particularly the tibialis anterior (TA) and gastrocnemius (Gas) muscles, suggesting these muscles are primary sources of apelin secretion in response to running. Transgenic mice with skeletal muscle specific knockout of apelin with running did not exhibit antidepressant effects and enhancement in adult hippocampal neurogenesis, highlighting the dispensable role of myokine apelin in mediating the antidepressant effects of running. Furthermore, overexpression of apelin in the TA and Gas muscles via adeno-associated viral infection mimicked beneficial effects of running on reducing depression-like behavior and enhancing hippocampal neurogenesis and synaptic NMDA receptor-mediated neurotransmission. This was accompanied by increased phosphorylation of NMDA receptors. Additionally, knockdown of APJ in glutamatergic neurons of the ventral hippocampus diminished the antidepressant effects of running, highlighting the importance of apelin-APJ-mediated hippocampal plasticity. Since production of apelin is reduced by ageing, it was also found that muscle apelin deficiency in the transgenic mice accelerated age-related muscle atrophy and motor coordination impairment, but showed no effect on cognitive deficits or emotional dysregulation. These findings highlight the critical role of apelin in mediating the antidepressant effects of voluntary running and suggest its potential involvement in the muscle-brain crosstalk axis in depression.

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Peer-reviewed papers

Formolo, Douglas A., **Jiasui Yu**, Kangguang Lin, Hector W. H. Tsang, Haining Ou, Georg S. Kranz, and Suk-Yu Yau. "Leveraging the Glymphatic and Meningeal Lymphatic Systems as Therapeutic Strategies in Alzheimer's Disease: An Updated Overview of Nonpharmacological Therapies." ***Molecular Neurodegeneration*** 18 (1): 26. https://doi.org/10.1186/s13024-023-00618-3.

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* These authors contributed equally to this work.

Conference

Jiasui Yu, Suk-yu Yau. "Apelin as a potential mediator for the antidepressant effects of physical exercise in a mouse model of depression". (Oral presentation). Brain & Mental Health Conference 2024, Hong Kong SAR, China. *Oral session #1*.

Jiasui Yu, Suk-yu Yau. "Apelin as a novel mediator of antidepressant effects of physical exercise". (Poster presentation). 35th World Congress Collegium Internationale Neuro-Psychopharmacologicum, Tokyo, Japan. *Poster 2-2, C000410*.

Jiasui Yu, Suk-yu Yau. "Potential role of apelin in mediating the antidepressant effects of physical exercise". (Poster presentation). Annual Conference of the Society of Neuroscience (SfN) 2023, Washington DC, US. *Poster #6189*.

Jiasui Yu, Suk-yu Yau. "Muscle myokine apelin mediates the antidepressant effects of physical exercise in a mouse model of depression". (Poster presentation). The Hong Kong Polytechnic University Academy for Interdisciplinary Research (PAIR) Conference 2023, Hong Kong SAR, China. *Ref #177.*

Suk-yu Yau, Kai Le, Ping Wang, Can Huang, **Jiasui Yu**, Gang Chen. "Effects of chronic treatment with extracted active ingredients from Chinese traditional medicine formula: Yueju on alleviating depression in animal models". (Poster presentation). Forum of European Neuroscience Societies (FENS) 2024, Vienna, Austria. *PS05-28AM-043.*

Awards and Special Events

2023 Best Poster Presentation Award of Mental Health Session, The Hong Kong Polytechnic University Academy for Interdisciplinary Research (PAIR) Conference 2023, The Hong Kong Polytechnic University, Hong Kong SAR, China.

2023 The Abstract Entitled "Potential Role of Apelin in Mediating the Antidepressant Effects of Physical Exercise" was selected by the Society of Neuroscience (SfN) for **Press Conference**, the Annual Conference of the SfN 2023, Washington DC, US.

Acknowledgements

I would like to express my deepest gratitude to everyone who has supported and assisted me throughout my PhD journey. First and foremost, my heartfelt thanks go to my supervisor, Dr. Sonata Suk-yu Yau, for her unwavering guidance and support. Her insights and encouragement have been invaluable, and her passion for research motivates me to continue my studies. I am also deeply grateful to Prof. Aimin Xu for his suggestions and support, and to Prof. Li Zhang for his constructive comments and suggestions. Special thanks to Prof. Kangguang Lin for his steadfast support.

My sincere thanks go to Dr. Julia M. Rosa, Dr. Vic K. Sun, Dr. Kai Le, and Dr. Zafar Iqbal for their technical guidance and suggestions on experimental design, as well as to Dr. Malegaddi Devaki for her assistance with the study. I also appreciate Dr. Leiluo Geng for providing essential technical suggestions, particularly for transgenic mouse models, and Dr. Runhua Wang for his technical suggestions on cryostat.

I would also like to express my gratitude to Dr. Huihui Guo for her insightful comments and encouragement. My appreciation extends to Mr. Douglas A. Formolo, Ms. Tong Cheng, Mr. Akhlaq Hussain, Mr. Thomas H. Lee, Mr. Ahadullah, and Ms. Yvette Yip for their valuable suggestions and assistance in my research study. I would like to acknowledge the technical expertise of Mr. Zhiping Song, Mr. Jiyang Li, Ms. Chunfang Cheng, Ms. Jiaqi Li, Ms. Yunxiao Zhong, Ms. Natalia P. Formolo, Ms. Yiting Zhao, Ms. Chunfang Cheng, Ms. Ping Wang, Ms. Yujie Ma, Mr. Can Huang, Ms. Jing Liao, and Ms. Lishuo Chao. I also appreciate the support from Mr. Dennis Mok and Mr. Kelvin Wong in the Department of Rehabilitation Sciences.

Special thanks to Dr. Na Li, Dr. Xiaoxiao Li, Dr. Haicui Wu, Dr. Huihui Xiao for their useful advice and helpful support when I was at the Shenzhen Research Institute (SZRI). I am thankful for the technical support provided by Dr. Dan Wang, Mr. Xin Liu, Mr. Haitao Liang, and Mr. Qing Yang at the SZRI.

I am profoundly grateful to my parents for their unwavering support, and to my brother for his encouragement. Lastly, I would like to thank my girlfriend, Ms. Pingyi Zou, for her love and support throughout this journey.

Last but not least, I would like to thank the Department of Rehabilitation Sciences for supporting my entire PhD study. I also appreciate the support from the University Research Facility in Behavioral and Systems Neuroscience, the University Research

Facility in Life Sciences, and the Centralized Animal Facilities. Finally, thank you to the Hong Kong Polytechnic University.

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Abbreviations

5-HT	Serotonin
AAV1	Adeno-associated virus type 1
AAV-shAPLNR	Transgenic mouse model of ventral hippocampal knockdown of apelin receptor in glutamatergic neurons
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPA	A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	5' AMP-activated protein kinase
APJ	Apelin receptor
APLN	Apelin gene
APLN-floxed	LoxP flanked apelin alleles
APLN-mKO	Transgenic mouse model of skeletal muscle knockout of apelin
APLNR	Apelin receptor gene
Bax	Bcl-2-associated protein x
BBB	Blood–brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CA	Cornu ammonis
Ca^{2+}	Calcium cation
Calpain	Calcium-activated neutral proteases
Camk2a	calcium/calmodulin-dependent protein kinase II alpha subunit
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CHD	Coronary heart disease
CK2	Casein kinase 2
CMV	Cytomegalovirus
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
CUMS	Chronic unpredictable mild stress
CUS	Chronic unpredictable stress
CWIRS	Chronic water-immersion restraint stress
DAG	Diacylglycerol
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DTT	Diffusion tensor tractography
ECT	Electroconvulsive therapy

EDL	Extensor digitorum longus
EDTA	Ethylenediamine tetraacetic acid
eEPSC	Evoked excitatory postsynaptic currents
E-LTP	Early long-term potentiation
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
FA	Fractional anisotropy
Flox	Apelin-flox background mice
FNDC5	Fibronectin type III domain-containing protein 5
FST	Forced swimming test
FV	Fiber volume
Gas	Gastrocnemius
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GluA1	Glutamate receptor 1
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK-3β	Glycogen synthase kinase 3
HDAC4/5	Histone deacetylases 4 and 5
HFD	High-fat diet
HIFT	High-intensity function training
HIIT	High intensity interval training
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
i.c.v.	Intracerebroventricular
IGN	Immature granule neurons
IL-6	Interleukin 6
IP3	Inositol trisphosphate
IPAQ	International physical activity questionnaire
IPC	Intermediate progenitor cells
KLF2	Kruppel-like factor 2
LBMS	Lower body muscle strength
LPS	Lipopolysaccharide
LSD	Fisher's least significant difference test
LTD	Long-term depression
LTP	Long-term potentiation
MAP1LC3B-II	Microtubule-associated protein 1A/1B light chain 3B-II
MAPK	Mitogen-activated protein kinase
MCK	Muscle-specific creatine kinase
MCK-Cre	Transgenic mice expressed the Cre recombinase gene driven by the MCK promoter
MDD	Major depressive disorders
MEF2	Myocyte enhancer factor 2

METH	Methamphetamine
MGN	Mature granule neurons
MK-801	Dizocilpine
ML	Molecular layer
MMSE	Lower mini-mental state examination
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
NMDA	N-methyl-D-aspartate
NORT	Novel object recognition test
NOS	Nitric oxide synthase
NPC	Neural progenitor cell
NRF2	Nuclear factor-E2-related factor 2
NSCs	Neural stem cells
OFT	Open field test
OHDA	6-hydroxydopamine
p2A	Porcine teschovirus-1 2A
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PGC-1α	Peroxisome-proliferator-activated receptor-gamma coactivator-1α
PHLPP1	pH domain and leucine rich repeat protein phosphatase 1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
Plant.	Plantaris
PLCb	Phospholipase C beta
pN	Neuroblasts
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PSD-95	Postsynaptic density-95
qPCR	Quantitative polymerase chain reaction
Quadri.	Quadriceps
rA	Radial astrocytes
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAMP	Senescence-accelerated mouse
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sed	Sedentary

sEPSC	Spontaneous excitatory postsynaptic currents
sgRNA	Single guide ribonucleic acid
SGZ	Subgranular zone
Sol	Soleus
SPT	Sucrose preference test
SQSTM1	Sequestosome 1
SST	Sucrose splash test
STEP	Striatal-Enriched protein phosphatase
TA	Tibialis anterior
TAE	Tris-acetate EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris buffer saline with Tween-20
Theta-BS	Theta burst stimulation
TNF-α	The tumor necrosis factor-α
TrkB	Tropomyosin receptor kinase B
TXNIP	Thioredoxin-interacting protein
ULK1	Unc51-like autophagy-activating kinase 1
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter 1. General Introduction

Major depressive disorder (MDD) is a severe condition with profound social and economic repercussions. It stands as the primary cause of years lived with disability (YLD) globally, contributing to over 10% of the 777 million YLD cases in 2010 [1]. The World Health Organization (WHO) estimated that more than 250 million individuals worldwide were affected by depression [2]. The first-line treatment for MDD generally includes antidepressant medications, with selective serotonin reuptake inhibitors (SSRIs) being prescribed to about two-thirds of patients [3,4]. However, 20% to 40% of patients exhibit resistance to these pharmacological interventions, and the therapeutic onset is often delayed, taking 4-12 weeks to alleviate symptoms [5,6]. Additionally, depressive disorders are associated with a high suicide rate, accounting for nearly 1.5% of all deaths worldwide [7]. This underscores the urgent need for more effective antidepressant treatments.

Muscle atrophy, particularly sarcopenia, represents a significant age-related disorder characterized by a progressive decline in skeletal muscle mass, strength, and function [8]. Sarcopenia not only impairs physical performance and mobility but is also intricately associated with an accelerated decline in cognitive abilities [9]. Recent research indicates that individuals with sarcopenia exhibit a 64-82% increased likelihood of experiencing depression, a correlation that persists independently of age, sex, cognitive performance, and physical activity levels [10]. Despite this, investigations into the shared pathophysiological mechanisms between sarcopenia and depression remain limited. Resistance training has proven highly effective in significantly enhancing muscle mass and strength among older adults diagnosed with sarcopenia [11]. These improvements are attributed to enhanced neuromuscular junction function, increased muscle protein synthesis, and satellite cell activation [12] . Aerobic exercise also contributes to muscle health by improving mitochondrial function and reducing oxidative stress. Exercise is recognized as an effective intervention for reducing depressive symptoms. A meta-analysis found that various forms of exercise, including aerobic activities, strength training, and yoga, significantly reduce depressive symptoms [13]. The antidepressant effects of exercise on cerebral function are mediated by mechanisms inherent to the muscle-brain crosstalk, such as the modulation of adult

neurogenesis, cognitive function, and metabolism, potentially facilitated by myokine signaling [14].

Our research team has identified that the therapeutic impact of physical exercise on alleviating symptoms of depression are mediated by various mediators, including adipokines secreted by adipose tissues (e.g., adiponectin), hepatokines released by the liver (e.g., FGF21), and myokines secreted by muscles (e.g., irisin, interleukin 6) [15]. While the precise mechanisms of muscle-to-brain communication remain elusive, the role of muscles as a secretory organ, particularly through myokine mediation, is increasingly evidenced [16].

Apelin, a novel myokine linked to sarcopenia [17], has also been shown to produce antidepressant outcomes [18,19]. Aging is associated with both the onset of sarcopenia and a decline in apelin expression within muscle tissues. Ito et al., [20] demonstrated that serum and muscle apelin levels are significantly reduced in aged mice compared to their younger counterparts, suggesting that apelin may play a vital role in the development of age-related diseases. Exercise-induced apelin expression has been reported to mitigate sarcopenia by enhancing muscle protein synthesis and improving mitochondrial function in aged mice [17]. Further studies have corroborated that exercise significantly elevates serum apelin levels, which is beneficial for muscle health and metabolic regulation [21], suggesting that exercise is a trigger for activation of apelin expression.

Apelin, identified as a promising therapeutic target for treating depression, also manifests neuroprotective properties crucial for safeguarding neurons and exhibits significant influence across a spectrum of neurological disorders. Z. Yan et al. [22] suggests that reduced apelin levels may be implicated in depression, and enhancing apelin signaling could potentially alleviate depressive symptoms. Additional studies demonstrated that apelin exerts its antidepressant effects by binding to receptors (APJ) in the hippocampus, but not the hypothalamus, by modulating pathways such as receptor-gamma coactivator-1α (PGC-1α)/PPARγ [23,24]. Considering the similar antidepressant effects of physical exercise, it is hypothesized that exercise may alleviate depression by upregulating apelin levels.

The hippocampus is highly plastic and responsive to environmental stimuli, with physical exercise enhancing hippocampal function, thereby improving cognitive function and emotional regulation [25]. Additionally, apelin is emerging as a potential

therapeutic target for neuropsychiatric disorders. The antidepressant effects of Apelin have been demonstrated by its ability to alleviate hippocampal neuroinflammation and prevent neuronal damage in hippocampus of rodent models [23,26,27]. Despite these findings, the mechanisms by which apelin mediates the antidepressant effects and enhances hippocampal neuroplasticity in response to physical exercise remain to be elucidated. Physical exercise has been shown to exert antidepressant effects, partly mediated by enhanced adult neurogenesis in the dentate gyrus [28–30]. Additionally, exercise promotes dendritic complexity, increases spinal density, and enhances synaptic plasticity within hippocampal subregions. Therefore, this study aims to elucidate the role of apelin in mediating these antidepressant effects of exercise, specifically investigating how apelin enhances hippocampal neuroplasticity through molecular and cellular mechanisms.

Chapter 2. Literature Review

2.1. Neuropathology of depression

2.1.1. Neurobiology of depression

Depression, or the wide array of depressive symptoms, encompasses a broad spectrum of emotional, cognitive, and physical manifestations, severely compromising an individual's daily functioning [31,32]. These symptoms extend beyond fleeting feelings of sadness, persisting relentlessly and permeating various facets of life, including mood, cognitive processes, and physical well-being [32]. The global incidence of depression continues to rise steadily, presenting a pressing concern in public health. Recent epidemiological investigations have reported the prevalence rates of depression worldwide, revealing alarming figures of 12% for lifetime prevalence [33], imparting a substantial burden on individuals and society at large. Remarkably, the heaviest burden rests upon those grappling with major depressive disorders (MDD), estimated to number in the 308 million globally [2].

Depression is a multifaceted psychiatric disorder with a profound and growing impact on global health, underscoring the urgent necessity for continuous clinical research and the development of effective therapeutic interventions. MDD is a widespread and debilitating form of depression, diagnosed when individuals exhibit five or more specific symptoms persisting for at least two weeks [34]. These symptoms span affective, cognitive, and somatic domains. Key features include a persistent depressed mood or markedly diminished interest or pleasure (anhedonia). Additionally, MDD symptoms may involve changes in appetite or weight, disruptions in sleep patterns, psychomotor agitation or retardation, feelings of fatigue or loss of energy, pervasive feelings of worthlessness or excessive guilt, diminished ability to think or concentrate, and recurrent thoughts of death or suicide [35]. Together, these symptoms characterize the multifaceted clinical presentations of MDD, reflecting its complex impact on emotional, cognitive, and physical functioning. These diverse symptoms reflect the complex interplay of genetic, biological, environmental, and psychological factors contributing to the pathophysiology of MDD, necessitating a multifaceted approach to treatment and a personalized medicine framework for effective management [36].

Over the past few decades, the understanding of the onset of depression has undergone significant transformations, leading to a shift in hypotheses and research paradigms. Initially, the monoamine hypothesis stood as the prevailing theory, attributing depression to imbalances in neurotransmitters. The brain's serotonergic, dopaminergic, and noradrenergic neurons, pivotal for regulating functions such as memory, behavior, and mood, were implicated in the pathogenesis of depression [37–39]. Reduced levels of neurotransmitters like serotonin, dopamine, and norepinephrine were associated with depressive symptoms [37,40], suggesting that a scarcity of these neurotransmitters, coupled with impaired receptor function, might contribute to the development of depression. However, this view has evolved to incorporate a more complex understanding of depression's etiology.

Recent research in depression has significantly shifted focus towards receptor hypotheses, with particular emphasis on the glutamatergic system and the potential therapeutic efficacy of ketamine as an N-Methyl-D-Aspartate (NMDA) receptor antagonist. Investigations have elucidated key mechanisms underlying ketamine's rapid antidepressant effects, highlighting its influence on improving synaptic plasticity [41,42], and modulating NMDA receptor activity in excitatory neurons [41–44]. Specifically, ketamine facilitates the disinhibition of excitatory neurons by preferentially targeting GABAergic interneurons in critical regions such as the cortex and hippocampus, leading to increased glutamatergic transmission [45,46]. This disinhibition is crucial for the rapid amelioration of depressive symptoms, positioning ketamine as a promising treatment for refractory depression. Moreover, depression is characterized by a complex interplay of multiple receptor systems, including 5-methyl-4-isoxazolepropionic acid (AMPA), serotonin, glucocorticoid, and dopamine receptors [47–49]. Dysregulation in these receptor functions, along with alterations in gene expression and epigenetic modifications, contribute to the onset and persistence of MDD. Understanding these intricate molecular and cellular mechanisms offers potential pathways for novel therapeutic interventions and underscores the importance of a multifaceted approach to treating this debilitating condition.

Another important development is the recognition of the kindling hypothesis, which posits that with each successive depressive episode, future episodes become increasingly susceptible to spontaneous activation, relying less on external life stressors [50,51]. This has significant implications for the treatment and management of depression, emphasizing the critical importance for early and efficacious interventions to mitigate the risk of recurrence.

Additionally, external environmentally induced emotional dysregulation has emerged as a prominent framework, elucidating the interplay between stressful life events and individuals predisposed for depressive symptoms [52–54]. Endocrine system disorders, such as the hypothalamic-pituitary-adrenal (HPA) axis, stand as significant contributors to the development of MDD [55–57]. These hormonal irregularities are linked to depression symptoms like sleep disturbances and weight fluctuations, exerting influence over the body's stress response and potentially exacerbating MDD symptoms [58,59].

Inflammation is increasingly recognized as a crucial factor in the etiology of MDD, with elevated levels of inflammatory markers such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) frequently observed in affected individuals [60–62]. Chronic stress-induced immune dysregulation perpetuates this inflammatory cascade, significantly contributing to MDD pathophysiology [63]. Cytokines, including IL-6 and TNF-α, modulate neuronal function, synaptic plasticity, and neurotransmitter systems, thereby influencing both the onset and progression of depressive symptoms [64,65]. These cytokines can cross the blood-brain barrier and interact with neural substrates, contributing to neuroinflammation and altering neurotransmitter metabolism, neuroendocrine function, and synaptic plasticity [66]. The resultant neurobiological changes manifest as depression-related behavioral symptoms, including disrupted sleep, anhedonia, and reduced appetite. This cytokine-induced behavioral phenotype highlighting the potential of anti-inflammatory strategies as therapeutic interventions for MDD [67,68]. Understanding the nuanced effects of inflammation in depression paves the way for innovative treatments targeting the immune system to mitigate depressive symptoms and improve patient outcomes.

Adult neurogenesis, the process of generating new neurons in the mature brain, is increasingly recognized as a focal point in depression research [69]. This phenomenon is closely linked with the production of brain-derived neurotrophic factor (BDNF), a key neurotrophin essential for neuronal survival, differentiation, and synaptic plasticity [70,71]. The hypothesis suggests that the interplay between cortisol, the stress hormone, and BDNF under stress contributes to the pathophysiology of MDD by impairing adult hippocampal neurogenesis [72,73]. Antidepressants and therapies like electroconvulsive therapy (ECT) function by counteracting the effects of cortisol and inflammation, potentially by elevating BDNF levels, which play a crucial role in neurogenesis and dendritic sprouting [74,75].

Overall, the perspective on depression has moved from a singular focus on neurotransmitter levels to a holistic and multifaceted approach that considers genetic, environmental, and neurobiological factors as integral components contributing to the onset and progression of the disorder. This integrated viewpoint acknowledges the intricate complexity of depression and the interplay of various pathophysiological mechanisms.

2.1.2. Hippocampal neuropathology in depressive disorders

The hippocampus, located in the medial temporal lobe, is integral to the limbic system [76]. It plays critical roles in memory consolidation, particularly in transitioning from memory and in spatial navigation [77]. The hippocampus is structurally composed of the dentate gyrus (DG) and the Cornu Ammonis (CA), which is further divided into regions CA1 through CA4 [76]. These interconnected regions, along with the entorhinal cortex, form a sophisticated neural circuit essential for various aspects of memory processing.

In rodent models of depression, the hippocampus exhibits structural and functional changes similar to those seen in humans under chronic stress. Studies by Canonica & Zalachoras [78] highlight dendritic retraction, synaptic plasticity deficits, and CA3 region atrophy as consequences of chronic stress. Chronic stress also disrupts adult neurogenesis in the dentate gyrus, a process vital for hippocampal function and mood regulation. This disruption mirrors the reduced hippocampal volume consistently found in depressed patients compared to healthy individuals [79]. These volumetric reductions are attributed to prolonged exposure to stress hormones, neuroinflammation, and decreased neurotrophic support, collectively impairing neuroplasticity and contributing to hippocampal neuronal loss in depression.

The neurobiology of depression is complex and involves a network of interconnected pathways that affect hippocampal function. Alterations in neurotransmitter systems, such as the serotonergic, glutamatergic, and GABAergic systems, have been observed in depression [80]. These changes can disrupt hippocampal circuitry and synaptic plasticity, leading to mood dysregulation and cognitive impairments commonly seen in depressive disorders. Microglia, the immune cells of the central nervous system, are crucial for maintaining brain homeostasis through synaptic pruning, debris clearance, and

inflammation modulation [81]. In the context of hippocampal neurogenesis, microglia regulate the niche supporting the proliferation and differentiation of neural progenitor cells [82]. However, dysregulated microglial function, characterized by an exaggerated inflammatory response, is implicated in the pathophysiology of depression [83]. This dysregulation disrupts the balance required for neurogenesis and contributes to cognitive impairments observed in depressive disorders.

Depression is also associated with alterations in several physiological markers within the hippocampus. In rodent models, chronic stress has been shown to affect levels of BDNF, leading to decreased neuroplasticity [84]. Additionally, there are changes in the expression of glucocorticoid receptors, which are critical in the stress response, and alterations of neurotransmitter systems, including serotonin and glutamate [85,86]. These changes can lead to impaired hippocampal function and are indicative of the neurobiological underpinnings of depression. At the core of hippocampal pathology in depressive disorders is the interplay between stress, neuroinflammation, and neuroplasticity [87]. Chronic stress activates the HPA axis by elevated levels of glucocorticoids, which can have neurotoxic effects on the hippocampus [88]. Additionally, pro-inflammatory cytokines exacerbate neuroplasticity deficits and further contribute to the pathogenesis of depression [89].

2.2. The role of exercise in hippocampal neuroplasticity

2.2.1. Adult hippocampal neurogenesis

Hippocampal neurogenesis refers to the generation of new neurons in the hippocamp, which is particularly significant in the dentate gyrus [90]. The stages of hippocampal neurogenesis include the proliferation of neural stem cells, their differentiation into neuronal precursors, maturation into neurons, and eventual integration into existing neural circuitry [91]. These stages collectively enhance the plasticity and functional capacity of the hippocampus, highlighting its importance in cognitive processes and mood regulation [92,93].

Neural stem cells (NSCs), residing in the subgranular zone of the dentate gyrus, have the remarkable ability to self-renew and generate progenitor cells (Fig. 2.1). These progenitor cells proliferate and differentiate into immature neurons, a critical phase of hippocampal neurogenesis. Immature neurons are identified by specific-expressed

markers including doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM), which are indicative of their developing state [94]. As these immature neurons mature, they extend axons and dendrites, form synapses, and begin to exhibit the electrical properties characteristic of neurons. They are guided by a variety of extrinsic and intrinsic signals, including neurotrophic factors like BDNF and environmental cues [95].

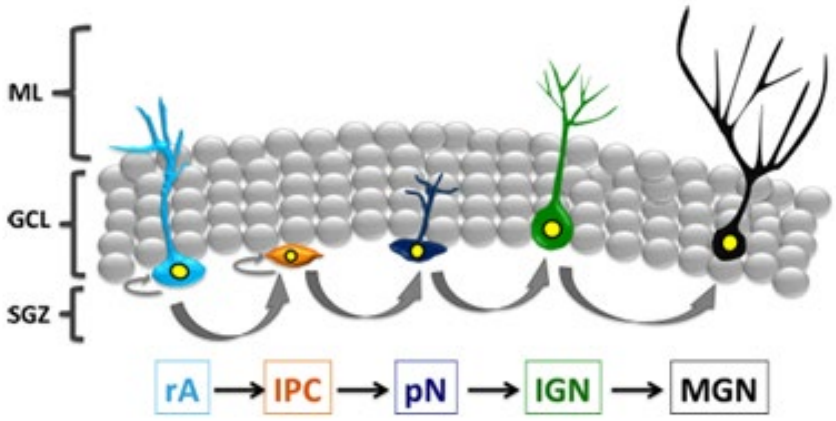

Figure 2.1 Adult hippocampal neurogenesis in the dentate gyrus.
Neural progenitors in the subgranular zone (SGZ) differentiate through various stages before integrating into the granule cell layer (GCL) and the molecular layer (ML). rA (radial astrocytes): Neural stem cells located in the SGZ, depicted in light blue. IPC (intermediate progenitor cells): These cells, shown in orange, undergo further division and differentiation. pN (neuroblasts): Represented in dark blue, these neuroblasts migrate and start to form dendritic processes. Immature granule neurons (IGN): depicted as green cells, extend their dendrites into the molecular layer as they develop.. Nature granule neurons (MGN): Finally, these fully mature neurons, depicted in black, integrate into the granule cell layer. The progression from radial astrocytes to mature granule neurons involves significant morphological changes, including dendritic growth and synaptic integration, which are essential for hippocampal neurogenesis and function. Figure was adapted form Campos et al. [96].

Exercise has been identified as a potent non-pharmacological stimulator of hippocampal neurogenesis. Aerobic exercise has demonstrated the ability to promote the growth and longevity of new neurons within the hippocampus [97]. The mechanisms underlying exercise-induced neurogenesis are multifaceted, involving an increase in BDNF [98]. Additionally, exercise promotes vascularization and increases blood flow to the

brain, providing a nourishing environment conducive to neurogenesis [99]. Molecularly, exercise stimulates the expression of genes linked to neuroplasticity, for instance, exercise increases BDNF levels, promoting neurogenesis, improving synaptic plasticity, and enhancing neuronal survival [98]. Furthermore, exercise increases both angiogenesis and neurogenesis in the hippocampus by promoted the expression levels of vascular endothelial growth factor (VEGF) [100]. Physical activity also influences neurotransmitter systems, which play a role in modulating neurogenesis. For example, serotonin (5-HT) levels increase by exercise, and this neurotransmitter has the benefits role in the regulation of neurogenesis through the 5-HT3 receptor [101]. Additionally, exercise-induced activation of the endocannabinoid system has been suggested to contribute to the proliferation of neural progenitor cells [102].

In summary, exercise-induced hippocampal neurogenesis is a dynamic process influenced by a cascade of molecular and cellular events. The increase in neurotrophic factors, enhanced vascularization, and regulation of neurotransmitter systems collectively create a supportive environment for the formation and integration of new neurons in the hippocampus [103,104]. These changes not only enhance cognitive function but also offer protective effects against neurodegenerative diseases and mood disorders.

2.2.2. Hippocampal synaptic plasticity

Synaptic plasticity in the hippocampus is a fundamental neurophysiological mechanism underlying learning and memory, which involves the ability of synapses and the communication points between neurons [105]. This plasticity enables the hippocampus to encode, store, and retrieve information, making it essential for cognitive processes [106]. The principle of synaptic plasticity is exemplified by Hebbian theory, which posits that 'neurons that fire together, wire together,' implying that simultaneous activation of neurons results in significant increases in synaptic strength [107]. Conversely, "neurons that fire out of sync, lose their link," suggesting that a lack of simultaneous activation weakens synaptic connections [106].

Long-term potentiation (LTP) is an enduring increase in synaptic strength following high-frequency stimulation of a synapse, representing a cellular mechanism underlying learning and memory. Conversely, long-term depression (LTD) is a long-lasting decrease in synaptic strength induced by low-frequency stimulation of a synapse [108]. At the

molecular level, synaptic plasticity involves changes in the quantity and type of neurotransmitter receptors at synapses, alterations in neurotransmitter release, and structural modifications such as the growth of new synaptic connections or the pruning of existing ones[109]. Additional study has indicated that BDNF also plays a essential role in modulating synaptic transmission and promotes LTP [110].

The hippocampus is particularly plastic, not just in early development but throughout life. This plasticity is not uniform across the hippocampus; different regions and pathways within the hippocampus exhibit distinct neural plasticity properties. For example, the CA1 region is known for its role in the consolidation of long-term memories, partly due to its capacity for robust LTP and LTD [111]. The dentate gyrus (DG), the few regions in brain where neurogenesis, contributes to the plasticity of the hippocampus. These newborn neurons in the DG integrate into existing circuits and form new connections, which is crucial for pattern separation and the formation of distinct memories. The CA3 region has a high level of plasticity, partly due to its extensive recurrent collaterals and inputs from the DG. This region is involved in the initial encoding of memories and can rapidly change its synaptic strength to form new memories [112].

Physical exercise facilitates hippocampal synaptic plasticity by promoting neurogenesis and BDNF expression [98]. Additionally, exercise enhances synaptic efficiency and induces related molecular changes within the hippocampus [113]. Physical exercise has been reported to induce structural changes in synapses to enhance signal transmission, including increased synaptic connections and dendritic spine modifications [114]. Notably, some studies has indicated that physical exercise upregulated the NMDA receptor expression, increased their sensitivity to neurotransmitters such as glutamate, thus promoting intraneuronal communication [25]. Furthermore, exercise enhances mitochondrial efficiency and energy metabolism. The upregulation of 5' AMP-activated protein kinase (AMPK) and peroxisome-proliferator-activated PGC-1α, induced by exercise, is thought to support mitochondrial biogenesis, aiding synaptic function and plasticity [115]. Additionally, exercise modulates peripheral hormones and metabolites to enhance brain function. Factors such as myokines, cytokines, metabolites, hormones, and neuropeptides, released or produced during physical activity, play roles in enhancing synaptic plasticity with effects on both early and late phases of LTP [116]. However, the detailed mechanisms underlying the peripheral hormones' action remain to be further investigated.

2.3. The muscle-brain crosstalk

2.3.1. The association between sarcopenia and depression

Sarcopenia, an age-related condition characterized by the gradual decline in muscle mass and strength, presents significant health challenges for older adults [9,10]. In older adults, sarcopenia is associated with numerous adverse outcomes, including increased risks of falls, functional impairments, extended hospitalizations, higher rates of hospital readmissions, and elevated mortality [117]. The prevalence of sarcopenia varies considerably, influenced by differences in measurement methodologies, populations surveyed, and diagnostic criteria. The Asia Working Group for Sarcopenia (AWGS) reports even broader prevalence rates across Asian populations, ranging from 2.5% to 45.7%. These variations underscore the necessity for standardized diagnostic protocols and tailored interventions to mitigate the burden of sarcopenia [118]. Addressing sarcopenia through early detection and targeted therapeutic strategies is crucial for improving health outcomes and well-being in the aging population.

Emerging research has consistently highlighted the intricate relationship between sarcopenia and depression. K.-V. Chang et al. [10] found a significant association between these conditions, suggesting a deeper link that warrants further investigation to establish causality. Complementing this, Z. Li et al. [119] observed a high prevalence of depression among sarcopenic patients. This connection was further reported that sarcopenia was linked to depressive symptoms among elderly Chinese community-dwellers [120], with sarcopenic individuals being over twice as likely to exhibit depressive symptoms. The role of sarcopenia in mental health is further complicated by studies like that of Zhong et al. [121], which posits depression as a potential precursor to sarcopenia, emphasizing the importance of early intervention. The Western China Health and Aging Trends (WCHAT) study identified sarcopenia as an independent risk factor for developing depressive symptoms, particularly due to low muscle mass [9].

The multifaceted nature of sarcopenia's impact on mental health is evident across various populations. Jin et al. [122] found that older Korean adults with both low muscle mass and function have a significantly increased risk of experiencing depressive symptoms. Similarly, Hamer et al. [123] reported that sarcopenic obesity, especially when accompanied by decreased grip strength, increases the risk of developing depression over

time. Zhang et al. [124] concluded that severe sarcopenia elevates the risk of depressive symptoms, particularly in women, with low gait speed being a key factor.

Not only elder, the middle-age Japanese individuals was observed a strong link between sarcopenia and depression [89]. Similarly, Both Heo et al. [125] and Kahl et al. [126] found that lower appendicular skeletal muscle mass was significantly associated with depressive symptoms in middle-aged men, but not in women. Muscle atrophy is potentially associated with depression in younger age groups, although there are no symptoms of sarcopenia. To support it, a Mendelian randomization study [127] suggested a bidirectional causal relationship between depression and sarcopenia-related traits, where depression is linked to lower muscle mass and decreased muscle strength, and vice versa. Fang et al. [128] found that in young individuals, body composition, specifically lower muscle mass and higher fat mass, is associated with increased depression, emphasizing the potential benefits of exercise and a healthy diet in reducing the risk of depression. A similar study reported that participants with poor lower muscle strength had around 2 times higher ratios of depressive symptoms [129]. Additionally, Ren et al. [130] discovered that among Chinese female college freshmen, higher handgrip strength is associated with a lower risk of depression, indicating that muscle strength may serve as an independent protective factor against depression.

Although public health and research interest in sarcopenia has burgeoned recently, the role of sarcopenia in the genesis and symptom severity of depression remains largely unexplored; however, no studies have examined the shared pathophysiological mechanisms for sarcopenia and depression that may potentially provide evidence for constituting the links between ageing skeletal muscle and depressive symptoms.

2.3.2. The association between sarcopenia and cognitive decline

Sarcopenia, is significantly associated with an increased prevalence of cognitive decline, with older adults exhibiting a higher risk compared to their non-sarcopenic counterparts [131]. Chen et al. [132] found that quadriceps strength is positively correlated with visuospatial and motor abilities in individuals aged 60 and above, highlighting the interconnected nature of muscle health and cognitive function. Taekema et al. [133] confirmed that diminished executive function is linked to a more pronounced decline in walking speed across all age groups. Xu et al [134] and Liu et al [135] suggested that higher

oxidative stress and inflammatory markers in sarcopenic elders contribute to cognitive impairment.

Kwak et al. [136] further established this connection by evaluating neural tracts in elders with sarcopenia. They observed decreased fractional anisotropy (FA) as well as fiber volume (FV) in eight neural tracts, with corticospinal tract FVs correlating with hand-grip power, and cingulum and fornix FVs correlating with memory function. These findings indicate significant deterioration of neural tracts in sarcopenic individuals.

A meta-analysis involving 26 studies and 18,788 participants found that older adults with sarcopenia face a markedly increased risk of cognitive impairment [9]. In this study, they revealed that Lower Mini-Mental State Examination (MMSE) scores in the sarcopenia group underscored correlation between muscle weakness and cognitive decline. This aligns with previous systematic reviews and highlights the importance of screening and preventing sarcopenia to enhance cognitive health and quality of life [137].

Animal studies also support this connection between muscle atrophy and cognitive impairment. In 5×FAD mice, a model of Alzheimer's disease (AD), a four-week administration of angiotensin II reduced gastrocnemius muscle fiber size and mass, correlating with cognitive impairment due to increased cortical amyloid-β deposition and inflammation [138]. In SAMP8 mice, a strain for age-associated pathologies, age-related kyphosis and spatial working memory decline paralleled progressive skeletal muscle dysfunction [139]. Research on 12-month-old APP/PS1 mice, another Alzheimer's model, showed that elevated myostatin levels in the gastrocnemius muscle correlated with muscle atrophy and memory impairment. Attenuating myostatin expression improved grip strength, muscle mass, and memory performance, implicating the ubiquitin-proteasome pathway [140].

Even in non-aged animal models, muscle atrophy and cognitive decline are linked. Kim et al. [141] demonstrated that physical inactivity in mice resulted in cognitive deficits and depression disorders with reduced neurogenesis. Nemoto et al. [142] reported that tail suspension, which induces muscle atrophy, accelerated neurocognitive disorders and reduced the population of Ki-67^+/DCX^+ cells in the hippocampus, indicating impaired neurogenesis. These findings underscore a significant correlation between muscle atrophy and cognitive impairment, though further research is required to explore the detailed mechanisms.

2.3.3. The effects of exercise on sarcopenia and depression

Lifestyle interventions, particularly exercise and physical activity, are widely recognized as foundational treatments for sarcopenia in the elderly. Systematic reviews have consistently demonstrated that exercise can significantly improve muscle strength (such as handgrip and knee extension), and physical performance measures (like gait speed and mobility) among older adults with sarcopenia [12]. These findings are crucial as they suggest that exercise can enhance the functional abilities of older adults, potentially reducing the risk of falls and improving their quality of life [143–146].

Among the various forms of exercise, resistance training has been highlighted as particularly effective. It has been shown to be superior to other types of exercise, such as mixed training (combining resistance, balance, and aerobic exercises) [147] and whole-body vibration training [148], in improving mobility outcomes. Resistance training is recommended as a primary intervention to mitigate the detrimental effects of sarcopenia [149]. Furthermore, resistance training has been shown to improve knee extension strength, which is essential for physical activity such as standing up from a seated position and climbing stairs [150,151]. Usual gait speed, another critical factor for independence, has also been improved through resistance training, as well as balance and agility, which are vital for preventing falls.

While the individual benefits of exercise on sarcopenia and depression are well-documented, the exercise effects on populations suffering from sarcopenia and depression are of particular interest. However, our screening finds only one study investigated the physical exercise effects on depression in Korea sarcopenia subjects with Alzheimer's disease (AD) [152]. The study included 40 elderly women with mild AD and sarcopenia, who were randomly allocated to either an exercise intervention group or a control group. The intervention comprised resistance training sessions conducted three times per week over a 12-week period. The findings were significant: participants in the exercise group demonstrated notable improvements in depression scores, muscle strength, and gait speed. These results underscore the potential of resistance training as a dual-purpose intervention, effectively mitigating depressive symptoms while simultaneously enhancing muscle strength in elderly patients with both sarcopenia and AD. The study provides compelling evidence for incorporating resistance exercise into therapeutic regimens for this vulnerable population, highlighting its role in improving both physical and mental health outcomes.

Even without direct evidence from exercise interventions, the role of physical activity in alleviating depression in patients with sarcopenia was also found. The path analysis model used in the study [153] demonstrated that lower levels of physical activity in 26.2% of the sarcopenia participants, measured by the international physical activity questionnaire (IPAQ), were associated with higher depressive symptoms, suggesting that enhancing quality of life and addressing malnutrition may alleviate depression in sarcopenic elderly. A study showed that Korean older adults with insufficient physical activity had 1.201 times higher ratio of depression symptoms compared to those with sufficient physical activity [129]. Additional study showed the low levels of physical activity were significantly associated with sarcopenia among the Thai older, with an odds ratio of 1.96 [154]. These studies emphasize the importance of regular exercise in potentially mitigating sarcopenia and its related depressive symptoms.

Myokines, a group of cytokines and peptides produced by muscle fibers during contraction, have emerged as key mediator in the crosstalk between muscles and various organs, including the brain [155]. These molecules play a role in biological functions from modulating metabolism to influencing cognitive processes [156,157]. The secretion of myokines is induced by physical activity, leading to autocrine, paracrine, and endocrine effects that can impact not only the muscle itself but also distant tissues and organs [158]. Among the myokines, interleukin 6 (IL-6), cathepsin B, and irisin have been particularly noted for their association with muscle and brain health. For instance, irisin, which is released from skeletal muscle during physical exercise, has been found to induce adult hippocampal neurogenesis [159] and also play a role in energy homeostasis [160]. During exercise, IL-6 is secreted by muscles and acts as a myokine that can increase up to 100-fold in the bloodstream [161], potentially aiding in immune response and reducing inflammation [162,163]. It also contributes to the brain by possibly downregulating neuroinflammation and protecting against injury [164,165], with its production linked to the duration of exercise rather than body temperature changes [166]. L-lactate, another myokine produced during physical exercise, serves as an energy source for neurons and promotes brain health by increasing VEGF-A expression [167] and supporting synaptic function [168]. It enhances neuroplasticity and memory [169], and regular exercise-induced lactate contributes to mitochondrial biogenesis and metabolic adaptability [170,171].

The dysregulation of myokine secretion, particularly irisin [172], BDNF [173], and FGF21 [174,175], has been correlated with the progression of depressive disorders in older

adults. This suggests a potential target for treating depression, especially in the context of sarcopenia. However, although over 600 myokines have been identified in recent studies [176], their bioactivity and the full extent of their physiological roles are yet to be comprehensively understood. Understanding the dynamic changes in myokine levels and their interactions with the brain following muscle-strengthening exercises could pave the way for developing interventions that address both muscle aging and depressive symptoms. Such research could lead to novel treatment protocols that leverage the muscle-brain axis to improve mental health.

2.4. Apelin: a sarcopenia-related myokine that mediates the muscle-brain axis

2.4.1. Functions of Apelin and its receptor APJ

Apelin, initially identified as an endogenous neuropeptide in stomach of bovine by Tatemoto et al. [177], plays a crucial role in various physiological processes through its interaction with the apelin receptor (APJ), a G protein-coupled receptor encoded by the APLNR gene. The human APLN gene, located on the X chromosome at Xq25-q26.1, encodes a 77-amino acid precursor peptide known as pre-pro-apelin.

Following enzymatic cleavage, pre-pro-apelin generates several bioactive fragments ranging from 12 to 55 amino acids, each exhibiting distinct receptor binding affinities and effects on APJ receptor trafficking [178–180] (Fig. 2.2). Among these fragments, apelin-13 is recognized as the most potent activator of APJ, compared to apelin-17 and apelin-36, along with other shorter variants [181]. The differential activities of these apelin isoforms highlight their diverse roles in modulating cardiovascular function, fluid homeostasis, energy metabolism, and neuroendocrine signaling [182,183].

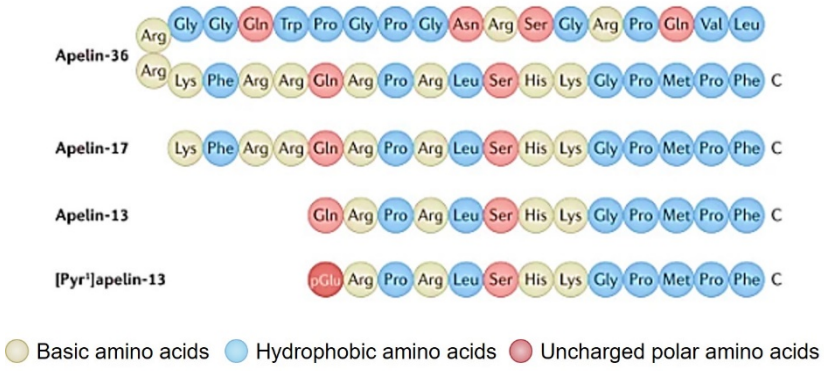

Figure 2.2 Distinct amino acid sequences of apelin isoforms' structures.

Apelin-36: The longest isoform, consisting of 36 amino acids. Key features include a sequence of glycine (Gly) residues interspersed with proline (Pro) and arginine (Arg) residues. Apelin-17: An isoform consisting of 17 amino acids. It starts with lysine (Lys) and contains a mix of hydrophobic and polar residues. Apelin-13: This shorter isoform contains 13 amino acids and has a similar sequence to the C-terminal portion of Apelin-17. [Pyr1] apelin-13: A modified form of Apelin-13, with a pyroglutamate (pGlu) at the N-terminus. Each circle represents an amino acid, color-coded based on its properties: basic amino acids (blue), hydrophobic amino acids (tan), uncharged polar amino acids (pink), and others as specified in the legend. Figure is adapted from Chapman et al. [184].

The apelin receptor (APJ), encoded by a gene on chromosome 11, exhibits approximately 50% structural homology with the angiotensin II type-1 receptor (AT1), despite lacking affinity for angiotensin II [185,186]. Beyond its interaction with apelin, APJ serves as a receptor for another hormonal peptide known as Apela/Elabela/Toddler, prominently expressed in human kidneys and pluripotent cells [187,188].

Apelin and its receptor APJ are extensively distributed throughout the body, exerting functions beyond the CNS in various peripheral tissues [189,190]. In the CNS, apelin influences synaptic plasticity and neuroprotection. Additionally, the apelin/APJ signaling pathways are integral to numerous physiological processes, highlight apelin/APJ as crucial regulators across multiple organ systems, underscoring their significance in both health and disease contexts [191–194].

APJ activates diverse intracellular signaling cascades through interactions with various G proteins ($G_{\alpha i}$, $G_{\alpha q}$, $G_{\beta\gamma}$; Fig. 2.3) [195,196]. $G_{\alpha i}$-mediated signaling pathways involve phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and protein kinase C (PKC)/extracellular signal-regulated kinases 1 and 2 (ERK 1/2), influencing processes such as apoptosis, cell proliferation, neuroinflammation, and oxidative stress. Additionally, $G_{\alpha i}$ signaling inhibits cyclic adenosine monophosphate (cAMP) production via protein kinase A (PKA) downregulation [197]. $G_{\alpha q}$ activation leads to phospholipase C beta (PLCβ) stimulation, resulting in diacylglycerol (DAG) and inositol trisphosphate (IP3) production, which initiates the PKC cascade and intracellular calcium release [198].

Apelin/APJ signaling exhibits heterogeneity by interacting with multiple G proteins, resulting in diverse effects across different tissues and cells [199]. Apelin and its isoforms, as well as toddler, can activate different signaling cascades depending on the context [200]. Beyond these interactions, APJ can form heteromers with other receptors such as AT1, κ-opioid, and bradykinin 1, influencing downstream signaling pathways in complex ways [201]. The biological effects mediated by APJ are intricately linked to the coupling preferences of $G_{\alpha i}$ and $G_{\alpha q}$ proteins, where $G_{\alpha i}$-mediated pathways, such as ERK phosphorylation, are notably activated by apelin-13, which exhibits specific binding affinities favoring $G_{\alpha i2}$ coupling [202,203]. Current evidence on preferential APJ signaling is limited, but it is suggested that different conformations of activated APJ, induced by various apelin isoforms, may result in distinct functional outcomes [204].

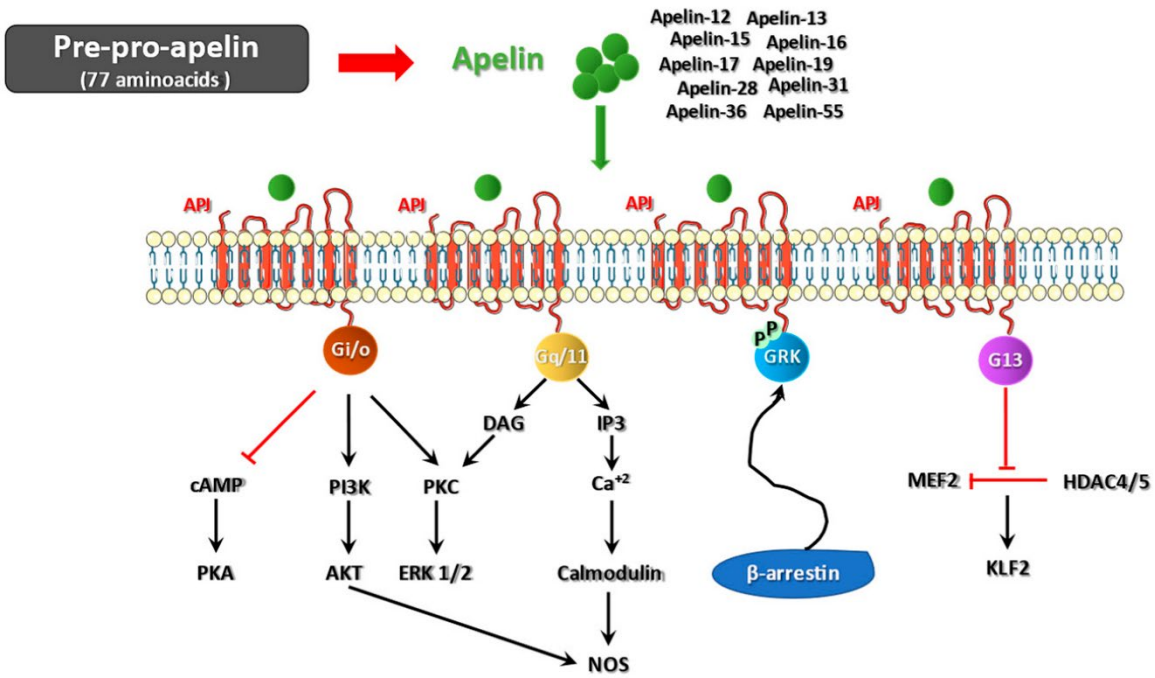

Figure 2.3 Apelin signaling pathways with APLNR (APJ) activation.

$G_{i/o}$ Pathway: Apelin binding to APLNR activates the $G_{i/o}$ protein, inhibiting cAMP production and activating PI3K, PKA, and AKT. Additionally, extracellular signal-regulated ERK 1/2 contributes to cellular responses. $G_{q/11}$ Pathway: Apelin binding activates $G_{q/11}$ protein, leading to phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis into DAG and IP3. DAG activates PKC, while IP3 triggers intracellular Ca^{2+} release, interacting with calmodulin and activating nitric oxide synthase (NOS). G13 Pathway: Apelin binding activates G13 protein, influencing transcription factors myocyte enhancer factor 2 (MEF2) and Kruppel-like factor 2 (KLF2) by inhibiting histone deacetylases 4 and 5 (HDAC4/5). G protein-coupled receptor kinase (GRK) and β-arrestin participate in receptor internalization and signal transduction modulation. Figure is adapted from Fibbi et al.[205].

2.4.2. Effects of physical exercise on the apelin/APJ pathway

Several clinical trials have reported that physical exercise does not increase the levels of apelin in the blood. After signal boat running [206], as well as high intensity cycling sprints [207], levels of apelin were raised in healthy subjects. Conversely, neither maximal nor submaximal treadmill change apelin levels in healthy participants [208]. Additionally, a notable decrease in apelin was recorded post-marathon [209], endurance exercise [210], and long-term high-intensity training [211,212] (Table 2.1). The observed variations in research

outcomes may stem from the disparate methodologies of physical activity employed, the duration of the exercise, time point of sample collection, and the athletic proficiency of the participants. Therefore, the effect of exercise on apelin in metabolism requires further research.

Loss of apelin signaling results in premature aging and sarcopenia, indicating its importance in muscle maintenance. Recent studies found that a high intensity interval training (HIIT) improved skeletomuscular hypertrophy via elevated mitochondrial function through apelin/APJ system [213]. Exercise-induced elevation of plasma apelin levels is associated with muscle strength and exercise capacity, highlighting the protective effects of apelin in muscular health [17]. For athletes, apelin levels have been observed to increase transiently after a single bout of exercise. This suggests that apelin may contribute to muscle protection by enhancing peak athletic performance and possibly aiding in recovery and adaptation to physical stressors [206]. On the molecular level, exercise induces the levels of apelin in muscle tissue, which activate signaling pathways that promote muscle growth and protection. This involves the activation of AMPK and PGC-1α, which play roles in mitochondrial biogenesis and energy metabolism, processes that are essential for muscle health and protection [214].

The protective role of maternal exercise on offspring is mediated by the apelinergic system. Studies indicate that sedentary maternal conditions can detrimentally impact offspring skeletal muscle development by downregulating apelin signaling and impairing mitochondrial biogenesis, highlighting the importance of physical activity during pregnancy for proper muscle development [215]. Increased circulating apelin levels from maternal exercise enhance brown adipogenesis and protect offspring from high-energy diet-induced metabolic dysfunction [216]. Apelin also mitigates obesity-related placental dysfunction by promoting mitochondrial biogenesis, mimicking the beneficial effects of exercise on placental health [217]. Furthermore, maternal exercise activates muscle-based thermogenesis in offspring through the apelin-AMPK signaling pathway, suggesting lasting metabolic health benefits for the next generation [218]. Additionally, in diabetic mothers, maternal exercise combined with insulin glargine administration improves testicular function in offspring, indicated by increased apelin-13 and APJ expression [219]. These studies collectively emphasize the crucial role of maternal physical activity and apelin signaling in influencing the developmental and metabolic outcomes of offspring,

offering potential strategies for addressing metabolic disorders and enhancing fetal development.

For the intestinal homeostasis, exercise enhances the function of the intestinal epithelium by upregulating the apelin-APJ signaling pathway, which activates AMPK and stimulates mitochondrial biogenesis, leading to improved gut health [220]. For the cardiovascular protection, exercise training enhanced [221] and promoted myocardial hypertrophy [222], a response inhibited by apelin binding through β-arrestin. This suggests that exercise-induced increases in apelin could modulate receptor signaling and prevent pathological cardiac remodeling. Additional study demonstrated that apelin administration enhanced atrial conduction velocity and refractoriness, thereby reducing the susceptibility to atrial fibrillation in mice subjected to intense exercise [198]. Wang [223] found that chronic exercise helps prevent myocardial inflammation, apoptosis, and cardiac dysfunction after a stroke by maintaining APJ expression levels and reducing phosphorylated-STAT3 activation, thus protecting cardiomyocytes. This suggests that the brain also has a response to exercise through apelin-mediated cardiovascular protection. However, there is no additional evidence on how exercise protects the brain and central nervous system through apelin.

Table 2.1 Levels of apelin in the blood after exercise interventions

Participants	Gender (sample size)	Age (years)	Time points of the blood collection	Grouping and exercise intervention	Apelin levels change	References
Type 2 diabetes mellitus	♂ (68), ♀ (179)	64.10	48 hrs after any physical activity	Obese group (physical activity > 2 h/week, N = 69)	↑	224
				Overweight group (physical activity > 2 h/week, N = 126)	↑	
				Normal-weight group (physical activity > 2 h/week, N = 52)	↔	
Older obese females	♀ (30)	65.40	3 days before the first training, and 4 days after the last training	Descending stair walking for 12 weeks (N = 15)	↑	225
				Ascending stair walking for 12 weeks (N = 15)	↔	
Males profess-sional soccer players	♂ (58)	22.90	Pre, immediately post, and 30 min after the exercise	Maximum incremental treadmill running test	↑	206
Healthy obese young males	♂ (40)	27.08	48 hrs after the last exercise	8-week endurance exercise	↓	210

(*Cont'd*)

Amateur male runners	♂ (74)	40.90	4 hrs before, and 24 hrs and 72 hrs after the race	Marathon	↓	[226]
Over-weight and obese males	♂ (60)	37.01	Pre, and 48 hrs after the end of exercise	8 weeks resistance training (N = 15)	↓	[227]
		36.00		8 weeks resistance training + spirulina (N = 15)	↓	
Males with obesity	♂ (60)	27.60	12 hrs before and 72 hrs after the exercise	12 weeks high-intensity function training (HIFT, N = 15)	↓	[211]
				12 weeks of HIFT + spinach-derived thylakoid (N = 15)	↓	
Males with obesity	♂ (44)	27.00	12 hrs before the first exercise session and 72 hrs after the last session	12 weeks of CrossFit training	↓	[212]
Healthy subjects	♂ (7)	22.43	pre, post, 1 hr post and 24 hrs pos exercise	Aerobic exercise on treadmill	↔	[208]
	♀ (5)	23.20				

↑ increased; ↓ decreased; ↔ no changed.

2.5. Potential roles of the apelin and APJ in the CNS.

APJ undergoes significant post-translational modifications, which profoundly impact its function and localization. Phosphorylation and glycosylation influence APJ's turnover rate and functional properties [228]. Within the central nervous system (CNS), apelin and APJ are expressed in various cell types, such as neurons, oligodendrocytes, and astrocytes, indicating diverse roles in neuronal signaling and support functions [229]. Apelin is widely distributed throughout the brain, with notable presence in regions critical for neurological functions [18,230], highlighting the potential role of apelin in a wide array of brain functions, from motor control and sensory processing to emotional regulation and cognitive processes.

The apelinergic system is increasingly recognized for its involvement in various neurological disorders. Although the mechanism by which apelin crosses the blood-brain barrier remains unclear, its ability to do so underscores its therapeutic potential [204].

Apelin-13, a specific isoform of the apelin peptide, exhibits potent neuroprotective properties in experimental models of cerebral ischemia/reperfusion injury [231], AD [232], and Parkinson's disease (PD) [233]. Remarkably, apelin-13 has been shown to rescue dopaminergic neurons, highlighting its potential as a therapeutic agent [234,235]. Therefore, the apelin and APJ are being extensively investigated for its role in regulating neuronal function and its therapeutic effects of neurological disorders [27].

2.5.1. Synaptic plasticity

Apelin-13 exerts neuroprotective effects by enhancing neuronal synaptic plasticity. In Parkinsonism rats, apelin-13 not only attenuates motor impairments but also prevents alterations in synaptic plasticity-related molecules, including postsynaptic density-95 (PSD95) and glutamate receptor 1 (GluA1), within the striatum, highlighting the effects in maintaining synaptic integrity [236]. This protective role of apelin on synaptic plasticity is also observed in the hippocampus of Parkinsonism rats, where it prevents impairment of early LTP (E-LTP) and upregulates the PSD-95 and dopamine receotor1 in hippocampal synaptosome extraction [237]. In the Alzheimer's disease rat model, apelin has been showed to mitigate neuroinflammation and alleviated learn and memory deficits via activating the molecular signaling cascade of the BDNF-tropomyosin receptor kinase B (TrkB) [238]. Furthermore, in a study where cognitive deficits and neuronal loss were

induced by scopolamine, apelin-13 provided protection against these deleterious effects, highlighting its potential therapeutic role in neurodegenerative diseases [239]. Collectively, these findings suggest the potential role of apelin in enhancing synaptic plasticity and providing neuroprotection in different models of neurodegeneration. However, the specific mechanisms require further investigation.

2.5.2. Excitotoxicity

Excitotoxicity, which is a form of neuronal injury induced by overactivation of receptors for the excitatory neurotransmitter glutamate, such as NMDA receptors [240]. Research indicates that apelin safeguards hippocampal neurons from the human immunodeficiency virus (HIV)-induced excitotoxic damage and modulates NMDA receptor activity, thereby mitigating the harmful effects of excitotoxic injury [241,242]. A deficiency in apelin can accelerate the progression of neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), highlighting its importance in neuronal health [243]. In the retina, apelin prevents NMDA-induced neuronal death through activating the Akt/ERK1/2 signaling pathways and inhibiting the expression of the TNF-α [244]. Administration of an APJ agonist also prevents the loss of retinal cells following NMDA exposure [245]. Additionally, the apelin and APJ signaling pathway has been implicated in modulating seizure activity and the endocytosis of the NMDA receptor GluN2B subunit, further underscoring its potential as a therapeutic target for neurological disorders characterized by excitotoxicity [246]. Collectively, these findings indicate apelin's potential as a therapeutic agent in various neurological conditions.

2.5.3. Apoptosis

Apelin, particularly in the form of apelin-13, exhibits robust anti-apoptotic properties in neurons [247], as demonstrated in mouse cortical neuronal cultures and *in vivo* models [248,249]. Intracerebroventricular injection of apelin-13 has been shown to significantly reduce ischemia and reperfusion injury by suppressing neuronal apoptosis. This anti-apoptotic effect is primarily mediated through the activation of the APJ/PI3K/Akt signaling pathway, as evidenced in PC12 cells [250]. The Akt pathway inhibits apoptosis by preventing procaspase activation, and alterations in mitochondrial

membrane potential [251–253]. The activation in the apelin/PI3K/Akt pathway thus preserves mitochondrial integrity and prevents cell death [254,255].

Moreover, the mitogen-activated protein kinase (MAPK) pathway is involved in apelin's anti-apoptotic effects. Specifically, apelin-13 upregulates the ERK1/2 pathway, promoted expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) [256]. This signaling cascade enhances cell survival under oxidative stress conditions by reducing the Bax/Bcl-2 ratio and suppressing caspase-3 expression [257]. Furthermore, apelin-13 has been demonstrated to decrease brain edema and suppress neuronal apoptosis through the downregulation of caspase-3 levels [258].

Preclinical evidence robustly supports the neuroprotective benefits of apelin-13, primarily by suppressing apoptosis [259]. *In vitro* studies have demonstrated that pre-treatment with apelin-13 diminished 6-hydroxydopamine (6-OHDA)-induced neurotoxicity as well as apoptosis by suppressing caspase-3 activation [233]. Furthermore, apelin-13 inhibits MPP^+-induced apoptosis by enhancing ERK1/2 phosphorylation without affecting p38 phosphorylation [260]. The activation of ERK1/2 via APJ coupling to the Gi2 protein, with phospho-ERK1/2 expression further enhanced by β-arrestin protein, underscores the specificity of apelin-13 in upregulating ERK1/2 [202,261].

Therefore, apelin-13 plays a critical neuroprotective role by suppressing apoptosis by activating the PI3K/Akt/ERK1/2 signaling pathways, highlighting its potential therapeutic applications in mitigating neuronal damage and neurodegenerative processes. This dual-pathway activation underscores the complexity and efficacy of apelin-13's neuroprotective mechanisms, making it a promising candidate for future research in the context of neurological disorders.

2.5.4. Autophagy

Apelin-13 has been shown to significantly increase AMPK phosphorylation in models of ischemic stroke, thereby contributing to its neuroprotective effects [262]. In the context of traumatic brain injury, apelin-13 appears to mitigate damage by modulating autophagy [263]. Specifically, *in vitro* studies demonstrate that apelin-13 activates autophagy by stimulating the AMPK/mTOR/ULK1 signaling pathway [264]. In H9C2 cells, apelin-13 promotes the development of autophagic vesicles and lysosomes, increases the expression of Beclin-1 and MAP1LC3BII/I, and stimulates the phosphorylation of AMPKα while

concurrently suppressing mTOR phosphorylation [265]. These actions underscore the essential role of apelin-13's interaction with APJ and the AMPK/mTOR/ULK1 pathway in its cytoprotective functions [195].

Conversely, apelin-13 has been observed to inhibit autophagy in specific contexts, such as in methamphetamine (METH)-induced PC-12 cells, where it reduces the expression of Beclin-1 and MAP1LC3II [250]. In models of ischemia/reperfusion injury, apelin inhibits autophagy by activating the PI3K/Akt/mTOR signaling pathway, highlighting its dual regulatory capacity [254]. *In vivo* evidence further supports these findings, with intranigral injection of apelin-13 enhancing autophagy and providing neuroprotection in MPTP-treated mice, as indicated by increased levels of MAP1LC3B-II and reduced p62 [266].

Overall, apelin exhibits a complex, heterogeneous role in autophagy regulation, demonstrating both promotive and inhibitory effects depending on the cellular and pathological context. This dual functionality suggests that apelin's modulation of autophagy is finely tuned and context-dependent, offering potential therapeutic avenues for a range of neurodegenerative and injury-related conditions. Further research is warranted to fully elucidate the mechanisms in apelin's bidirectional influence on autophagy and its broader implications for neuroprotection and cellular homeostasis.

2.5.5. Neuroinflammation

The research corpus elucidates the multifaceted role of apelin, particularly its bioactive fragment apelin-13, in modulating inflammatory responses across various pathological states. Studies by Yu et al. [267] and Xu et al. [268] establish a foundational understanding of apelin's correlation with inflammation, particularly in obesity-related type 2 diabetes and diabetic peripheral neuropathy. These findings collectively suggest that elevated apelin levels may serve as a biomarker for neuroinflammation and related neuropathological conditions.

Apelin-13 emerges as a potent inhibitor of neuroinflammatory damage, especially in ischemic conditions. Its ability to downregulate pro-inflammatory cytokines and curtail microglial activation within the ischemic penumbra is noteworthy [249,269]. This is supported by evidence of its efficacy in improving neurological outcomes post-stroke, as indicated by reduced brain edema and apoptosis [270,271]. The therapeutic potential of apelin-13 is

further underscored by its modulation of key inflammatory markers such as the glial fibrillary acidic protein (GFAP), indicating a broad-spectrum anti-inflammatory effect [249].

Cerebral ischemia/reperfusion injury, characterized by the overproduction of reactive oxygen species (ROS), highlights the critical role in the endogenous antioxidant defense system [272]. The activation of this pathway via the AMPK/Glycogen synthase kinase 3 (GSK-3β)/NRF2 axis by apelin-13 suggests a novel approach to mitigating ischemic stroke through both anti-inflammatory and antioxidant mechanisms [273]. Moreover, the involvement of the nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) in neuroinflammation and its modulation by apelin-13 provide deeper insights into the peptide's neuroprotective strategies [274,275]. Apelin's suppression of microglial activation and inhibition of key inflammatory pathways, including ERK and PI3K/Akt, underscore its potential effects for subarachnoid hemorrhage and related neuroinflammatory disorders [26].

In neurodegenerative diseases, apelin-13 suppress the expression of inflammatory factors and improve cognitive deficits [238]. The blockade of its effects by the TrkB receptor antagonist K252A further delineates the specificity of its action within the neuroinflammatory cascade [238]. In the broader context of depression, the role of apelin and APJ in mitigating stress and inflammation is well-documented [276,277]. The ability of apelin-13 to reverse lipopolysaccharide (LPS)- and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated neuroinflammation and regulate microglial polarization highlights its potential as a novel therapeutic approach for inflammation-associated mood disorders [275].

2.5.6. Oxidative stress

Apelin-13 has emerged as a significant neuromodulator with antioxidant properties. *In vivo*, it suppresses ROS generation, indicating its role as a neuroprotective agent [278]. Apelin-13 mitigates the harmful effects of chronic neurotoxin exposure, such as methamphetamine (METH) [250]. Furthermore, against ischemic and reperfusion-induced neuroinflammation and oxidative stress by activating the AMPK/GSK-3β/NRF2 pathway, which upregulates endogenous antioxidant enzymes [262].

Beyond its antioxidative actions, apelin-13 also exerts antidepressant effects and enhances cognitive functions such as recognition and memory. These benefits are

attributed to activate the PI3K/ERK1/2 signaling pathways in models of stress-induced depression and memory impairment [279]. *In vitro* studies further highlight the neuroprotective effects of apelin-13. It reduces ROS production and maintains mitochondrial membrane potential in SH-SY5Y cells exposed to 6-OHDA [233]. Additionally, in Aβ-treated SH-SY5Y cells, apelin-13 lowers intracellular ROS and Ca^{2+} levels [280]. These findings suggest that the antioxidant action of apelin-13 could be a viable approach for combating Alzheimer's disease. However, the precise mechanisms by which the apelin and APJ axis exert its antioxidant effects and modulates mitochondrial function are not yet fully elucidated.

2.5.7. Neuropathology in depression

Recent clinical investigations have elucidated the role of apelin in mood regulation and its association with depression. A pivotal study examined the influence of the apelin and APJ pathway on coronary heart disease (CHD) and its subsequent connection to depression and anxiety [281]. This research identified specific gene polymorphisms, specifically apelin gene (APLN) rs2235310 and APJ gene (APLNR) rs9943582, are associated with a heightened risk of CHD and depression. Moreover, the APLNR rs2282623 variant was linked to an increased anxiety risk among CHD patients. These genetic variations, associated with reduced apelin expression, may predispose individuals to depression and anxiety within the CHD patient population. Further inquiry into the role of apelin-13 in mood disorders revealed significantly lower serum apelin-13 levels in adolescents diagnosed with MDD compared to their healthy counterparts, indicating a potential association between apelin-13 and depression in the younger demographic [282]. Complementary findings from Türkiye demonstrated an inverse relationship between salivary apelin levels and dysfunctional attitudes among adolescents, with increasing apelin levels correlating with a decline in negative attitudes as age progressed [283]. These observations underscore the significance of apelin in emotional regulation during formative years.

In preclinical models, alterations in apelin and APJ expression have been implicated in depression-like behaviors. Historical data indicates that both acute and chronic restraint stress elevate APJ mRNA expression in the hypothalamus, accompanied by increased glucocorticoid levels, suggesting APJ's involvement in stress response modulation [284].

Recent studies have explored the effects of Xiaoyaosan, a traditional Chinese medicinal formulation, on depression-like behaviors in chronically stressed mice [22]. Findings revealed that stress-induced behavioral changes were associated with decreased apelin expression and increased APJ expression in the hypothalamus, effects that were ameliorated by Xiaoyaosan and fluoxetine treatment. This suggests that stress-mediated reduction in apelin levels may disrupt its interaction with APJ, potentially leading to compensatory receptor upregulation. Further research has indicated that stress-induced depression models exhibit promoted apelin and APJ expression in the hippocampus, highlighting its role in stress response via apelin [18,285]. Notably, targeting apelin-13 to the hippocampus alleviated depression-like behaviors in stressed rats, normalized stress-response system overactivity, and improved BDNF and glucocorticoid receptor functioning [18]. These findings propose that exogenous apelin administration could serve as a therapeutic intervention for depression.

Recent advancements have highlighted the antidepressant properties of exogenous apelin in animal stress models. Intracerebroventricular infusion of apelin-13 has been shown to mitigate depression-like behaviors and cognitive impairments in stressed rats, with comparable efficacy to the antidepressant imipramine [19,286]. The therapeutic benefits of apelin-13 are attributed to the activation of the PI3K and ERK1/2 signaling pathways [19]. Additional study indicated that apelin-13 reducing neuronal death and enhancing neuroprotective pathways in stress rats, specifically the AMPK/PGC-1α/ Fibronectin type III domain-containing protein 5 (FNDC5)/BDNF signaling cascade [287]. The antidepressant effects of apelin are posited to arise from its capacity to modulate neuroinflammation. Empirical evidence suggests that apelin treatment curtails depressive behaviors and inflammatory responses in rats co-treated with chronic stress and lipopolysaccharide, as evidenced by the subdued activating the microglia and astrocytes, alongside reduced concentrations of pro-inflammatory markers such as TNF-α [277]. S. Zhou et al. [275] has indicated that apelin-13 administration diminishes the expression of M1 microglial markers, concurrently elevating the expression of M2 markers and anti-inflammatory agents. These findings collectively propose that the antidepressant properties of apelin-13 may be mediated through its regulatory influence on microglial activity and neuroinflammatory processes.

Conversely, some studies have reported that apelin may induce depression-like behaviors. Elevated serum levels of apelin were identified as an independent predictor for

depression and anxiety in peritoneal dialysis patients (N=40) [288]. Additionally, intracerebroventricular administration of apelin-13 induced depression-like behaviors [271]. A recent study showed that higher plasma apelin levels in older adults correlated with increased depressive symptoms, while apelin knock-out mice (Apelin$^{-/-}$) showed varied responses based on age and diet [204]. 6-month Apelin$^{-/-}$ mice on a high-fat diet (HFD) exerted anxiety and insulin resistance, whereas 12-month Apelin$^{-/-}$ mice on HFD displayed an antidepressant-like state. These findings highlight the complexity of apelin's impact on mood, necessitating further research to elucidate its therapeutic potential for mood disorders.

2.6. Research questions and hypothesis

We therefore hypothesize that physical exercise increases myokine apelin levels to restore impairment in hippocampal neuroplasticity, hence elicits antidepressant effects. Because it is largely unknown whether (1) apelin directly affect hippocampal neuroplasticity (including adult neurogenesis, dendritic complexity and synaptic plasticity which is involved in antidepressant response); (2) whether myokine apelin is a key peripheral mediator for physical exercise to promote hippocampal neuroplasticity; (3) whether the influence of apelin on hippocampal neurogenesis or plasticity is indeed related to antidepressant effect of physical exercises. Identifying peripheral myokines responsible for antidepressant effects of physical exercise can provide significant insights on how exercise promotes brain health in humans and will pave the way for therapeutic discovery for rehabilitation.

2.7. Research Objectives

Objective 1: To investigate whether voluntary running and chronic stress alter apelin expression in the muscle and the brain.

Objective 2: To investigate whether myokine apelin is a key mediator for the antidepressant effects of physical exercise.

Objective 3: To investigate whether apelin elicits antidepressant effected by promoting hippocampal function.

Objective 4: To investigate whether knocking out myokine apelin accelerates cognitive memory impairment in ageing mice.

Objective 5: To investigate the signaling pathway of apelin in the hippocampus to alleviate depression-like behavior.

Chapter 3. General Methods and Materials

3.1. Experimental animal and study design

To establish a developing but are mature enough to exhibit stable physiological and behavioral responses model, post-weaning stage C57BL/6J male mice (4-6 weeks), were obtained from and housed at the Centralized Animal Facility (CAF) and CAF Shenzhen of the Hong Kong Polytechnic University. The mice were group-housed under standard conditions, maintained on a 12-hour light-dark cycle with *ad libitum* access to standard rodent chow and water. All experimental procedures involving animals were conducted in accordance with guidelines approved by the Animal Subjects Ethics Committee and were supervised by a licensed researcher. Ethical approval for the experimental protocols was granted by the institutional research ethics committee under references 20-21/172-12S-R and 21-22/200-RS-R, ensuring compliance with established ethical standards for animal research.

3.1.1. Experimental design for young male wild type mice subjected to a 4-week voluntary running protocol

To elucidate the potential role of apelin in the antidepressant effects of voluntary running, young adult male wild type (WT) mice (6-week-old) were subjected to a 4-week voluntary running regimen (Fig. 3.1). Mice of running (Run) group were housed in pairs with access to a shared running wheel, while their sedentary (Sed) counterparts were housed without a running wheel (n = 7-8 per group). Behaviors were evaluated using established paradigms such as the Open field test (OFT), Sucrose Preference Test (SPT), Splash Test (SST), and Forced Swim Test (FST). Additionally, separate cohorts of mice were sacrificed for the assessment of expression levels of apelin and APJ in fresh tissues. Serum samples were collected from all mice to quantify circulating apelin levels.

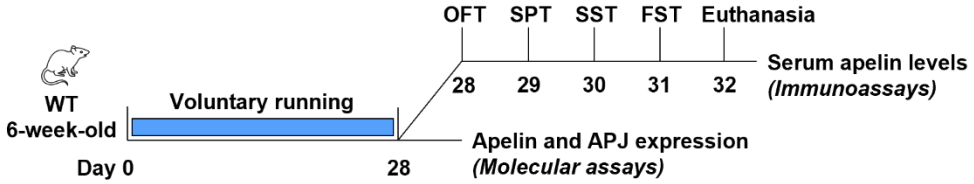

Figure 3.1 Experimental timeline for studying antidepressant effects of running in relation to expression of apelin and its receptor in the hippocampus.

Six-week-old WT male mice were subjected to behavioral assessments, including the OFT, SPT, SST, and FST, following a 28-day regimen of voluntary running. Additionally, separate cohorts of mice were used to collect fresh tissue samples for the quantification of apelin and APJ expression levels. Serum samples were also obtained to measure apelin levels via immunoassays.

3.1.2. Experimental design for young male stressed mice with voluntary running

To elucidate whether the antidepressant effects of a 4-week voluntary running regimen on depression-like behaviors induced by chronic unpredictable stress (CUS) are mediated by adult hippocampal neurogenesis, 6-week-old mice were treated with either 4 weeks of CUS (CUS group, n = 14), or 4 weeks of voluntary running concomitant with CUS (CUS+Exe group, n = 12), or maintained under sedentary conditions as a control (n = 10; Fig. 3.2). Behavioral assessments and molecular assays were conducted as previously described. Following behavioral tests, whole brains were harvested for immunohistochemical analysis. Additionally, muscle function was evaluated using the grip test (Fig. 3.2).

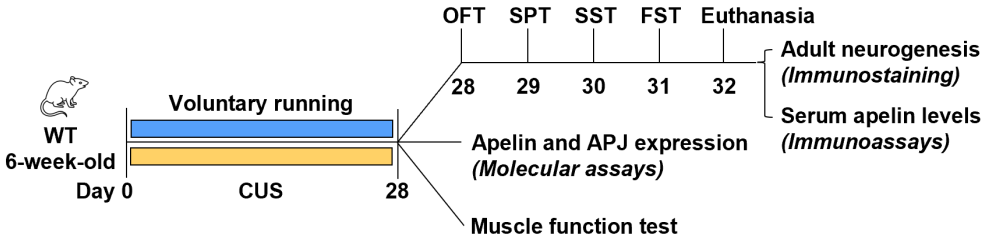

Figure 3.2 Experimental timeline for studying antidepressant effects and pro-neurogenic effects of voluntary running in stressed mice.

6-week-old WT mice were subjected to behavioral assessments, including the OFT, SPT, SST, and FST, following a 28-day regimen of voluntary running and CUS. Post-behavioral testing, whole brains were collected to examine adult hippocampal neurogenesis via immunostaining. Separate cohorts of mice were used to collect fresh tissue samples for the quantification of apelin and APJ expression levels. Serum samples were also obtained to measure apelin levels via immunoassays. Muscle function was assessed using the grip strength test.

3.1.3. Experimental design for muscle-specific apelin knockout mice subjected to voluntary running

To determine whether the antidepressant effects of running are mediated via apelin in skeletal muscle, we utilized 6-week-old male mice with muscle-specific knockout of apelin (APLN-mKO) and their Flox background counterparts (Flox). These mice were assigned to either a 4-week voluntary running regimen (APLN-mKO + Exe group, n = 8; Flox + Exe group, n = 9) or maintained under sedentary conditions as controls (APLN-mKO group, n = 8; Flox group, n = 7; Fig. 3.3). Behavioral tests and molecular analyses were conducted as previously described.

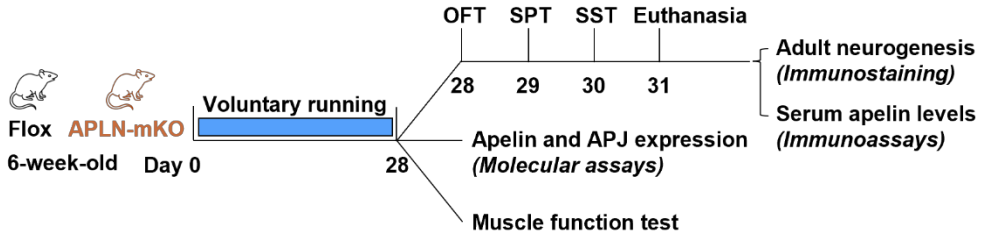

Figure 3.3 Experimental timeline for examining antidepressant effects of voluntary running in muscle-specific apelin knockout mice.

6-week-old APLN-mKO and Flox mice were subjected to behavioral assessments, including the OFT, SPT, and SST, following a 28-day regimen of voluntary running. Post-behavioral testing, whole brains were harvested to evaluate adult hippocampal neurogenesis through immunostaining. Separate cohorts of mice were utilized to collect fresh tissue samples for the quantification of apelin and APJ expression levels. Serum samples were obtained to measure apelin levels via immunoassays. Muscle function was tested by the grip strength test and the Rotarod test.

3.1.4. Experimental design for apelin overexpression in muscle of WT mice

To investigate the role of the myokine apelin in mediating antidepressant effects and promoting hippocampal plasticity, we utilized an adeno-associated virus (AAV) vector driven with the muscle creatine kinase (MCK) promoter to overexpress apelin with green fluorescent protein (GFP) in muscle tissue (AAV-APLN group, n = 7). Control mice received a vehicle virus expressing only GFP (AAV-GFP group, n = 8; Fig. 3.4). AAV-mediated GFP expression in muscle was confirmed through *in vivo* imaging. Behavioral tests and molecular analyses were performed following established protocols. Synaptic protein levels in hippocampal synaptoneurosome were

tested using western blot. Electrophysiological properties of hippocampal plasticity were tested by whole-cell recording.

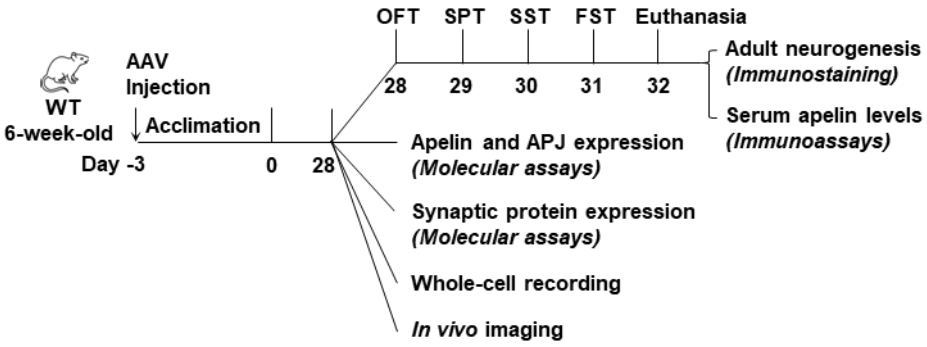

Figure 3.4 Experimental timeline for examining effects of muscle apelin overexpression on reducing depressive behavior and promoting hippocampal neurogenesis in adult mice.

6-week-old WT mice received intramuscular injections of AAV and were subsequently subjected to behavioral assessments, including the OFT, SPT, SST, and FST. Following these behavioral tests, whole brains were harvested to evaluate adult hippocampal neurogenesis via immunostaining. Separate cohorts of mice were used to collect fresh tissue samples for the quantification of apelin, APJ, and synaptic protein expression levels. Another group of mice was used to perform whole-cell patch-clamp recordings. Serum samples were obtained to measure apelin levels using immunoassays. AAV-mediated GFP expression in muscle was visualized through *in vivo* imaging.

3.1.5. Experimental design for apelin overexpression in muscle of mice with chronic unpredictable stress

To investigate whether overexpression of apelin in muscles replicates the antidepressant effects of running in mice subjected to CUS, 6-week-old WT male mice were treated with 4 weeks of CUS and intramuscular injection of AAV (Fig. 3.5). The group injected with AAV-APLN served as the CUS + AAV-APLN group (n = 12). Another subset, which received 4 weeks of voluntary running and was injected with AAV-GFP, served as the CUS + Exe group (n = 8). The remaining mice, injected with AAV-GFP and subjected only to CUS, served as the CUS group (n = 11). Mice in the control group did not receive CUS or running interventions but were injected with AAV-GFP (n = 10). Behavioral tests were conducted following established protocols.

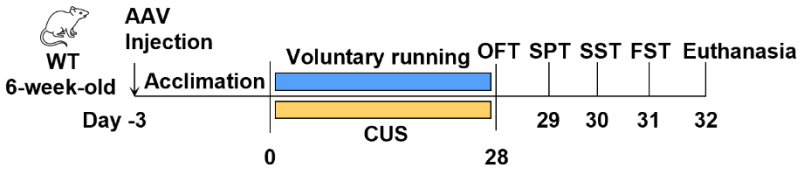

Figure 3.5 Experimental timeline for the effects of muscle overexpression of apelin in stressed mice.

Six-week-old WT male mice received intramuscular injections of AAV, CUS, and voluntary running, separately. Subsequently, these mice were subjected to behavioral assessments, including the OFT, SPT, SST, and FST.

3.1.6. Experimental design for APJ knockdown in glutamatergic neurons of the ventral hippocampus

To determine whether the antidepressant effects of running are mediated via APJ in glutamatergic neurons of the hippocampus, utilized AAV vector driven by the Cytomegalovirus (CMV) promoter, was used to express red fluorescent protein (RFP) along with miR30-shRNA targeting APLNR in the ventral hippocampus of calcium/calmodulin-dependent protein kinase II alpha subunit (Camk2a)-Cre mice. (AAV-shAPLNR group, n = 11). Control mice received a control virus expressing only RFP (AAV-RFP group, n = 12; Fig. 3.6). AAV-mediated RFP expression in ventral hippocampus was confirmed through immunofluorescence. These mice were assigned to either a 4-week voluntary running regimen (AAV-RFP + Exe group, n = 12; AAV-shAPLNR + Exe group, n = 11) or maintained under sedentary conditions as controls. To assess the survival of newly formed cells, mice were injected with bromodeoxyuridine (BrdU, MedChemExpress, USA, HY-15910) i.p. at a dose of 50 mg/kg, dissolved in 0.9% saline, 3 days prior to running. Behavioral tests and molecular analyses were performed following established protocols. Synaptic protein levels in hippocampal synaptoneurosome were tested using western blot.

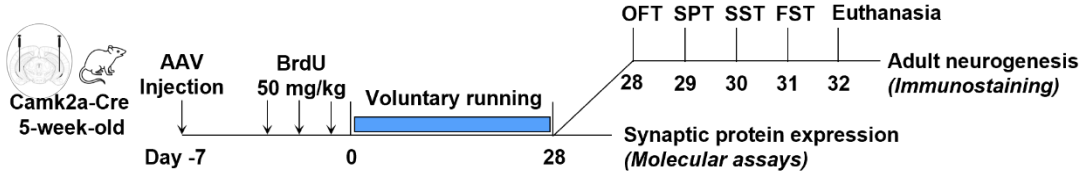

Figure 3.6 Experimental timeline for examining the antidepressant effects of voluntary running in mice with knockdown of APJ in hippocampal glutamatergic neurons

6-week-old Camk2a-cre mice received intra-ventral hippocampal injections of AAV and were subsequently subjected to behavioral assessments, including the OFT, SPT, SST, and FST. Post-behavioral testing, whole brains were harvested to evaluate adult hippocampal neurogenesis through immunostaining. Separate cohorts of mice were utilized to collect fresh tissue samples for the quantification of synaptic protein expression levels.

3.1.7. Experimental design for examining the aging effects of apelin knockout on muscle functions and cognitive learning and memory

To confirm whether muscle-specific knockout of apelin impairs age-related muscle weakness, cognitive deficits, and emotional dysregulation during aging, adult (6-month-old), middle-aged (12-month-old), and older (18-month-old) APLN-mKO mice were evaluated, using Flox background mice as controls (Fig. 3.7, n = 6 per group). Emotional behavioral tests and muscle function assessments were conducted following established protocols. Cognitive abilities were evaluated using the novel object recognition test (NORT) as well as the Y-maze test.

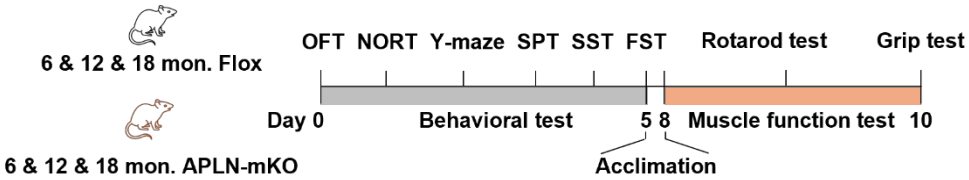

Figure 3.7 Experimental timeline for examining apelin knockout on muscle functions and cognitive learning and memory in ageing mice.

6-, 12-, and 18-month-old APLN-mKO and Flox male mice were subjected to behavioral assessments, including the OFT, NORT, Y-maze, SPT, SST, and FST. Following these behavioral evaluations, the mice underwent muscle function tests, including the Rotarod test, and Grip test.

3.2. Skeletal muscle-specific apelin knockout mice

3.2.1. Generation of APLN-mKO mice

All mice were provided by GemPharmatech Co., Ltd. (APLN-floxed: T009451; MCK-Cre: T036859) and maintained on a C57BL/6J background. To generate Apelin-floxed mice, we utilized the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology to modify the APLN gene, which has one transcript. According to the structure of the APLN gene, exons 1-3 of the APLN-201 transcript (ENSMUST00000039026.7) were targeted as the knockout region. This technique uses guide RNA to direct the Cas9 nuclease to the target gene, increasing homologous recombination efficiency and enabling precise genomic modifications.

We then established skeletal muscle-specific MCK-Cre mice. In these mice, Cre recombinase is driven by the MCK promoter, allowing Cre expression specifically in skeletal muscle tissue. Through breeding Apelin-floxed mice with MCK-Cre transgenic mice, we generated offspring carrying both MCK-Cre and APLN-flox alleles, leading to the development of skeletal muscle-specific knockout mice (APLN-mKO) (Fig. 3.8a). Since the APLN gene is located on the chromosome X, male apelin muscle-specific knockout mice have the genotype $APLN^{fl/Y}$ $MCK\text{-}Cre^{+}$, while control mice from the same litter without MCK-Cre expression are Apelin-floxed ($APLN^{fl/Y}$ $MCK\text{-}Cre^{-/-}$).

3.2.2. Genotyping

At postnatal 21 days (3 weeks old), offspring were separated from the mother's cage and group housed. At 4 weeks old, the offspring were ear-marked according to gender and ear punches (~2 mm^2) were collected from the pinna margins of external ear for DNA extraction. DNA samples were extracted from the ear punches using a lysis and neutralization protocol. Ear punches were added to the lysis buffer (30 µl per reaction) and incubated at 95°C for 30 minutes, followed by cooling at 4°C for 20 minutes. Neutralization buffer (30 µl per reaction) was then added.

The polymerase chain reaction (PCR) mixture contained the primers (2 µ) (Table 3.5), PCR master mix (10 µl, Vazyme Biotech, China, P112), and nuclease-free water (6 µl). Sample solution (2 µl) was added to the reaction mixture. The PCR protocol was as follows: Initial denaturation at 95°C for 3 minutes; followed by 35 cycles of

denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute; with a final extension step at 72°C for 5 minutes.

A 2% agarose gel (Sigma-Aldrich, USA, 1.01236) was prepared with 1× Tris-acetate EDTA buffer (TAE, 100 ml, Vazyme Biotech, China, TAE402) and Safe Green dye (10 µl, Biosharp, China, BS356A). The gel was submerged in an electrophoresis apparatus (Bio-Rad, USA, 1704401) filled with Tris-Acetate EDTA buffer. Amplified PCR products (7 µl) and the molecular marker (3 µl) were loaded into the gel wells. Electrophoresis was conducted at a voltage of 120V at least 30-40 minutes. The agarose gel was visualized using a Bio-Rad imaging system (SYBR Safe channel) to confirm the presence of specific bands.

The genotyping strategy depended on the primer design. For the APLN-floxed allele, primers were designed flanking the LoxP sites at the 5' and 3' arms (Fig. 3.8b). The analysis strategy is as follows: WT mice: PCR produced a single WT band. Heterozygote mice (female: $APLN^{fl/-}$): PCR produced both WT and targeted bands. Homozygote mice (male: $APLN^{fl/Y}$, female: $APLN^{fl/fl}$): PCR produced a single targeted band.

For the MCK-Cre allele, primers were designed flanking the 5' and 3' connectors (Fig. 3.8b). The analysis strategy is as follows: WT mice: PCR produced no bands. MCK-Cre Mice: PCR produced a single Cre band. For skeletal muscle-specific knockout of apelin (APLN-mKO), PCR produced both flox and MCK-Cre bands. The control group (Flox) produced only a flox band without the MCK-Cre band (Fig. 3.8c).

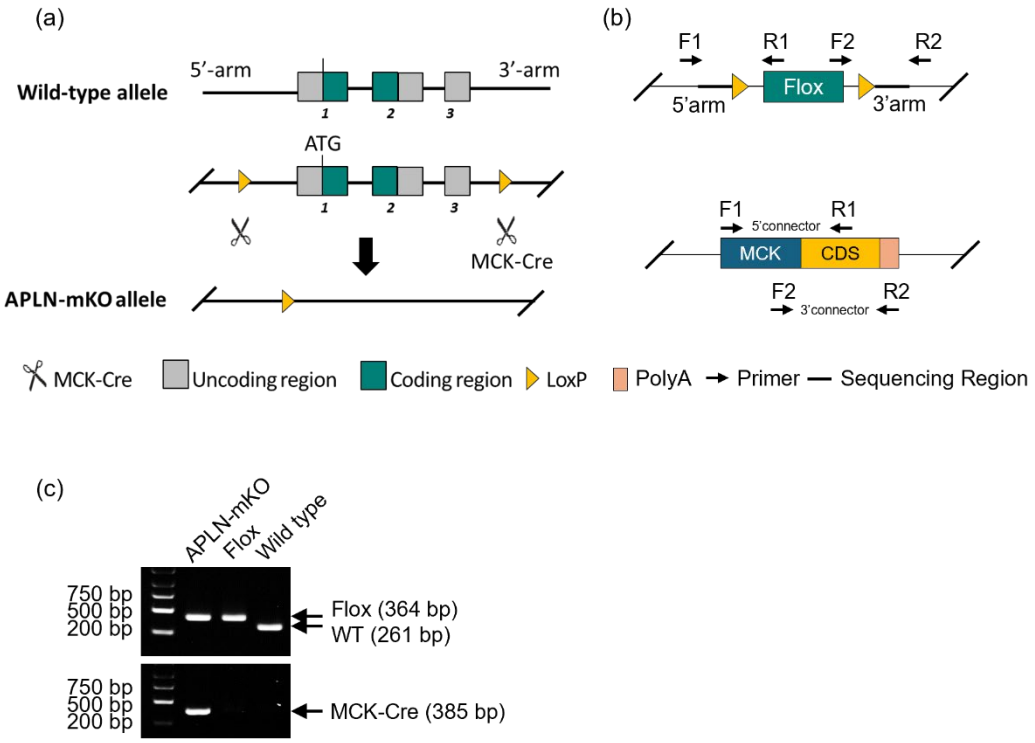

Figure 3.8 The animal model of skeletal muscle apelin knockout.

(a) Schematic representation of the APLN and WT alleles, including exons (numbered boxes), noncoding regions (gray boxes), and the APLN coding region (green boxes). The insertion sites of loxP upstream and downstream of exons 1-3 (yellow triangles) were targeted by Cre recombinase driven by the MCK promoter for apelin knockout. (b) Cre-mediated recombination of the floxed allele, indicating the positions of primers (F1, R1, F2, R2) used for genotyping. (c) Agarose gel electrophoresis results of genotyping among WT, Flox, and APLN-mKO mice.

3.3. Overexpression of apelin in the muscle by intramuscular injection of adeno-associated virus

3.3.1. Adeno-associated virus design

The adeno-associated virus in this experiment was provided by WZ Bio (WZ Biosciences Inc, China). The murine APLN gene sequence (NM_013912.4) was cloned into an adeno-associated virus type 1 (AAV1) vector to enable gene delivery and expression *in vivo* (Fig. 3.9a). The APLN gene was strategically positioned under the control of the MCK promoter to achieve specific expression in muscle tissue. Additionally, a secretion signal was incorporated to facilitate the extracellular release

of the apelin protein. To facilitate detection and quantification, the apelin protein was labeled with a FLAG-tag. This tagging enables precise immunodetection through FLAG-specific antibodies. The construct was designed to co-express apelin and green fluorescent protein (GFP) using the porcine teschovirus-1 2A (p2A) peptide (pAV-MCK-APLN-FLAG-(P2A)-GFP). The p2A peptide ensures equimolar expression of both proteins from a single transcript by promoting ribosomal 'skipping,' thereby allowing independent peptide release.

3.3.2. Intramuscular injection protocol

Six-week-old C57BL6/J mice were randomized into experimental and control groups. Each mouse was injected with 10^{11} viral particles of AAV1-APLN-GFP suspended in 50 µl of phosphate-buffered saline (PBS). Control mice were administered an AAV1 vector encoding a scramble peptide sequence in place of the apelin gene, denoted as AAV1-GFP-Ctrl. This control vector allowed for the differentiation between specific effects attributable to apelin expression and non-specific effects of the viral vector or injection procedure. In Chapter 4, having established that voluntary running enhances apelin expression in muscle, specifically in the gastrocnemius (Gas) and tibialis anterior (TA), we selected these muscle groups for apelin overexpression to investigate whether this could replicate the effects of voluntary running. Injections were precisely administered into muscles bilaterally to ensure consistent gene delivery and expression in these major muscle groups. The experimental protocol allowed for a four-week period post-injection, during which the expression of the apelin gene and its physiological impacts were monitored.

The preparation for intramuscular injections began with the induction of anesthesia in the mice. Anesthesia was administered via inhalation of isoflurane using a stereotaxic apparatus, ensuring precise and consistent delivery. Following the induction of anesthesia, the skin overlying the tibialis anterior, and gastrocnemius muscles were carefully shaved to remove hair and then disinfected with 70% ethanol to minimize the risk of infection during the injection procedure. The working solution (AAV in PBS, 10^{11} particles) was then intramuscularly injected into the mid-belly of the TA (12.5 µl) and the two bifurcated parts of the mid-belly of the Gas (6.25 µl per bifurcation section) using an insulin syringe (30 gauge, BD, USA, BHI1308) (Fig. 3.9b & c). The lower limb muscles on the opposite side underwent the same treatment (25 µl per side, 50 µl per mouse).

With inhalation of isoflurane (RWD Life Science, China, R510-22) administered to the nasal cavity using a stereotaxic, immediate post-injection observations included a mild swelling at the injection sites of the TA and Gas muscles, indicating successful administration of the viral solution. This swelling typically subsided within 30 minutes, suggesting that the injected volume and technique were well-tolerated by the muscle tissue. The mice were carefully monitored as they were returned to their home cages. Close observation ensured that the mice recovered smoothly from the anesthesia and the injection process, maintaining their health and well-being throughout the experimental period.

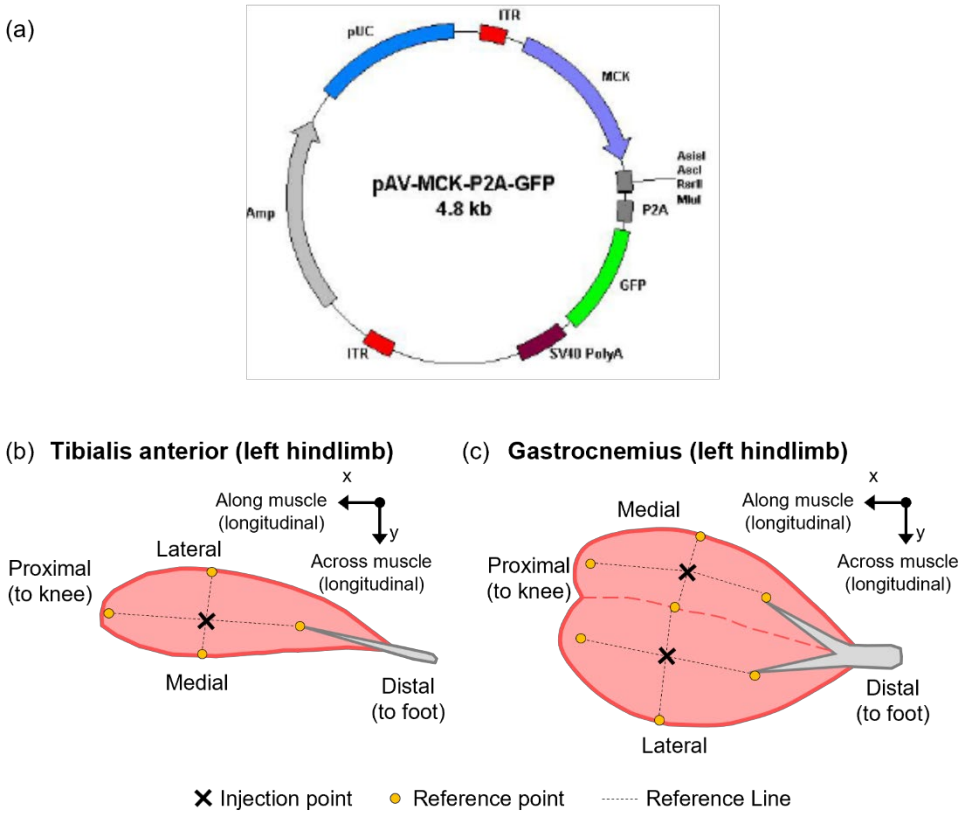

Figure 3.9 Plasmid vector construction for AAV and the schematic of intramuscular injection of AAV on TA and Gas muscle.

(a) Construction of the AAV plasmid for muscle-specific overexpression of apelin. (b) TA and (c) Gas muscle of the left hindlimb labeled the injection point.

3.3.3. Validation of intramuscular injections

To verify the specificity of the viral delivery to the TA and Gas muscles, a control injection of 0.4% Trypan Blue dye was performed in a 6-week-old WT mouse. The dye was injected into the TA and Gas muscles, and the absence of staining in the extensor

digitorum longus (EDL) and soleus (Sol) muscles after one day confirmed the targeted delivery and specificity of the injections to the intended muscle groups (Fig 3.10 a-f).

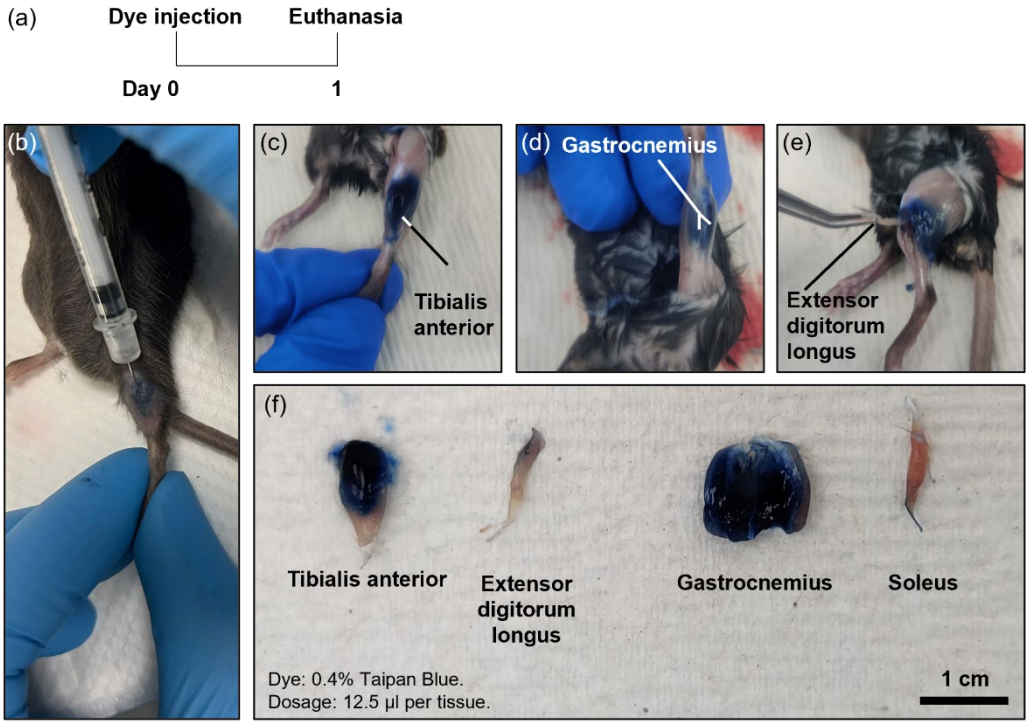

Figure 3.10 Validation of intramuscular injection in the gastrocnemius and tibialis anterior muscles.

(a) Timeline for the experiment. (n = 1) (b) Intramuscular injection of 0.4% Taipan Blue dye into the TA muscle. (c) Presence of 0.4% Taipan Blue dye in the TA muscle, (d) Gas muscle, but not in the (e) extensor digitorum longus (EDL) muscle 24 hours post-injection. (f) Comparison of dye distribution in excised muscles: TA, EDL, Gas, and soleus (Sol).

3.4. Knockdown of APJ in glutamatergic neurons of the ventral hippocampus

3.4.1. Generation and genotyping of Camk2a-Cre mice

Camk2a-Cre mice (005359, B6.Cg-Tg(Camk2a-cre)T29-1Stl/J) with a C57BL/6J background were obtained from the Jackson Laboratory. In these mice, Cre recombinase expression is driven by the Camk2a promoter, enabling specific Cre activity in Camk2a-expressing neurons [289].

The genotyping protocol is the same as above for APLN-mKO mice. For the Camk2a-Cre allele, primers were designed flanking the 5' and 3' connectors (Fig. 3.11a). The analysis strategy is as follows: WT mice: PCR produced no bands. Camk2a-Cre Mice: PCR produced a single Cre band with (Fig. 3.11b).

3.4.2. Adeno-associated virus design

The adeno-associated virus (AAV) used in this experiment was provided by WZ Biosciences Inc., China. The mir30RNA sequences targeting the mouse APLNR gene (NM_011784.3) were inserted into the AAV9 vector pAV-CMV-DIO-RFP-mir30RNA (Fig. 3.11c). This DIO vector is Cre-dependent, with the Cytomegalovirus (CMV) promoter driving the expression of red fluorescent protein (RFP) and mir30RNA in the presence of Cre recombinase (Fig. 3.11d). In the absence of Cre expression, the RFP-mir30RNA construct remains inactive.

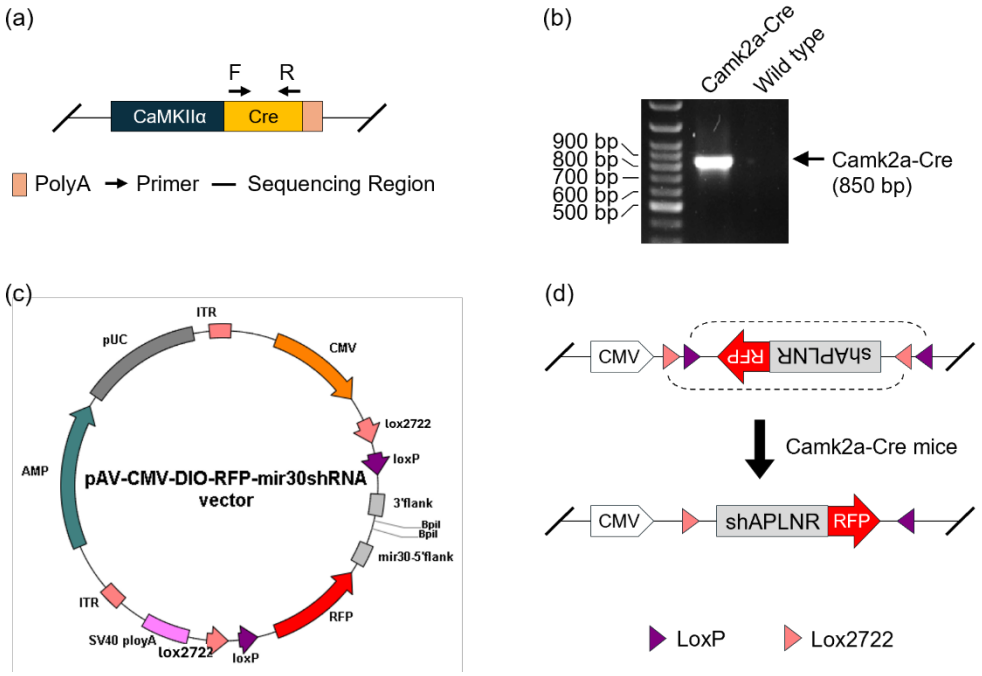

Figure 3.11 The animal model of APJ knockdown in Camk2a-Cre mice

(a) Schematic representation of the Cre-mediated recombination allele, showing the positions of the forward (F) and reverse (R) primers used for genotyping. (b) Agarose gel electrophoresis results in demonstrating the genotyping outcomes for wild-type (WT) and Camk2a-Cre mice. (c) Construction of the AAV plasmid for APJ knockdown in Camk2a-Cre mice. (d) Experimental design for Cre-dependent APLNR gene knockdown via AAV transduction in Camk2a-Cre mice.

3.4.3. Stereotactic injection protocol

Mice were anesthetized using an isoflurane anesthesia system (RWD Life Science, China, R650) with an initial concentration of 4% and a flow rate of 1 L/min. Upon confirmation of full anesthesia, the mice were positioned in a neonatal mouse adaptor (RWD Life Science, China, 68030) and secured on a brain stereotaxic apparatus (RWD Life Science, China, 68803). Their mouths were placed within an anesthetic mask, and the isoflurane concentration was adjusted to 0.5–1% to maintain anesthesia during the procedure. The fur between the eyes and behind the ears was shaved, and anesthesia depth was verified by the absence of a response to a toe pinch. This check was repeated every 20 minutes throughout the surgery. The head was cleaned with a cotton swab dipped in iodophor, and erythromycin eye ointment (Baiyunshan, China, BM-EEO-25G) was applied to protect the eyes from light exposure.

A midline incision was made between the eyes and the back of the skull, and the incision site was cleaned using a saline-moistened cotton swab. The mouse incisors were secured on the tooth rods of the adaptor, the anesthetic mask was repositioned, and ear rods were inserted into the cochlea with equal graduation marks on both sides. The skull surface was cleaned and dried. A 25-gauge needle (BD, USA, 301805) mounted onto the stereotaxic apparatus using a needle holder (RWD Life Science, China, 68218) was dipped in black ink to mark auxiliary points for guidance.

The cranial level was adjusted using a glass electrode tip, which touched the bregma point (Fig. 3.12a) set as the origin. The height differences between defined cranial points were measured and kept below 0.05 mm to ensure proper alignment. Adjustments to the ear bars and nose clip were made as necessary. Based on the Mouse Brain Atlas, the injection coordinates for the ventral hippocampus were determined as AP: -3.0 mm, ML: ±3.3 mm, and DV: -3.6 mm, consistent with previous publications (Fig. 3.12b). The target location was marked with the inked needle, and a Φ0.5 mm cranial drill (RWD Life Science, China, 78040) was used to create a craniotomy at the marked site. Bleeding was controlled using a cotton swab and absorbable sponge, and the craniotomy site was covered with a saline-soaked sponge.

The needle holder was replaced with a microinjection pump (RWD Life Science, China, R480) fitted with a drawn glass electrode (Φ1.14 mm OD, RWD Life Science, China, GC-3.5). A virus suspension (1100 nL aspirated at 800 nL/min, with an estimated 10% loss) was withdrawn from the refrigerator and loaded into the electrode. The tip of

the electrode was positioned at the bregma origin point. After removing the saline-soaked sponge, the needle was aligned with the target site, lowered to the intended depth, and briefly advanced 0.3 mm deeper before being returned to the target position to promote viral diffusion. A total of 500 nL of AAV was injected at a rate of 100 nL/min. The syringe was left in place for 10 minutes post-injection before being withdrawn and repositioned for injection into the contralateral site. The craniotomy was again covered with a saline-soaked sponge.

After the injections, the wound was closed using absorbable surgical sutures (radian 3/8, length 18 mm, Jinhuan, China, F404). Povidone-iodine was applied to the suture lines and surrounding skin. The mice were removed from the stereotaxic apparatus and placed on a heating pad for recovery. Once fully conscious, they were transferred to individual cages and monitored daily for four days following surgery.

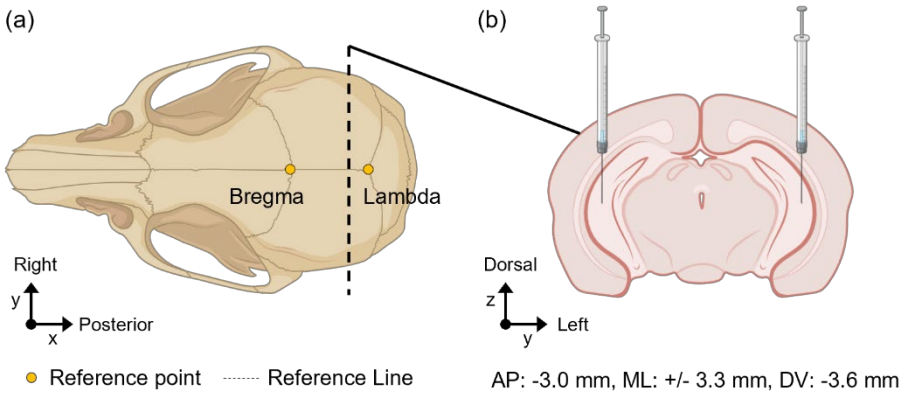

Figure 3.12 Schematic of intra-ventral hippocampal injection

(a) Dorsal view of the mouse skull sutures and stereotaxic surgery coordinates.

(b) Coronal section showing the injection site in the ventral hippocampus.

3.5. Voluntary running

As description in previous publication from our team [29,290,291], animals were group-housed. Following a 3-day acclimation period, mice in the running group were housed with two wheels (4 mice / 2 running wheels) for 4 weeks. The diameter of the running wheel is 14 cm, and magnets with a diameter of 5 mm were attached to the outside of the wheel. Wheels were fixed to the iron mesh cage cover, with the wheel at least 1 cm above the bedding. A magnetic detector (CKC TINNER) was attached outside the cage to record the number of wheel laps at 24hr intervals over the 28-day period.

3.6. Chronic unpredictable stress

The chronic unpredictable stress (CUS) procedure was performed on 6-week-old male mice housed in cages for a duration of 4 weeks (28 days) (Table 3.1). The protocol consisted of the following stressors:

(1) Restraint stress: Clean, sterilized 50 mL centrifuge tubes (Corning, 430291) were used, with 5 mm diameter holes drilled in the lid and bottom. Mice were restrained in the tubes, with their heads towards the bottom and tails through the lid holes. Once the mice were fully in the tubes, the lids were securely fastened without harming the mice. During restraint, the tubes were placed horizontally on the bedding in the home cage, ensuring the mice could breathe normally. After the stress period, the lids were carefully removed, and the mice were returned to their home cages.

(2) Inescapable foot-shock: Mice were placed in a mouse fear conditioning system (Med Associates, USA, VFC-008) for the administration of 0.5 mA shocks through the grid floor of the chamber. Each trial lasted 5 minutes, with shocks administered every 30 seconds, each lasting 1 second, totaling 9 shocks per trial.

(3) Exposure to the shock apparatus: On the day following the foot-shock trial, mice were returned to the same fear conditioning box for 1 hour without receiving shocks.

(4) Paired housing with intruder: Mice were housed individually and then paired with an unfamiliar male C57BL/6J of similar weight ($< \pm 2$ g) and age ($< \pm 2$ weeks) for 1 hour.

(5) Cage tilting: The cage was tilted at a 45-degree angle with food and water positioned at the elevated top, ensuring the mice could access them freely. The test duration did not exceed 24 hours.

(6) Wet bedding: 500 mL of water was poured onto 1 L of wood shavings bedding. The test duration did not exceed 24 hours.

(7) Food and water Deprivation: Food and water bottles were removed from the cage. The test duration did not exceed 24 hours.

(8) Empty bottle exposure: Following the 24-hour food and water deprivation, food and water bottles were returned to the cage, but the water bottle was empty. This test lasted for 1 hour. Afterward, the water bottle was filled and returned to the cage for normal drinking.

Table 3.1 The schedule of chronic unpredictable stress

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Restraint stress (0 .5 h)	Inescapable foot-shock (5 min – 2 mA)	Exposure to the shock apparatus (without shock-1h)	Paired housing (1 h)	Cage tilting (maximum 24 h)	Restraint stress (1 .5 h)	Inescapable foot-shock (5 min – 2 mA)
Week 2	Exposure to the shock apparatus without receiving shocks (1 h)	Moist bedding (maximum 24 h)	Restraint stress (2 h)	Food and water deprivation (maximum 24 h)	Exposure to an empty bottle (1 h)	Cage tilting (maximum 24 h)	Paired housing (1 h)
Week 3	Inescapable foot-shock (5 min – 2 mA)	Exposure to the shock apparatus without receiving shocks (1 h)	Moist bedding (maximum 24 h)	Food and water deprivation (maximum 24 h)	Exposure to an empty bottle (1 h)	Cage tilting (maximum 24 h)	Restraint stress (2 .5 h)
Week 4	Paired housing (1 h)	Inescapable foot-shock (5 min – 2 mA)	Exposure to the shock apparatus without receiving shocks (1 h)	Food and water deprivation (maximum 24 h)	Exposure to an empty bottle (1 h)	Moist bedding (maximum 24 h)	Restraint stress (3 h)

3.7. Behavioral test procedures

3.7.1. Sucrose preference test

As we previously performed [29,292] to assess anhedonia-like behavior, Individually housed mice were provided with both a bottle of drinking water and a bottle containing a 2% (w/v) sucrose solution for 24 hours. At the 12-hour mark, the positions of the bottles were swapped. The measurement of water and sucrose solution consumption was based on the change in weight of each bottle before and after the test. Sucrose

preference was calculated as the percentage of sucrose consumed relative to the total liquid intake, using the formula:

$$Sucrose\ preference\ (\%)=(\frac{Sucrose\ consumption}{Total\ liquid\ consumption})\times 100$$

3.7.2. Open field test

As previously performed [293,294], the open field test is employed to evaluate anxiety-like behavior and locomotor activity in mice. Animals were placed in the corner of an open field apparatus measuring 50 cm in length, 50 cm in width, and 40 cm in height, under illumination of 20-30 lux. Their activity was recorded using an automated monitoring system for 10 minutes. The apparatus was cleaned with 10% ethanol between tests. Data on the distance traveled and the duration spent in the center area were captured and analyzed using EthoVision XT (Noldus). The ratio of time spent in the center area to the total time spent was calculated for analysis.

3.7.3. Forced swim test

As previously performed [29,291–293], mice were placed in a Plexiglas cylinder (internal diameter = 10 cm; height = 20 cm) filled with water maintained at room temperature (depth = 13 cm). The test session lasted for 6 minutes, with swimming behavior analyzed during the final 4 minutes. Immobility time was recorded and analyzed using EthoVision XT (Noldus), and results were cross-checked by an observer in a blinded sample manner. Immobility was defined as floating on the water's surface while making only minimal movements to keep their heads above water.

3.7.4. Sucrose splash test

The sucrose splash test is utilized to assess anhedonia-like behavior in mice. Mice were individually housed in transparent cages and sprayed six times with a 10% sucrose solution on their dorsal fur [295]. The test duration was 5 minutes. Grooming behavior after splashing sucrose salutation will be recorded and analyzed in a sample blinded manner.

3.7.5. Y-maze task

Short-term spatial recognition memory is assessed using the Y-maze task [294]. The test was conducted with a Y-maze that has three opaque arms positioned at 120° angles to the other arm. Each identical arm (20 cm length × 6 cm width × 8 cm height)

was with a spatial visual cue of different symbols (△, □, or ×). A video recording device was placed directly above the Y-maze. The maze was covered with fresh bedding to facilitate mouse activity. The spatial memory test consisted of the training phase, and the testing phases.

(1) Training phase: Under dim light (20-30 lux), mice were placed in the Y-maze and allowed to explore freely for 10 minutes, with one arm blocked. This blocked arm was designated as the novel arm (N), the explored arm as the familiar arm (F), and the arm from which the mouse was introduced as the start arm (S).

Testing phase. After training, mice were returned to their home cages but kept separate from untested mice to prevent contact for 3 hours mice were then reintroduced to the maze from the start arm (S) and allowed to explore freely for 5 minutes. The bedding was replaced, and the maze cleaned with 10% alcohol and water between each mouse to remove olfactory queue.

During the test phase, the time spent on each arm was tracked and recorded using EthoVision XT (Noldus). The exploration ratio of the novel arm and the exploration index were calculated using the following formulas:

$$Exploration\ ratio\ in\ novel\ arm = \frac{N}{N + F}$$

$$Exploration\ index = \frac{N - F}{N + F}$$

where N is the time spent on the novel arm, F is the time spent on the familiar arm.

3.7.6. Novel object recognition test

The novel object recognition test (NORT) is employed to evaluate short-term object discrimination learning and memory [296]. The NORT was conducted under dim lighting (20-30 lux) in an open field apparatus. The objects used for exploration are of similar size and material, and free of distinct odors to avoid inherent preferences. To prevent climbing or sitting on the objects, they are smaller than mice and have vertical but non-sharp edges. In this study, we used cylindrical plastic empty containers and similarly sized but irregularly shaped plastic toys as exploration objects.

The NORT comprised four phases: the habituation phase, the training phase, the trial interval, and the testing phase. Two identical objects were used during the training phase, while one novel object and one familiar object were used during the testing phase.

The apparatus and objects were wiped with 10% ethanol between trials to remove excretions and scents left by the previous mouse.

(1) During the habituation phase, mice were placed in an open field apparatus for 15 minutes on the first day to acclimate to the environment.
(2) In the training phase on the second day, two identical objects (F1 and F2) were positioned in two corners on the same side of the apparatus, 6×6 cm from the corners. Mice, facing away from the objects, were released from the opposite side and allowed to explore for 10 minutes.
(3) During the trial interval, mice were returned to their home cages for 2 hours to prevent interaction with other tested mice.
(4) In the testing phase, two different objects (a familiar object, F, and a novel object, N) were placed in two corners on the same side of the apparatus, 6×6 cm from the corners. Mice, again facing away from the objects, were released from the opposite side and allowed to explore for 5 minutes.

The time spent exploring each object, defined as having the nose within a 2 cm radius of the object, was analyzed by EthoVision XT (Noldus). The exploration ratio on the novel object and the discrimination index between the objects during the test session were calculated as follows:

$$Exploration\ ratio\ on\ novel\ object = \frac{N}{N + F}$$

$$Discrimination\ index = \frac{N - F}{N + F}$$

where N is the exploration time on the novel object, F is the exploration time on the familiar object.

3.8. Skeletal muscle function test

3.8.1. Grip strength test

The grip strength test was employed for measuring forelimb strength. As previously described [291]. The procedure involved positioning each mouse to grasp a grid attached to a calibrated force gauge (KEW, Beijing, China) using its forelimbs. Once the mouse securely grasped the grid, it was gently pulled backward in a consistent manner. During each trial, the force exerted by the mouse on the grid was recorded in Newtons (N). To account for variations in body size and weight, the average force measurement from the five trials was normalized to each mouse's body weight.

3.8.2. Rotarod test

The Rotarod test is employed to evaluate motor coordination. The Rotarod apparatus (Rod dimensions: 3 cm, height: 16 cm, Ugo Basile, Italy, 47650) [291] comprised two phases: the training phase and the testing phase.

(1) The training phase spanned the first and second days, during which mice were placed on a rotating rod set at a constant speed of 4 rpm. The objective was for the mice to remain on the rod for 60 seconds without falling, turning around, or gripping the rod. Each training session lasted 5 minutes, with three sessions conducted daily, spaced 15 minutes apart. If a mouse fell off the rod within 3 seconds, it was reintroduced after the 15-minute interval.

(2) In the testing phase, an accelerating program was employed, gradually increasing the rotation speed from 8 to 80 rpm for 240 seconds. The trial concluded if the mouse fell off, clung to the rod and spun twice, or remained on the rod for over 240 seconds. The latency to fall (in seconds) was automatically recorded for each trial, and the average latency across three trials was computed for analysis. After each trial, the rod and bottom tray were sanitized using a solution of 75% ethanol and water.

3.9. *In vivo* imaging for validating muscle injection

The objective was to conduct *in vivo* imaging of whole animals to analyze fluorescence, specifically for detecting and quantifying GFP expression. To ensure clear and unobstructed visualization of fluorescence, the mice were anesthetized using isoflurane. Following anesthesia, the fur on the lower limbs of the mice was carefully shaved, facilitating unobstructed observation and accurate fluorescence detection. The anesthetized and shaved mice were positioned within the IVIS imaging system (Perkin Elmer, CA, IVIS Lumina X5). The system setup and operation were performed following the manufacturer's guidelines to ensure optimal imaging conditions. For the detection of GFP fluorescence, the excitation wavelength was set at 488 nm, utilizing a filter range of 445 to 490 nm. Emitted fluorescence was then detected at 510 nm, allowing for the specific capture of GFP signal. Images were acquired using Living Image software v2.50. The software facilitated the capture and analysis of fluorescence data, which was expressed quantitatively in terms of photons/second/square centimeter [297].

3.10. Tissue preparation for immunostaining

Mice were deeply anesthetized with isoflurane, and trunk blood was collected. This was followed by sequential perfusion with 0.1 M PBS containing 4% (w/v) paraformaldehyde (PFA) for 15 minutes. The brains were then extracted and placed in a 4% PFA solution in 0.1 M PBS at 4°C for overnight fixation, not exceeding 48 hours. Post-fixation, the brains were transferred to a 30% sucrose solution (w/v) in 0.1 M PBS for cryoprotection, which dehydrates and protects the brain tissue from ice crystal formation during freezing, thereby preserving tissue morphology. Initially, the brains floated in the sucrose solution to ensure thorough penetration of the cryoprotectant. Following cryoprotection, the brains were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, USA, 4583). Brain sections were then sliced using a cryostat (Leica, USA, CM 1950) set to a thickness of 30 μm. Sections were prepared in a 1-in-6 series and stored in cryoprotectant at -20°C until analysis.

3.11. Immunohistochemistry staining and cell quantifications

As previously described [291–294,298], antigen retrieval was performed by immersing sections in citric acid buffer (pH 6.0) at 95°C for 10 minutes. After antigen retrieval, sections were incubated overnight at room temperature with the following primary antibodies in blocking solution. The following primary antibodies were used: rabbit anti-Ki-67 (1:1000, Cell Signaling Technology, USA, #9449), mouse anti-DCX (1:200, Santa Cruz Biotechnology, USA, sc-271390). Sections were then washed in 0.01 M PBS and incubated for 2 hours with biotinylated secondary antibodies: goat anti-mouse (1:200, Vector Laboratories, USA, BA-9200-1.5) or goat anti-rabbit (1:200, Vector Laboratories, USA, BA-1000-1.5). The immunostaining was visualized by VECTASTAIN® ABC kit (Vector Laboratories, USA, PK-4000) and DAB peroxidase substrate kit (Vector Laboratories, USA, SK-4100). Sections were subsequently dehydrated in ethanol (Sigma-Aldrich, USA, 2856) and xylene (Sigma-Aldrich, USA, 534056) baths and coverslipped with DPX Mountant (Sigma-Aldrich, USA, 6522).

Manual counts were conducted on 5 to 6 coronal sections spanning per animal. The average count per section was multiplied by the total number of 30 μm sections within the specified plane [291,292]. For specificity, Ki-67- and DCX-positive cells were quantified separately in two distinct regions of the dentate gyrus: the subgranular zone of the dorsal dentate gyrus (bregma -1.34 to -2.54 mm) and the ventral dentate gyrus

(bregma -2.54 to -3.40 mm). Quantifications were performed in a sample-blinded manner using bright-field microscopy (Nikon, Japan) to minimize observer bias and ensure the reliability and reproducibility of the findings.

3.12. Immunofluorescence staining and cell quantifications

Immunofluorescence double staining of Camk2a, or GAD67 co-label with APJ were performed in free-floating approach. The antigen retrieval was performed by immersing sections in citric acid buffer (pH 6.0) at 95°C for 10 minutes. After antigen retrieval, sections were incubated 1 hour at room temperature in blocking solution. For the double staining of BrdU co-label with DCX, brain sections were incubated with 2N HCl at 37℃ for 30 minutes and then transferred to 0.1 M borate buffer (pH 8.5, Yuanye Bio, China, R26034) for 15 minutes in room temperature for antigen retrieval before the blocking. Sections were then incubated overnight at room temperature with the following primary antibodies in blocking solution. The following primary antibodies were used: mouse anti-Camk2a (1:200, Millipore, Germany, 05-532), mouse anti-GAD67 (1:200, Millipore, Germany, MAB5406), rabbit ani-APJ (1:100, Proteintech, USA, 20341-1-AP), mouse anti-BrdU (1:500, Cell Signaling Technology, USA, 5292), rabbit anti-DCX (1:1000, Abcam, UK, ab18723). Sections were then washed in 0.01 M PBS and incubated for 3 hours with fluorescence secondary antibodies: Fluorescein horse anti-mouse (1:200, Vector Laboratories, USA, FI-2000), Texas RedTM goat anti-rabbit (1:200, Vector Laboratories, USA, TI-1000), DyLight™ 488 goat anti-rabbit (1:1000, Thermo Fisher Scientific, USA, 35552), Alexa Fluor® 647 goat anti-mouse (1:1000, Jackson ImmunoResearch, USA, 115-605-003). Finally, the sections were cover-slipped with an anti-fade mounting medium containing DAPI (Beyotime, China, P0131), and immunofluorescence signals were observed using a Nikon AXE laser confocal microscope (Nikon, Japan).

$BrdU^+$ and DCX^+ cells were quantified following established protocols. Confocal images of the hippocampal dentate gyrus (DG) were acquired using a Z-stack approach, with optical sections taken at 4 μm intervals over 5-7 steps per brain section. Maximum intensity projections were generated for visualization. For the colocalization analysis of DCX^+ and $BrdU^+$ cells, quantification was performed in a blinded manner, focusing on the subgranular zone of the hippocampal DG.

3.13. Protein extraction

3.13.1. Tissue dissection and homogenate preparation

Mice were swiftly euthanized using isoflurane, and tissue was dissected as outlined in previous studies [292]. The dentate gyrus was isolated and homogenized from a pooled sample of seven mice from the same experimental group in RIPA buffer (Thermo Fisher Scientific, USA, 89901) with protease inhibitors (Roche, USA, 04693159001). Homogenization was performed on ice to maintain protein integrity. The samples were then centrifuged at 12,000 rpm for 8 minutes at 4 ℃. The supernatant containing the solubilized proteins was carefully collected and either kept on ice for immediate analysis or stored at -80°C for future use.

3.13.2. Preparation of synaptoneurosome fraction of hippocampal tissues

The hippocampi were immediately isolated and placed in ice-cold PBS. As previously described [292], the hippocampal tissue was transferred to a pre-chilled homogenizer containing Syn-PER reagent (Thermo Fisher Scientific, USA, 87793) with added protease inhibitors (Roche, USA, 04693159001) and phosphatase inhibitor (Thermo Fisher Scientific, USA, 78420). The homogenate was transferred to 1.5 mL microcentrifuge tubes and centrifuged at 1,200 rpm for 10 minutes at 4°C to remove nuclei and debris. The supernatant was collected and centrifuged at 12,000 rpm for 20 minutes at 4°C to collect the pellet as the synaptoneurosome, and the supernatant was collected as the cytosol fraction. The synaptoneurosome pellet was resuspended in ice-cold Syn-PER reagent adhering to the manufacturer's instructions.

3.13.3. Protein quantification and storage

Protein concentrations were measured using the BCA Kit (Thermo Fisher Scientific, USA, 23225), adhering to the manufacturer's guidelines to ensure accurate quantification for subsequent analyses.

3.14. Western blotting

As describe in previous publications [292,294], samples containing 30 μg of protein were denatured in Laemmli buffer (Bio-Rad, USA, 1610747) supplemented with 5% 2-mercaptoethanol (Bio-Rad, USA, 1610710) at 98°C for 15 minutes. The proteins were then separated by electrophoresis on 8%, 10%, and 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred

onto methanol-activated polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA, 1620177). Following the transfer, membranes were blocked for 1 hour at room temperature using 5% bovine serum albumin (Macklin Biochemical Technology, China, T6335) in TBS-T, pH 8.0. After blocking, membranes were incubated overnight at 4°C with primary antibodies diluted in the blocking buffer.

The following primary antibodies were used: mouse anti-apelin (1:200, Santa Cruz Biotechnology, USA, sc-293441), mouse anti-APJ (1:200, Santa Cruz Biotechnology, USA, sc-517300), rabbit anti-Synaptophysin (1:1000, Cell Signaling Technology, USA, 5461), rabbit anti-PSD95 (1:1000, Cell Signaling Technology, USA, 2507), rabbit anti-GluA1 (1:1000, Cell Signaling Technology, USA, 13185), rabbit anti-phospho-GluA1 (Ser831) (1:1000, Cell Signaling Technology, USA, 75574), rabbit anti-GluN2A (1:1000, Cell Signaling Technology, USA, 4205), rabbit anti-phospho-GluN2A (Tyr1246) (1:1000, Cell Signaling Technology, USA, 4206), rabbit anti-GluN2B (1:1000, Cell Signaling Technology, USA, 4207), rabbit anti-phospho-GluN2B (Tyr1472) (1:1000, Cell Signaling Technology, USA, 4208), rabbit anti-AMPKα (1:1000, Cell Signaling Technology, USA, 5831), rabbit anti-phospho-AMPKα (Thr172) (1:1000, Cell Signaling Technology, USA, 2535), rabbit DYKDDDDK Tag anti-FLAG (1:200, Cell Signaling Technology, USA, 14793), rabbit anti-Calpain-1 (1:1000, Abcam, UK, Ab39170), rabbit anti-Calpain-2 (1:1000, Abcam, UK, Ab39165), rabbit anti-CK2α (1:1000, Abcam, UK, ab76040), rabbit anti-CK2β (1:1000, Abcam, USA, ab76025), rabbit anti-GAPDH (1:5000, Cell Signaling Technology, USA, 5174), rabbit anti-α-tubulin (1:5000, Thermo Fisher Scientific, USA, 236-10501).

After primary antibody incubation, membranes were washed three times with TBS-T and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies: goat anti-rabbit (1:1000, Cell Signaling Technology, USA, 7074), goat anti-mouse (1:1000, Cell Signaling Technology, USA, 7076). Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, USA, 1705061) and imaged with a ChemiDoc MP Imaging System equipped with Image Lab Touch Software 3.0 (Bio-Rad). Quantification of band intensities was performed using ImageJ software (NIH).

3.15. Immunoassays for serum apelin

Blood samples were allowed to clot at room temperature for at least 1 hour. Subsequently, the clotted samples were centrifuged at 2,000g for 30 minutes at 4°C.

The resulting serum was carefully collected and stored at -80°C until further analysis. Serum apelin-12 levels were measured using the apelin-12 EIA kit (Phoenix Pharmaceuticals, EK-057-23), following the manufacturer's instructions.

3.16. RNA extraction and quantitative PCR

Total RNA was extracted from tissue samples using the ReliaPrep^{TM} RNA Tissue Miniprep System (Promega, USA, Z6112) following the manufacturer's protocol. Frozen tissue was transferred to LBA buffer containing 2% 1-thioglycerol. Subsequent RNA purification steps were conducted as per the non- and fibrous tissue extraction protocol. The homogenate of fibrous tissue was mixed with RDB buffer and centrifuged. The supernatant was collected, mixed with 100% molecular-grade isopropanol, and transferred to a rotating column. DNA was digested on-column with DNase I (Promega, USA, Z6112), and the RNA was washed repeatedly before eluting in 25 µl of nuclease-free water. RNA concentration and purity (260/280 and 260/230 ratios) were measured by spectrophotometry (Thermo Fisher Scientific, USA, VARIOSKAN LUX). 500-1000 ng of total RNA was reverse transcribed using the GoScript^{TM} Reverse Transcription System (Promega, USA, A6010) with oligo (dT) primers in a 20 µl reaction volume for cDNA synthesis.

Quantitative PCR (qPCR) was performed using synthesized cDNA. Each 15 µl reaction contained 1.5 µl of diluted cDNA (1:10 dilution), 1.6 µl of primers (forward & reverse primer), and 7.5 µl of 2X GoTaq qPCR Master Mix (Promega, USA, A6010), supplemented with nuclease-free water. Table 3.5 lists the specific primers utilized in this study. Reactions were set up using the Promega automated PCR system and run on 96-well PCR plates (Bio-Rad, USA, HSP9601). Gene expression levels were normalized to the internal control gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), and analyzed using appropriate qPCR software.

3.17. Whole-cell patch-clamp recording

Brains were collected from mice four weeks after intramuscular injection of AAV in both AAV-APLN and AAV-GFP groups. Brain slices were prepared as described in our previous publication[292,293], and transverse hippocampal slices (350 µm thick) were obtained using artificial cerebrospinal fluid (ACSF) with high Mg^{2+} and low Ca^{2+} concentrations for slicing. After a recovery period of at least 1 hour in ACSF at 33 °C,

whole-cell patch-clamp recordings were performed using an Axon Axopatch 700B amplifier, with data digitized by a DigiData 1550A system with Clampex 11.3 software.

Recording electrodes had a resistance of 5–7 MΩ. Synaptic responses were evoked by electrical stimulation of the Schaffer collaterals. Recordings were made from pyramidal neurons located in the CA1 region of the hippocampus. For spontaneous excitatory postsynaptic currents (sEPSCs), cells were held at -70 mV. Once whole-cell configuration was achieved in normal ACSF, AMPA receptor-mediated EPSCs were recorded by holding cells at -70 mV, while NMDA receptor-mediated EPSCs were recorded by holding cells at +40 mV, using a 0.05 ms pulse in voltage-clamp mode. The peak current observed at -70 mV represents the maximum AMPAR-EPSC, whereas the NMDAR-EPSC was measured at +40 mV after 50 ms of the stimulation pulse, when the AMPAR-EPSC had decayed. For input/output curves, Schaffer collaterals were stimulated at increasing intensities (20-60 μA).

3.18. Statistics

The data were presented as Mean ± S.E.M. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software). Depending on the experimental design, we employed t-tests (two-tailed, paired or unpaired), one-way ANOVA, and two-way ANOVA, followed by Tukey's and Fisher's least significant difference (LSD) post hoc tests, as appropriate. For datasets where the Shapiro-Wilk test for ANOVA residuals yielded $P > 0.05$, indicating no significant deviation from normality, an unpaired Welch's correction with t-test was applied. Statistical significance was determined with P values less than 0.05 (*), 0.01 (**), 0.001 (***), or 0.0001 (****). Additionally, logistic regression analysis was conducted to assess the correlation between serum apelin levels and behavioral outcomes, including time spent immobile in the FST, sucrose consumption ratio in the SPT, and grooming time in the SST.

3.19. Materials

3.19.1. Chemical reagents and solutions

Table 3.2 Solutions for western blotting, DNA electrophoresis, immunostaining, and whole-cell patch-clamp recordings

Solution	Composition
1 × Lysis buffer for DNA extraction	0.692 mM NaOH, 5.5 μM EDTA in ddH_2O
1 × Neutralization buffer for DNA extraction	1.45 mM Tris-HCl in ddH_2O
1 × Phosphate buffered saline (0.1 M, PBS)	0.1 M Sodium Phosphate (Na_2HPO_4 and NaH_2PO_4), 0.1 M Sodium Chloride (NaCl) in double-distilled water (ddH2O), adjust to pH 7.4
1 × PBS with Tween-20 (PBS-T)	17 mM NaH2PO4, 125 mM NaCl, 58 mM, Na2HPO4, (pH 7.4), 0.1 % Tween-20
1 × Separating gel	1.5 M Tris Base with HCl, pH 8.8, 0.4 % (w/v) SDS
1 × Stacking gel	1.0 M Tris Base with HCl (pH 6.8, 0.4 % (w/v) SDS
1 × Tris buffer saline with Tween-20 (TBS-T)	150 mM NaCl, 50 mM Tris Base with HCl, pH 7.4, 0.1 % Tween-20 in ddH_2O
1 × Transfer buffer	192 mM glycine, 25 mM Tris, and 20 % (v/v) methanol in ddH_2O
1 ×SDS running buffer	192 mM glycine, 25 mM Tris, and 0.1% (w/v) SDS in ddH_2O
ACSF	123mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.25 mM NaH2PO4·H2O, 1mM CaCl2, 6 mM MgCl2, 100 μM Picrotoxin
Blocking buffer for immunohistochemistry staining	5% normal goat serum, 0.25% tritonX-100 in 1 × PBS-T
Blocking buffer for western blotting	5% (w/v) non-fat dried milk in 1 × TBS-T
Cryoprotectant	30% glycerol and 30% ethylene glycol in PBS solution
The intracellular solution for whole-cell recording	135 mM CsMeSO4, 8 mM NaCl, 10 mM HEPES, 7 mM phosphocreatine, 0.3 mM Na3GTP, 2 mM Mg2ATP, 10 mM QX-314, pH 7.3

Table 3.3 Reagents for western blotting, DNA electrophoresis and immunohistochemistry

Reagent or material	Supplier	Catalogue number
10 × Tris Buffered Saline (TBS)	Bio-Rad, USA	1706435
10 × Tris/Tricine/SDS Running Buffer	Bio-Rad, USA	1610744
10% SDS Solution	Bio-Rad, USA	1610416
4 × Laemmli Sample Buffer	Bio-Rad, USA	1610747
PVDF membrane	Bio-Rad, USA	1620177
Quick Start Bovine Serum Albumin Standard	Bio-Rad, USA	5000206
Resolving Gel Buffer 1.5M	Bio-Rad, USA	1610798
Stacking Gel Buffer for PAGE, 0.5 M Tris-HCl, pH 6.8	Bio-Rad, USA	1610799
β-mercaptoethanol	Bio-Rad, USA	1610710
10000 × Safe green	Biosharp, China	BS356A
Anti-fade mounting medium containing DAPI	Beyotime, China	P0131
Adhesion-resistant slides	Citotest Labware, China	188105W
Square cover slides	Citotest Labware, China	0340-4020
Bovine Serum Albumin (BSA)	Macklin Biochemical Technology, China	B824162
Tween 20	Macklin Biochemical Technology, China	T6335
BrdU	MedChemExpress, USA	HY-15910
Apelin-12 EIA kit	Phoenix Pharmaceuticals, USA	EK-057-23
2-Step RT-qPCR System	Promega, USA	A6010
RNA extraction kit	Promega, USA	Z6112
Protease inhibitor cocktail	Roche, USA	5892970001
Isoflurane	RWD Life Science, China	R510-22
Normal goat serum	Servicebio Technology, China	G1208
Tissue-Tek® O.C.T. Compound	Sakura Finetek, USA	4583
Tris Buffered Saline (TBS, Powder)	Servicebio Technology, China	G0001
TritonX-100	Servicebio Technology, China	G3005
DPX Mountant for microscopy	Sigma-Aldrich, USA	6522
Agarose	Sigma-Aldrich, USA	1.01236
Bovine Serum Albumin	Sigma-Aldrich, USA	A8022

(*Cont'd*)

Ethanol	Sigma-Aldrich, USA	2856
Glycerol	Sigma-Aldrich, USA	G5516
Isopropanol	Sigma-Aldrich, USA	278475
Propylene glycol	Sigma-Aldrich, USA	1576708
Taipan Blue	Sigma-Aldrich, USA	T8154
Xylene	Sigma-Aldrich, USA	534056
Hight-sig ECL Wester Blotting Substrate	Tanon Bioscience, China	180-5001
100 bp DNA Ladder	Thermo Fisher Scientific, USA	15628050
BCA Protein Assay Kits	Thermo Fisher Scientific, USA	23225
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific, USA	26616 and 26619
Phosphatase inhibitor	Thermo Fisher Scientific, USA	78420
RIPA Buffer	Thermo Fisher Scientific, USA	89901
Synaptic Protein Extraction Reagent	Thermo Fisher Scientific, USA	87793
1 × Tris-Acetate EDTA (TAE)	Vazyme Biotech, China	TAE402
2 × Taq Master Mix (Dye Plus)	Vazyme Biotech, China	P112
ABC-HRP Kit, Peroxifdase	Vector Laboratories, USA	PK-4000
DAB Substrate Kit	Vector Laboratories, USA	SK-4100
Methanol	Yonghua Chemical, China	M105002
0.1M Borate Buffer, pH 8.5	Yuanye Bio, China	R26034

3.19.2. Antibodies for western blotting and immunohistochemistry

Table 3.4 The list of antibodies

Antibodies	Supplier	Catalogue number
Rabbit anti-Calpain-1	Abcam, UK	Ab39170
Rabbit anti-Calpain-2	Abcam, UK	Ab39165
Rabbit anti-CK2α	Abcam, UK	Ab76040
Rabbit anti-CK2β	Abcam, UK	Ab76025
Rabbit anti-DCX	Abcam, UK	ab18723
Goat anti-mouse, HRP-conjugated antibodies	Cell Signaling Technology, USA	7076
Goat anti-rabbit, HRP-conjugated antibodies	Cell Signaling Technology, USA	7074
Mouse anti-BrdU	Cell Signaling Technology, USA	5292
Rabbit anti-AMPKα	Cell Signaling Technology, USA	5831
Rabbit anti-GAPDH	Cell Signaling Technology, USA	5174
Rabbit anti-GluA1	Cell Signaling Technology, USA	13185
Rabbit anti-GluN2A	Cell Signaling Technology, USA	4205
Rabbit anti-GluN2B	Cell Signaling Technology, USA	4207
Rabbit anti-Ki-67	Cell Signaling Technology, USA	9449
Rabbit anti-phospho-AMPKα (Thr172)	Cell Signaling Technology, USA	2535
Rabbit anti-phospho-GluA1 (Ser831)	Cell Signaling Technology, USA	75574
Rabbit anti-phospho-GluN2A (Tyr1246)	Cell Signaling Technology, USA	4206
Rabbit anti-phospho-Glun2B (Tyr1472)	Cell Signaling Technology, USA	4208
Rabbit anti-PSD95	Cell Signaling Technology, USA	2507
Rabbit anti-Synaptophysin	Cell Signaling Technology, USA	5461
Rabbit DYKDDDDK Tag anti-FLAG	Cell Signaling Technology, USA	14793
Alexa Fluor® 647 goat anti-mouse antibody	Jackson ImmunoResearch, USA	115-605-003
Mouse anti-Camk2a	Millipore, Germany	05-532
Mouse anti-GAD67	Millipore, Germany	MAB5406
Rabbit ani-APJ	Proteintech, USA	20341-1-AP

(*Cont'd*)

Mouse anti-apelin	Santa Cruz Biotechnology, USA	sc-293441
Mouse Anti-APJ	Santa Cruz Biotechnology, USA	sc-517300
Mouse anti-DCX	Santa Cruz Biotechnology, USA	sc-271390
DyLight™ 488 goat anti-rabbit antibody	Thermo Fisher Scientific, USA	35552
Rabbit anti-α-tubulin	Thermo Fisher Scientific, USA	236-10501
Fluorescein horse anti-mouse antibody	Vector Laboratories, USA	FI-2000
Goat anti-mouse, biotinylated antibody	Vector Laboratories, USA	BA-9200-1.5
Goat anti-rabbit, biotinylated antibody	Vector Laboratories, USA	BA-1000-1.5
Texas Red™ goat anti-rabbit antibody	Vector Laboratories, USA	TI-1000

3.19.3. Sequences for qPCR and genotyping

Table 3.5 The list of sequences of primer

Exp.	Gene	Forward primer	Reverse primer
qPCR	Apelin, pair 1	GTTTGTGGAGTGCCACTG	CGAAGTTCTGGGCTTCAC
	Apelin, pair 2	CGAGTTGCAGCATGAATCTGAG	TGTTCCATCTGGAGGCAACATC
	APJ, pair 1	GCTGTGCCTGTCATGGTGTT	CACTGGATCTTGGTGCCATTT
	APJ, pair 2	GCATTATCGTGGTGCTTGTAGTGA	GCAAACTGCCCAGCATGTAGA
	GAPDH	CCCATCACCATCTTCCAGGAGC	CCAGTGAGCTTCCCGTTCAGC
	HPRT	CTGGTGAAAAGGACCTCTCG	TGAAGTCTCATTATAGTCAAGGGCA
Genotyping	Ckmm-iCre-5'connector	CCCAGCAGGATCACATAGGCAG	AGCCCAGGATCTGCACACAGA
	Ckmm-iCre-3'connector	CAAGGATGACTCTGGGCAGAG	AGCCAGAAGTCAGATGCTCAAG
	APLN-flox-5'arm	GAGTTCTCTCTGCCATTCTGGAAG	AGCTTGATTGGCAACTGCATG
	APLN-flox-3'arm	AGCAAGTGTGGTGAAGTGTGCA	GTTGGTCCACATTCATAGCGATC
	Camk2a-Cre	GGGGAGGTAGGAAGAGCGATGA	CATCGACCGGTAATGCAG

Chapter 4. Voluntary Running Alleviates Chronic Stress Induced Depression-like Behavior in Association with Increased Apelin Levels in the Hippocampus

4.1. Introduction

Depressive disorders represent a significant global health burden, characterized by persistent low mood, anhedonia, and cognitive impairments [2]. The development of these disorders is complex, involving hereditary [299], acquired factors [300], and neuropathic factors [301]. Among the neurobiological underpinnings, dysregulation of neuroplasticity, neuroinflammation, and neurotransmitter systems are prominent [302]. Emerging evidence suggests a bidirectional relationship between sarcopenia, the age-related muscle weakness, and depression [9,121,127]. Recent clinical trials reported that sarcopenia not only exacerbates physical disability but also contributes to the progression of depressive symptoms [127,153,154]. This interplay underscores the importance of understanding the molecular mechanisms linking muscle health and mental health.

Apelin, a myokine associated with sarcopenia [17,21], has garnered attention for its potential antidepressant effects [282,283,287]. Notably, decreased serum levels of apelin have been involved in the pathogenesis of sarcopenia, which is concurrently associated with depression in the elderly [303]. Recent studies have illuminated apelin's direct regulatory the antidepressant effects on the hippocampus, a critical area for memory and emotional regulation. In a rat model of depression induced by chronic water-immersion restraint stress (CWIRS), apelin-13 has been shown to exert antidepressant effects and enhance recognition memory, mediated through the activation of PI3K and ERK1/2 signaling pathways in hippocampus [18]. Furthermore, intrahippocampal administration of apelin-13 could mitigating depressive symptoms [19,285], and improving cognitive function and reducing neuronal damage in AD models through the PGC-1α/PPARγ signaling pathway [23]. These findings are crucial as they highlight the hippocampus's role in mediating the beneficial effects of apelin in mood and cognition.

Exercise is well-documented for its positive effects on both sarcopenia and depression [126]. Regular physical activity enhances muscle protein synthesis, improves mitochondrial function, and elevates serum apelin levels [17,213]. In addition, our previous

publication have indicated that physical exercise counteracts stress, enhances hippocampal-dependent learning and memory, and alleviates depressive phenotypes by restoring adult neurogenesis (neurogenesis in the dentate gyrus), dendritic complexity, and synaptic plasticity in the hippocampus of rodent models of depression [28,291,304]. Despite these benefits, the specific involvement of apelin in the antidepressant effects of running remains unclear. This gap in knowledge necessitates further investigation to elucidate the underlying mechanisms.

In this chapter, we aim to test the antidepressant effects of voluntary running using a depression model induced by chronic unpredictable stress (CUS), to elucidate the potential role of muscle-secreted apelin as a critical mediator for antidepressant effects of physical exercise vis its enhancing effects on hippocampal adult neurogenesis and synaptic plasticity. The CUS model is a widely accepted and predictive animal model of depression [305,306], characterized by its ability to induce depressive-like behaviors through chronic exposure to unpredictable stressors. Utilizing this model, we will explore whether voluntary running can elevate hippocampal apelin and APJ levels and subsequently ameliorate depressive symptoms. This investigation aims to elucidate the role of apelin in the neurobiological mechanisms underlying the antidepressant effects of exercise.

4.2. Results

4.2.1. Antidepressant effects of voluntary running in association with enhanced expression levels of apelin and APJ in the hippocampus.

In order to investigate whether the antidepressant effects of voluntary running are linked to change in apelin, young adult male mice (6-week-old) were subjected to 4-week voluntary running (Fig. 4.1a). Depression-like behaviors were assessed using behavioral paradigms including the sucrose preference test (SPT), sucrose splash test (SST), and forced swim test (FST). The running group exhibited a higher sucrose preference in the SPT (Fig. 4.1b; $P < 0.01$) and displayed increased grooming behavior during the SST (Fig. 4.1c; $P < 0.01$), indicating reduced anhedonia-like behaviors. Additionally, the runner mice demonstrated a decrease in the time spent immobile in the FST (Fig. 4.1d; $P < 0.001$) compared to the sedentary mice, suggesting that 4-week voluntary running reduced depression-like behavior. Notably, locomotor activity in the running group, as assessed by the OFT, was decreased (Fig. 4.1e; $P < 0.001$). However, the absence of a change in the time spent on the center area of the OFT (Fig. 4.1f; $P > 0.05$) suggests that 4-week voluntary running had no effects on anxiety-related behaviors.

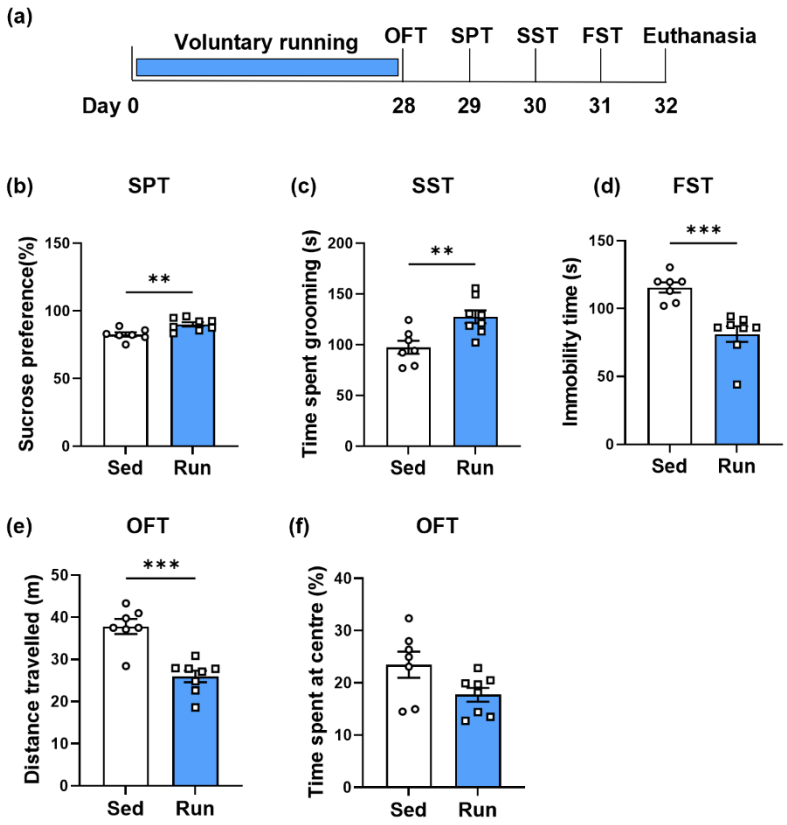

Figure 4.1 Antidepressant effects of 4-week voluntary running in mice.
(a) Study timeline with interventions and behavioral tests conducted on sedentary and exercised mice. Sed: sedentary mice; Run: runner mice. Running reduced (b) sucrose preference in the SPT, and increased (c) grooming time in SST, indicating reduction in anhedonia-like behavior. Running reduced (d) immobility time in FST, indicating reduced depression-like behavior. Running also reduced (e) locomotor activity as shown in reduction in total distance traveled in OFT but did not affect anxiety-like behavior as indicated by no significant changes in (f) the time spent in the center of the arena in OFT. Data are presented as mean ± S.E.M. Statistical significance assessed using unpaired t-test, is denoted as $^{**}p < 0.01$, $^{***}p < 0.001$. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test, FST: forced swim test.

To confirm the influences of the voluntary running on muscle mass, we monitored changes in body weight over the 4-week duration and measured lower limb muscle tissue mass after mice were sacrificed. Following 4 weeks of voluntary running, there was no change in body weight gains (Fig. 4.2b; $P > 0.05$). However, running mice showed an increase in muscle mass in the lower limb muscles (Fig. 4.2c; $P < 0.05$), including the soleus (Sol), extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius (Gas), when compared to sedentary mice. Particularly, significant increases in muscle mass were observed in both the Gas (Fig. 4.2d; $P < 0.01$) and Sol (Fig. 4.2e; $P < 0.01$) muscles, which are part of the plantar flexors. Conversely, no significant changes were observed in the TA (Fig. 4.2f; $P > 0.05$) and EDL (Fig. 4.2g; $P > 0.05$) muscles, which belong to the plantar extensors. These findings indicate that running substantially increased hindlimb muscle mass.

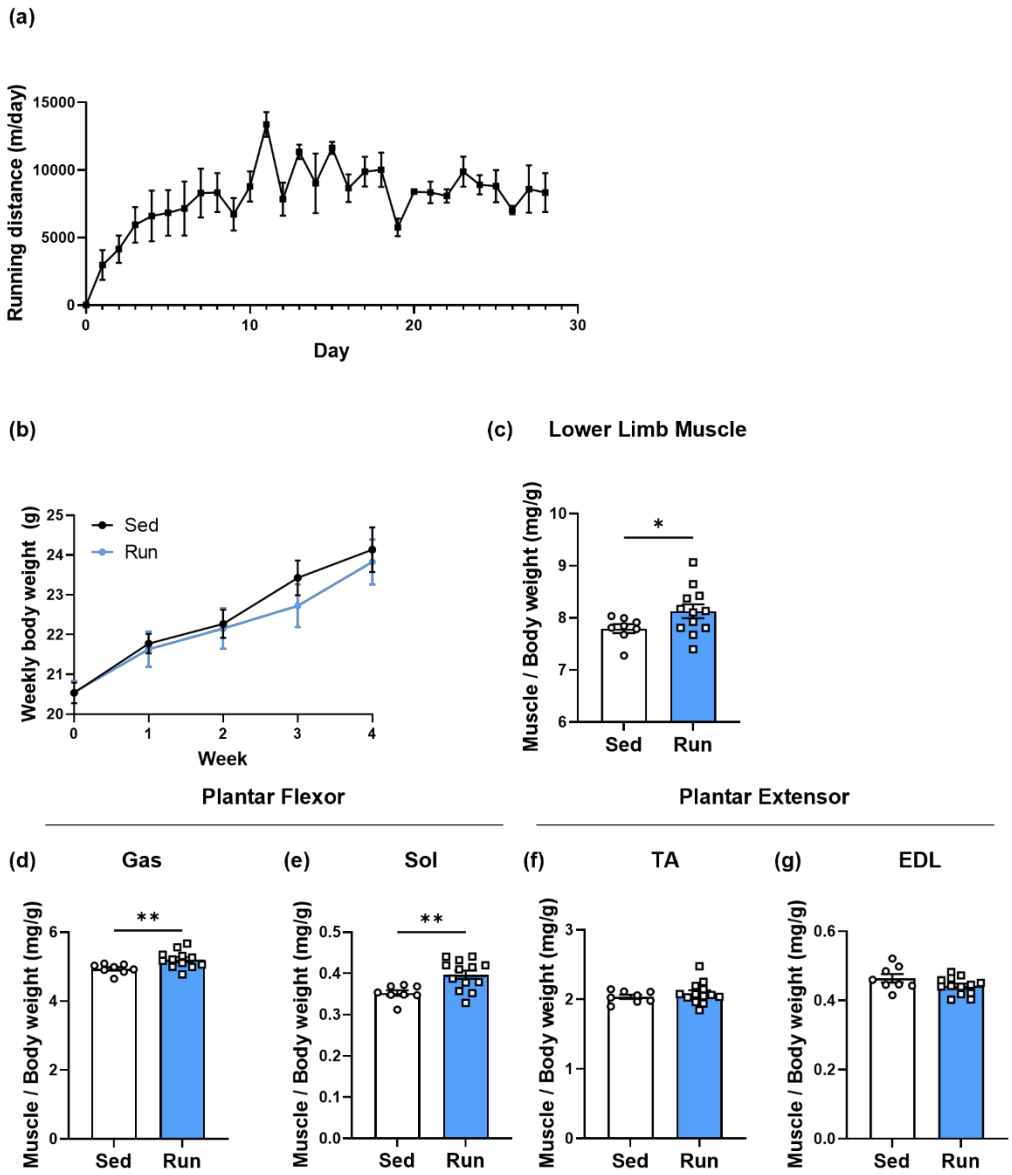

Figure 4.2 Chronic voluntary running increased the muscle mass in plantar flexor. (a) The daily running distance in meters, with each point representing a day's performance. (n = 16) (b) Trajectory of body weight gain throughout the four-week period of voluntary running on sedentary and runner mice. Sed: sedentary mice; Run: runner mice. Voluntary running increased (c) the ratio of lower limb muscle mass to total body weight. Voluntary running increased the absolute weights of (d) Gas, and (e) Sol muscle, but did not affect (f) TA, and (g) EDL muscle, with Gas and Sol serving as indicators of lower limb plantar flexor mass, and TA and EDL representing lower limb plantar extensor mass. Data are presented as mean ± S.E.M. Unpaired t-test: *$p < 0.05$, **$p < 0.01$. Gas: gastrocnemius, Sol: soleus, TA: tibialis anterior, EDL: extensor digitorum longus.

In order to identify the primary organ secreting apelin in response to running, we subsequently investigated the alterations in apelin levels in muscles and other peripheral organs in response to voluntary running. The APLN mRNA levels were assessed by using molecular assay qPCR (Fig. 4.3a-b). Voluntary running increased APLN mRNA levels in the Gas ($P < 0.001$) and TA ($P < 0.05$) muscles, whereas APLN mRNA levels in the quadriceps (Quadri., $P > 0.05$), the EDL ($P = 0.8734$), Sol ($P > 0.05$), and plantaris (Plant., $P > 0.05$) showed no significant increase. In peripheral organs, 4-week voluntary running did not significantly elevate APLN mRNA levels in the heart ($P > 0.05$), adipose tissue ($P > 0.05$), lung ($P > 0.05$), kidney ($P > 0.05$), and liver ($P > 0.05$).

Furthermore, we examined the expression levels of the apelin receptor (APJ) mRNA in the lower limb muscles (Fig. 4.3b). Consistent with the findings for APLN mRNA expression, running increased APJ mRNA levels among the Gas ($P < 0.05$) and TA ($P < 0.05$) muscles after 4 weeks of voluntary running, with an increasing trend in the soleus ($P > 0.05$). However, there was no increase of APJ mRNA expression in the EDL ($P > 0.05$). Notably, 4 weeks of voluntary running significantly decreased serum apelin levels (Fig. 4.3c, $P < 0.05$).

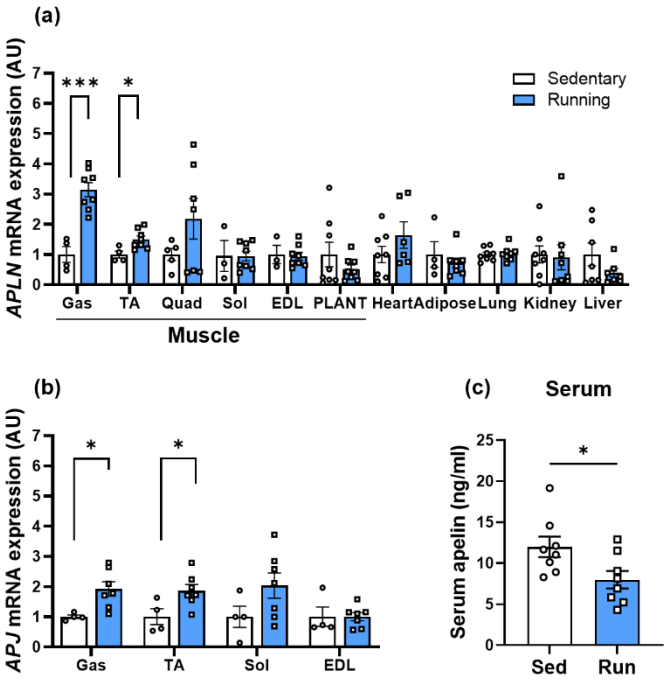

Figure 4.3 Altered apelin and APJ expression levels in the lower limb muscles and serum by chronic voluntary running.

(a) 4-week voluntary running promoted APLN mRNA expression levels in skeletal muscle tissues gastrocnemius (Gas) and tibialis anterior (TA), but did not affect quadriceps (Quadri.), soleus (Sol), extensor digitorum longus (EDL), and plantaris (Plant.), and non-muscle tissues (including the heart, adipose tissue, lung, kidney, and liver). Voluntary running increased (b) APJ mRNA expression in the lower limb muscle and decreased (c) serum apelin concentrations. Data are presented as mean ± S.E.M. Unpaired t-test: *p < 0.05, ***p < 0.001.

Furthermore, voluntary running showed significant effects on altering hippocampal APJ protein expression levels. Running significantly increased APJ protein levels (Fig. 4.4b, $P < 0.05$), while apelin levels remained unchanged (Fig. 4.4d, $P > 0.05$) in the hippocampus. This suggests an enhanced apelin signaling within the hippocampus following running. Despite the absence of significant changes in apelin and APJ mRNA expression levels (Fig. 4.4d & e, $P > 0.05$), our findings indicate that 4 weeks of voluntary running increased apelin secretion in both Gas and TA muscles and enhanced apelin and APJ signaling in the hippocampus.

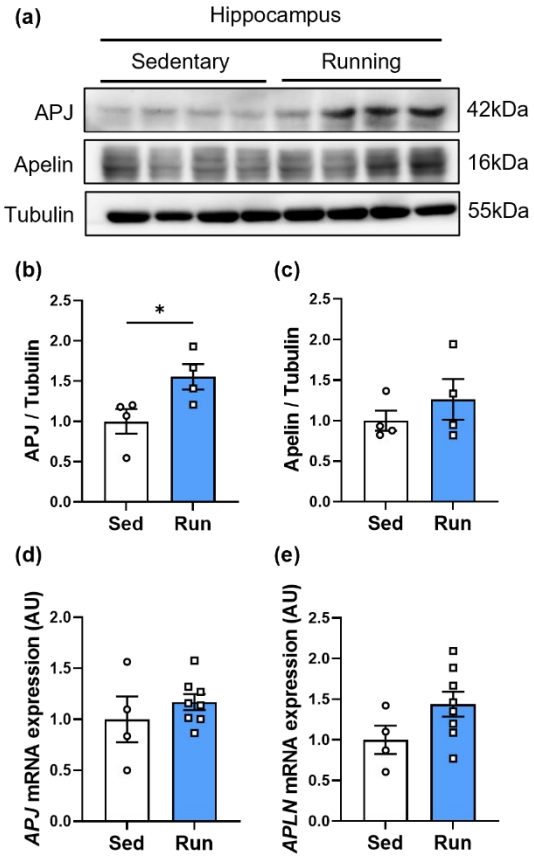

Figure 4.4 Chronic voluntary running enhanced apelin and APJ protein expression in the hippocampus.

(a) Western blotting analysis showing differential expression of apelin and APJ proteins in the hippocampi of sedentary and runner mice; Sed: sedentary; Run: runner mice. Voluntary running increased (b) the APJ protein but did not affect (c) the apelin protein levels in hippocampus. Voluntary running has no effects on (d) APJ mRNA and (e) APLN mRNA expression levels in the hippocampus. Data are presented as mean ± S.E.M. Unpaired t-test: *p < 0.05.

4.2.2. Voluntary running reduced depression-like behavior in correlation with elevated hippocampal APJ and apelin expression in stressed mice.

We first demonstrated the potential effects of 4-week voluntary running on alleviating depression-like behaviors induced by chronic unpredictable stress (CUS). Young adult male mice (6-week-old) were subjected to CUS with concurrent voluntary running (Fig. 4.5a). Chronic stress reduced grooming time in the SST (Fig. 4.5b, $P < 0.05$), and increased the time spent immobile in the FST (Fig. 4.5b, $P < 0.001$) in stressed mice when compared to control mice. Conversely, voluntary running significantly increased grooming time during the SST (Fig. 4.5c, $P < 0.001$) and reduced immobile time in the FST (Fig. 4.5c, $P < 0.001$), indicating 4-week voluntary running decreased antidepressant-like behaviors.

Furthermore, chronic stress did not change sucrose preference (Fig. 4.5d, $P > 0.05$, Control vs. CUS), whereas voluntary running increase sucrose preference in stressed mice (Fig. 4.5d, $P < 0.05$, CUS vs. CUS+Exe), suggesting a significant antidepressant effects of running in stressed mice. However, one-way ANOVA analysis indicated no significant change in either the duration in the center arena or the locomotor activity of the OFT (Fig. 4.5e & f, $F(2, 33) = 2.270$, $P > 0.05$).

To investigate the potential link between peripheral apelin levels and antidepressant effects of physical running, serum apelin levels were measured by EIA kits. The results revealed that 4-week voluntary running mitigated the decrease in serum apelin levels in stressed mice (Fig. 4.5g, $P < 0.05$, Control vs. CUS; $P < 0.05$, CUS vs. CUS+Exe). Furthermore, there was a negative correlation between serum apelin levels and immobility time in the FST (Fig. 4.5h, $P < 0.005$, $r^2 = 0.3650$), and a positive correlation with grooming time in the SST (Fig. 4.5i, $P < 0.01$, $r^2 = 0.3278$). Additionally,

there was a trend towards a positive correlation with sucrose preference in the sucrose preference test (SPT) (Fig. 4.5j, P = 0.0908, r^2 = 0.1432), suggesting a potential link between serum apelin levels and depression-related behaviors.

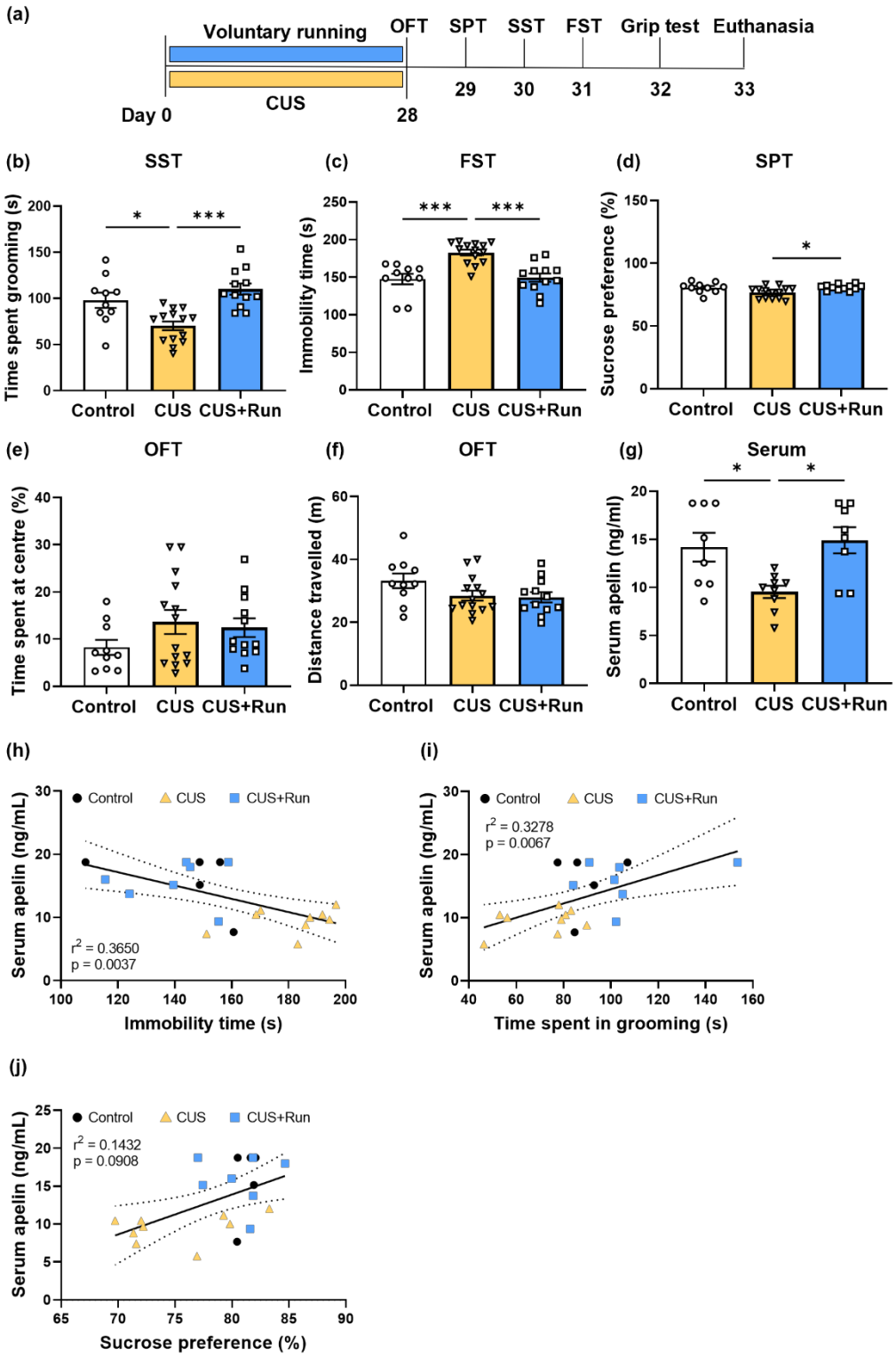

Figure 4.5 Chronic running reduced depression-like behavior and enhanced serum apelin levels in stressed mice.

(a) Experimental timeline of the 4-week chronic unpredictable stress and voluntary running. CUS: chronic unpredictable stressed mice; CUS+Run: CUS runner mice. Voluntary running exhibited antidepressant effects as shown by (b) increased self-grooming time in SST, (c) reduced immobility time in the FST, (d) increased sucrose preference in the SPT in the stressed mice. However, there was no change in (e) the time spent in the center, and (f) distance traveled of the OFT. (g) Chronic running restored the decrease in serum apelin levels in stressed mice (n = 8 per group). (h) Linear regression analysis showed a negative correlation between immobility time in FST and serum apelin levels. (i) Linear regression analysis showed a positive correlation between grooming time in SST and serum apelin levels. (j) Linear regression analysis showed a positive correlation between sucrose preference in SPT and serum apelin levels. Data are presented as mean ± S.E.M. Statistical significance as determined by One-way ANOVA with Tukey's post hoc test, and linear regression analysis, is indicated by *p < 0.05, **p < 0.01, ***p < 0.001. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test, FST: forced swim test.

Chronic stress did not change apelin levels (Fig. 4.6b; P > 0.05) and its receptor APJ (Fig. 4.6c; P > 0.05) in the hippocampus in the stressed mice; however, voluntary running significantly elevated the expression levels of both apelin and APJ in the stressed mice (Fig. 4.6b, P < 0.01; Fig. 4.6c, P < 0.05). These findings collectively demonstrate that voluntary running increased hippocampal APJ and apelin expression in stressed mice.

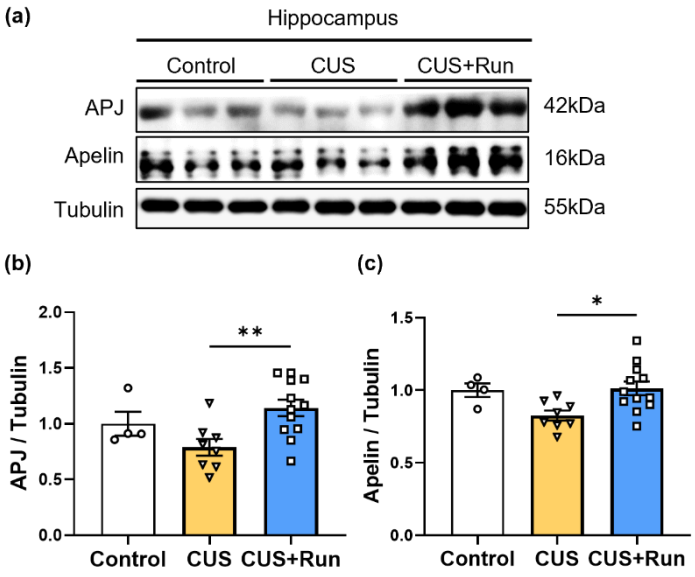

Figure 4.6 Voluntary running increased apelin and its receptor APJ protein expression in the hippocampus of stressed mice.

(a) Western blotting results of apelin, APJ and Tubulin protein levels in the hippocampus. CUS: chronic unpredictable stressed mice; CUS+Run: CUS runner mice. Chronic voluntary running increased (b) APJ protein and (c) apelin protein levels in hippocampus. Data are presented as mean ± S.E.M. A one-way ANOVA followed by Tukey's post hoc test was used, with significance levels of *$p < 0.05$ and **$p < 0.01$.

We further explored the impact of voluntary running on hippocampal neurogenesis utilizing immunohistochemical markers: Ki-67 for proliferating cells (Fig. 4.7a) and doublecortin (DCX) for immature neurons (Fig. 4.7b). Four weeks of voluntary running elevated the number of Ki-67^+ cells in the hippocampal dentate gyrus (DG) of stressed mice (Fig. 4.7e, $P < 0.001$). Post hoc analysis indicated no significant difference in Ki-67^+ cell count in the dorsal DG between CUS and CUS runner mice (Fig. 4.7c, $P > 0.05$), whereas running markedly enhanced the number of Ki-67^+ cells in the ventral DG (Fig. 4.7d, $P < 0.0001$).

Regarding immature neurons, chronic stress did not significantly affect the number of DCX^+ cells (Fig. 4.7f-h, $P > 0.05$). In contrast, running significantly increased the number of DCX^+ cells in both the ventral (Fig. 4.7g, $P < 0.0001$), dorsal (Fig. 4.7f, $P < 0.001$), and entire DG regions (Fig. 4.7h, $P < 0.0001$) in stressed mice. These findings highlight the promoting effects of voluntary running on hippocampal neurogenesis, particularly within the ventral DG in stressed mice. To explore the potential link between peripheral apelin levels and adult hippocampal neurogenesis, we conducted a correlation analysis. Our results demonstrated a positive correlation between serum apelin levels and DCX^+ cells in the dentate gyrus (DG) (Fig. 4.7i, $P < 0.005$, $r^2 = 0.4209$). Additionally, there was a trend towards a positive correlation with Ki-67^+ cells in the DG (Fig. 4.7j, $P = 0.0789$, $r^2 = 0.2041$). These findings suggest that serum apelin levels may be associated with adult hippocampal neurogenesis.

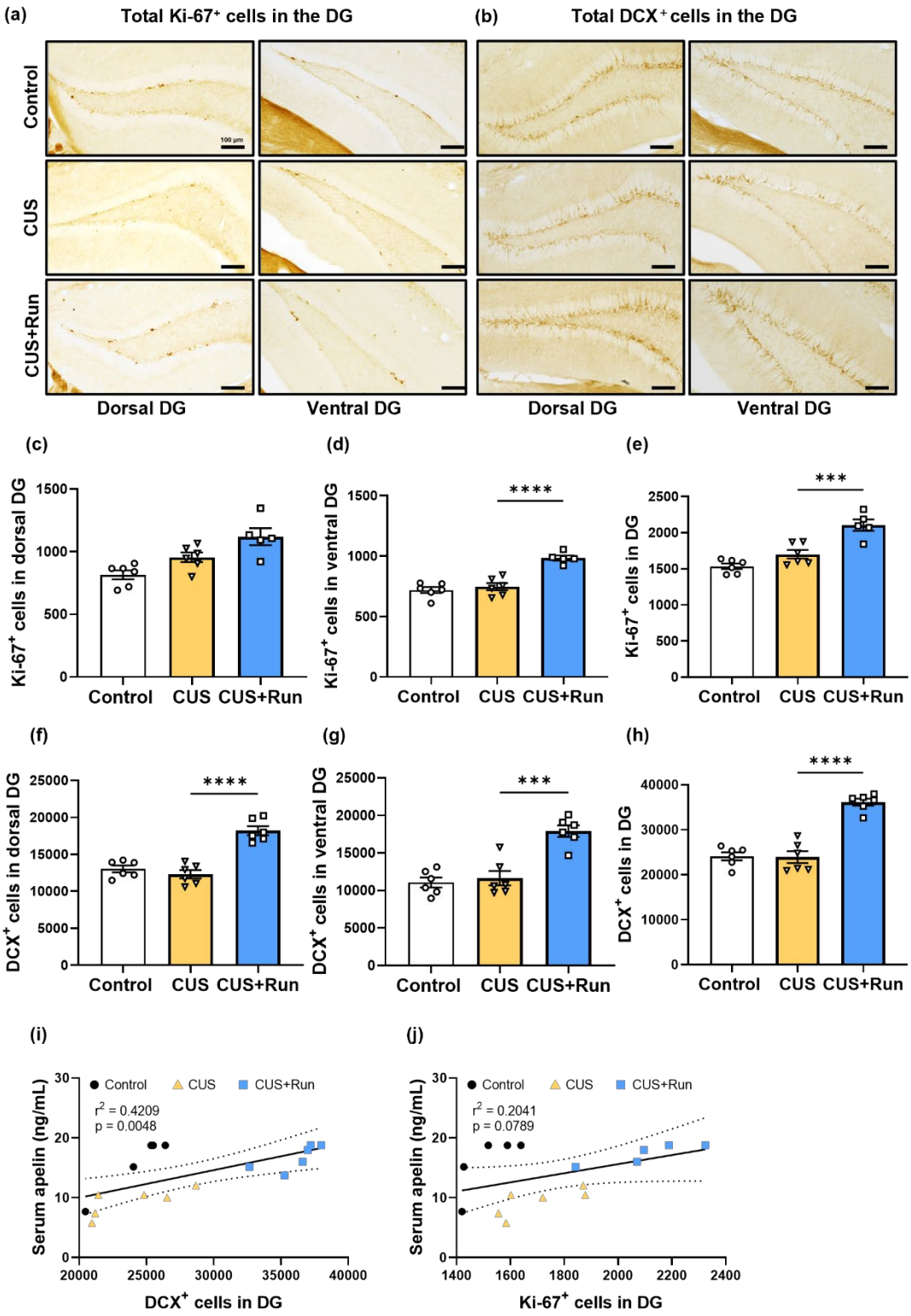

Figure 4.7 Chronic voluntary running promoted adult hippocampal neurogenesis in stressed mice.

(a) and (b) immunohistochemical staining for Ki-67 and DCX in the DG of adult mice under CUS and its combination with running. CUS: chronic unpredictable stressed mice; CUS+Run: CUS runner mice. Voluntary running promoted the number of Ki-67^{+} cells

in (c) the dorsal DG, (d) the ventral DG, and (e) the entire DG of stressed mice. Similarly, running increased the number of DCX^+ cells in these (f-h) DG regions of stressed mice (scale bars, 100 μm in 100×). (i) Linear regression analysis showed a positive correlation between DCX^+ cells in DG and serum apelin levels. (j) Linear regression analysis showed a positive correlation between Ki-67^+ cells in DG and serum apelin levels. Data are presented as mean ± S.E.M. Statistical significance, as assessed by One-way ANOVA with Tukey's post hoc test, and linear regression analysis: ***$p < 0.001$, ****$p < 0.0001$. DCX: doublecortin, DG: dentate gyrus.

To eliminate the possibility that depressive-like behaviors were associated with muscle function loss in stressed mice, we examined changes of muscle mass, muscle strength, and muscle apelin expression levels. Neither CUS nor voluntary running significantly affected body weight gain (Fig. 4.8a, $P > 0.05$). In terms of muscle mass, CUS did not alter muscle weight (Fig. 4.8b, $P > 0.05$). However, voluntary running increased the muscle weight of the lower limbs in stressed mice (Fig. 4.8b, $P < 0.05$). Neither CUS nor voluntary running had significant effects on the muscle weight of the plantar flexors and extensors (Fig. 4.8d-g, $P > 0.05$, Control vs. CUS and CUS vs. CUS+Exe). Voluntary running increased gastrocnemius muscle weight compared to the control group (Fig. 4.8d, $P < 0.05$).

Regarding muscle strength, post hoc analysis revealed that voluntary running significantly increased four-limb muscle strength (Fig. 4.8c; $P < 0.01$) in stressed mice. We further assessed whether CUS affects apelin expression in the muscles. The results indicated that CUS decreased APLN mRNA levels in the Gas muscles (Fig. 4.8h, $P < 0.05$) but had no effect on the TA muscles (Fig. 4.8i, $P > 0.05$). Notably, 4 weeks of running increased APLN mRNA expression levels in both the Gas muscle (Fig. 4.8h, $P < 0.01$) and TA muscle (Fig. 4.8i, $P < 0.05$) in stressed mice. These findings suggest that CUS did not affect muscle strength and mass, whereas voluntary running significantly enhanced both muscle strength and apelin expression levels.

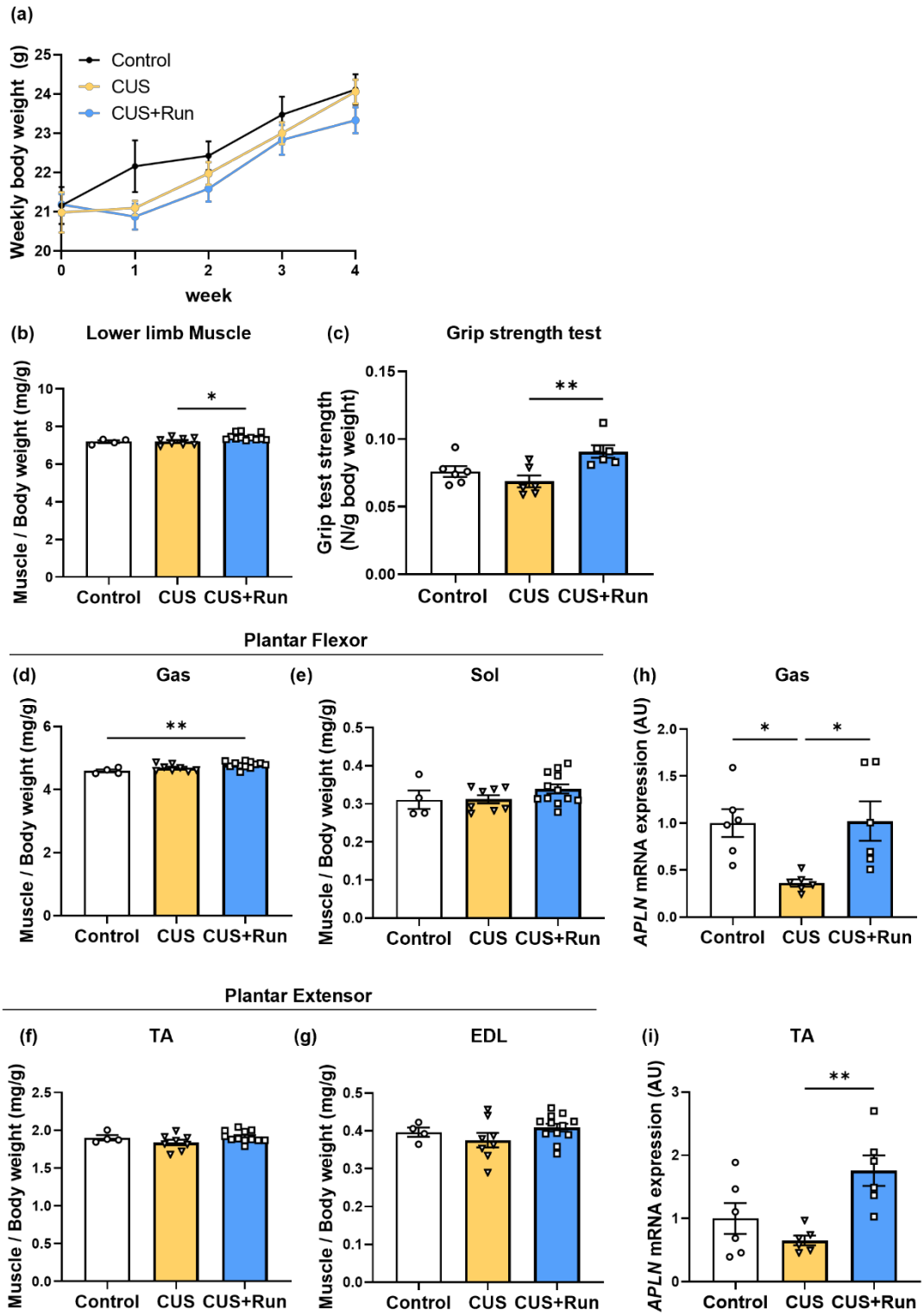

Figure 4.8 Chronic voluntary running promoted muscle function and muscular APLN mRNA levels in stressed mice.

(a) Changes of body weight gain throughout the four-week period of voluntary running on sedentary and runner mice. CUS: chronic unpredictable stressed mice; CUS+Run: CUS runner mice. Chronic running increased (b) the lower limb muscle mass and

enhanced (c) four-limb grip strength in stressed mice. Running increased Weights of (d) Gastrocnemius (Gas) compared to the control mice but did not affect (e) Sol, (f) TA, and (g) EDL, with Gas and Sol as markers of plantar flexor mass and TA and EDL as indicators of plantar extensor mass. Running increased APLN mRNA expression levels in (h) Gas and (i) TA muscles of stressed mice. Data are presented as mean ± S.E.M. A one-way ANOVA followed by Tukey's post hoc test was used, with significance levels of *$p < 0.05$ and **$p < 0.01$. Gas: gastrocnemius, Sol: soleus, TA: tibialis anterior, EDL: extensor digitorum longus.

4.3. Discussion

In this chapter, we demonstrated that 4-week voluntary running increased the expression levels of apelin and its receptor APJ in the hippocampus and elevated serum apelin concentration. Changes of apelin levels were positively correlated with antidepressant effects. We found that stress significantly decreased protein expression of apelin and APJ in the hippocampus. On the other hand, 4-week voluntary running rescued depression-like behaviour, in concurrent with increased apelin and APJ levels and enhanced neurogenesis in the hippocampus. Additionally, we identified that the lower limbs skeletal muscles are the primary sources of apelin secretion in mice with voluntary running. These findings indicate that muscle-secreted apelin may play a crucial role in mediating the anti-depressant effects and pro-neurogenic effects of voluntary running

4.3.1. The antidepressant effects of voluntary running

Voluntary running in terms of unlimited access to running wheels in the holding cages is frequently used in rodent studies, with a benefit over forced running due to its less stressful and more voluntary nature. The antidepressant effects of voluntary running have been well-documented, and our findings consistently showed that voluntary wheel running reduced depression-like behaviors including increased sucrose preference in the SPT, promoted grooming time of the SST, as well as reduced immobility time at the FST. These results align with previous studies showing that chronic running reduced immobility time in the FST [307]. Similarly, voluntary running for 14-21 days has been shown to decreased immobility time [29,308], with effects that are comparable to the antidepressant amitriptyline.

Anhedonia and reduced exploratory interest are significant symptoms of depressive disorder, also observed in patients suffering from cognitive disorder [309,310]. Indicators such as sucrose preference and grooming behavior reflect an animal's ability to experience pleasure, with increased sucrose preference and grooming time linked to reduced anhedonia Consistent with our observations of the anti-anhedonic effects of voluntary running, previous studies have revealed that aerobic exercise significantly improves depression-like behavior in rats, evidenced by increased sucrose preference [311,312]. Further, voluntary running has been reported to mitigate the effects of maternal separation stress on depressive-like behaviors in adult male rats, as evidenced by

increased grooming activities in the splash test [313]. This evidence strongly supports the notion that running can serve as a beneficial intervention for anhedonia in rodents.

We found that voluntary running has no significant effect on the anxiety-like behavior in the OFT, consistent with findings that voluntary wheel running is primarily used to study motricity in rodent research rather than anxiety-related behaviors [314]. However, our results show that the 4-week voluntary running decreased the travel distance in the OFT. This aligns with research suggesting that while voluntary wheel running promotes endurance-like adaptations in skeletal muscles, it may also lead to acute fatigue post-running [315]. The decrease in locomotor activity could be related to the central fatigue hypothesis, where prolonged exercise affects the central nervous system and reduces subsequent physical performance [316]. The effects of voluntary running on locomotor activity can rely on the duration and intensity of the exercise, as well as the specific strain of rodent used. Typically, C57Bl/6J male mice run nearly 4 km per day [317,318]. In this study, however, mice exhibited an average running distance at least double the norm for this strain (Fig. 4.2a). The two-fold difference in recorded running activity can be attributed to factors such as the lubrication level of the running wheel axle, the number of mice using the wheel, and the sensitivity of the detector. Despite the excessive number of runs recorded due to these factors, the antidepressant benefits associated with voluntary running remained unaffected.

By using the CUS mouse model, we re-emphasized the antidepressant effect of voluntary running. CUS is widely used to simulate depression-like behavior in rodents due to its ability to evoke behaviors analogous to human depression symptoms [319]. Our findings align with previous literature indicating that higher immobility time in the FST suggests CUS can reduce passive coping strategies in mice, a behavior often linked to depressive states [320]. The reduction in grooming behavior after CUS observed in our study is supported by previous research [321,322], although changes in sucrose preference in the CUS model were not significant. These results illustrate that CUS induces depressive-like and anhedonia-like behaviors, and the reversal effect of voluntary running further illustrates the importance of physical exercise as an antidepressant treatment.

In our CUS model, no anxiety-like behaviors were observed in the OFT, even though chronic stress has been reported to induce anxiety. The effectiveness of the CUS model in inducing anxiety-like behaviors is controversial; some studies report an

increase in anxiety-like behaviors, while others, like ours, report no significant change [323,324]. This discrepancy could be attributed to the complexity of stress responses, where not all stress paradigms uniformly lead to heightened anxiety, depending on factors such as the type of stressors used and their predictability. In sum, while our study supports the CUS as a model for inducing certain depression-like behaviors in mice, it also highlights the variability in response to chronic stress.

4.3.2. Voluntary running promotes adult hippocampal neurogenesis and APJ protein expression in stressed mice.

Our study reveals that voluntary running has a differential impact on serum apelin levels in healthy and CUS mouse models. Apelin, a bioactive peptide critical for cardiovascular homeostasis, energy metabolism, and fluid balance, is influenced by various physiological conditions [214]. In healthy mice, 4-week voluntary running reduced serum apelin levels. Conversely, in the CUS mouse model, the same running regimen prevented the expected decline in serum apelin levels. In Chapter 2, we found that long-term exercise, such as 8-week endurance exercise and marathon, down-regulates apelin levels in the blood [209,210]. This may reflect a physiological adaptation to regular exercise, where apelin, known to be involved in energy metabolism and cardiovascular homeostasis, is modulated in response to increased physical activity [21,214]. On the other hand, voluntary running improved serum apelin levels in CUS models, suggesting a protective or restorative effect on stress-induced alterations in apelin signaling. This aligns with previous research indicating that running can increase serum apelin levels, which may benefit metabolic diseases such as diabetes [21]. These findings underscore the potential of voluntary running as a non-pharmacological intervention to modulate apelin levels, with implications for managing metabolic disorders and stress-related conditions.

Voluntary running counteracted the effects of CUS not only by elevating serum apelin levels but also by improving apelin and APJ protein expression in the hippocampus. APJ, is highly expressed in the brain, particularly in the hippocampus [241,325]. Notably, we found that 4-week voluntary running upregulated apelin and APJ expression levels in the hippocampus, but CUS had no significant effects. Pre-clinical research suggests that environmental stress can modulate apelin and APJ signaling in the brain. For example, short-term forced swimming stress have been demonstrated to increase apelin and APJ expression levels in the hippocampus [18,285]. But they also

showed that exogenous apelin-13, a specific isoform, reversed stress-induced depression-like behavior. Furthermore, stressed rats show increased apelin protein expression in the medulla [326]. Anne-Marie O'Carroll [284] has showed that acute stress upregulates the expression levels of APJ in the hypothalamus. Recent studies have indicated that significant depression-like behavior is associated with upregulated APJ levels but decreased apelin in the hypothalamus [22]. It is hypothesized that elevated APJ levels may be an endogenous stress coping mechanism during stress-induced pathogenic processes [21], but the specific mechanisms require further investigation.

Adult hippocampal neurogenesis, the process of generating new neurons in the hippocampus, is closely linked to the behavioral neurobiology of stress-related disorders [327]. In our study, voluntary running increased the population of Ki-67$^+$ and DCX$^+$ cells in DG of stressed mice, although chronic stress alone had no effect on hippocampal neurogenesis. Previous research by our team has reported that running promotes neurogenesis and is a potent stimulator of BDNF, supporting the proliferation and differentiation of new neurons [29,328].To further explore the association between apelin and hippocampal neurogenesis, we conducted a correlation analysis between serum apelin levels and markers of hippocampal neurogenesis. Our analysis revealed a positive correlation, indicating that peripheral apelin may serve as a potential indicator of hippocampal neural regeneration. In parallel, we found that CUS did not affect the number of the number of Ki-67- and DCX-positive cells in the DG, nor did it alter the protein expression of apelin and its receptor APJ in the hippocampus. Conversely, voluntary running upregulated both adult neurogenesis and the expression of apelin and APJ proteins. These findings collectively indicate that apelin is a potential indicator of neurogenesis.

The upregulation of apelin and its receptors could be part of this neurogenic response, as apelin has been implicated in neuroprotection and neurotrophic processes [329]. Recent research highlights the potential neuroprotective and neurogenic roles of apelin. For instance, apelin has been demonstrated to reduce neuroinflammation and promote the proliferation and differentiation of native NSCs following spinal cord injury [330]. This suggests that apelin could have a similar role in the brain, possibly influencing neurogenic niches to support new neurons' growth and survival. Another study showed that apelin-13 counteracted stress and protected neurons from neurotoxicity induced by glutamate exposure, with its neuroprotective action associated

with the PGC-1α-FNDC5-BDNF pathway. The results may have suggested therapeutic potential of apelin for promoting brain health and protection against cognitive decline and depression [287]. Given these findings, it's reasonable to hypothesize that apelin could play a significant role in adult neurogenesis, contributing to brain plasticity and resilience. However, further research is necessary to fully elucidate how apelin influences neurogenesis in the adult brain and its therapeutic role in neurological conditions.

Our results showed that voluntary running increased mRNA expression levels of APLN and APJ in the lower limb muscles, specifically the Gas and TA muscles, following voluntary running, suggesting localized upregulation of the apelinergic signaling in response to physical activity [214]. It has been previously reported that 4-week treadmill running [17] and single bout of endurance maternal exercise [215] increased APLN mRNA expression in TA and Gas muscles. This muscle specific expression is consistent with the notion that skeletal muscle acts as an endocrine organ, capable of expressing myokines in an autocrine [17] and paracrine manner [215,331]. The corresponding increase in muscle mass further supports the role of apelin as a growth factor, promoting muscle hypertrophy and potentially enhancing muscle function [206]. Interestingly, our CUS model did not affect muscle strength and mass, yet it inhibited apelin expression in the gastrocnemius. This may indicate a stress-induced disruption of the apelinergic system in regulating muscle physiology, suggesting apelinergic signaling pathway could be a target for therapeutic intervention [332]. Increase in muscle strength in CUS mice with exercise suggests that physical activity may counteract some of the detrimental effects of stress on muscle biology [333].

The question of whether running-induced muscle apelin secretion is associated with hippocampal neurogenesis and antidepressant effects remains unknown. The apelin and APJ pathway has been implicated in depressive symptoms and is known to have neuroprotective roles [328]. Moreover, running is repeatedly recognized for its benefits on promoting mental health particularly its antidepressant effects [334]. Therefore, it is plausible to hypothesize that muscle-derived apelin may influence brain plasticity and contribute to running-induced antidepressant effects, potentially through mechanisms involving hippocampal neurogenesis. In conclusion, our study highlights the impact of voluntary running on muscle-secretory apelin in antidepressant response, and the therapeutic potential of targeting the apelin and APJ pathway. Further research

is warranted to explore this novel role of muscle-secreted apelin in mediating the antidepressant effects of physical exercise and its signaling pathway in the brain.

Chapter 5. Myokine Apelin Mediates Physical Exercise-induced Adult Neurogenesis in the Hippocampus

5.1. Introduction

Major depressive disorder (MDD), commonly known as depression, is a widespread and serious mood disorder characterized by persistent feelings of sadness and a significant reduction in interest or pleasure in activities that were previously found enjoyable or rewarding [31,54]. This mood disorder is the culmination of an intricate interplay between genetic predispositions, environmental pressures, and psychological resilience [31,58]. Depression has been linked to imbalances in neurotransmitters such as serotonin, which regulate brain development, neural connections, motivation, reward, and mood [46,47]. Additionally, depression is associated with structural abnormalities in the hippocampus, amygdala, and prefrontal cortex— key brain regions that play crucial roles in mood regulation, stress response, and executive function [335,336]. Depression is also linked to decreased adult neurogenesis (the formation of new neurons) and neuroplasticity (the brain's ability to reorganize and form new neural connections), which may affect how the brain responds to environmental stimuli and stressors [29,290,291].

Emerging research suggests muscle atrophy as a potential harbinger of depression, as evidenced by clinical studies linking sarcopenia in older adults with depressive symptoms [119,120,126]. In addition, recent studies across various age groups suggest a bidirectional link between muscle health and depression, indicating that lower muscle mass and strength are significantly associated with increased depressive symptoms [89,125,126,128], while robust muscle strength may offer protective benefits against depression [129]. However, the specific mechanisms in the muscle-brain crosstalk in depression remain elusive.

Physical activity is acknowledged as a treatment method for both depression and sarcopenia. Recent reviews of multiple studies consistently demonstrate that physical exercise has a positive impact on muscle strength, including handgrip, knee extension, and lower body strength, as well as physical performance measures like walking speed and mobility, especially in older adults with sarcopenia [144–146]. There are differences in findings regarding the effects of exercise on muscle mass across different studies. Specifically, resistance training appears to be more effective than other types of exercise, such as mixed training (which includes resistance, balance, and aerobic exercises) and

whole-body vibration training, in improving mobility outcomes such as knee strength, walking speed, balance, and agility [144]. Myokines, which are produced and released by muscle cells when they contract during physical activity, have a critical role in communication among muscles and the brain [176]. Importantly, changes in the secretion of myokines, including BDNF, irisin, FGF21, and apelin, have been linked to the development and progression of depression in older adults [174,337,338]. These myokines may have antidepressant effects by influencing pathways like the HPA axis [55,67]. However, the specific roles of myokines in depression associated with sarcopenia have not been fully explored. More research is necessary to understand how myokines protect the brain in the context of sarcopenia-related depression.

Apelin, as a myokine, has been shown to be stimulated by exercise, and be able to alleviate the muscle weakness associated with aging [17]. Previous research has shown that antidepressant effects of exogenous apelin (apelin-13) are related to alleviating neuroinflammation via reducing pro-inflammatory M1 markers [275], and inhibiting neuronal death in the hippocampus [287]. However, whether apelin upregulates hippocampal plasticity remains unknown. In Chapter 4, we found an association between the antidepressant effects of voluntary running and increased apelin levels in the serum, along with elevated expression levels of apelin and APJ in the muscle and hippocampi. However, whether the antidepressant effects of running are mediated by muscle-secreted apelin is not clear. Therefore, in this chapter, we aimed (1) to reveal whether the myokine apelin is a key mediator for running-mediated antidepressant effects using approaches of knocking out or overexpressing apelin in the muscles, and (2) to elucidate the critical role of muscle-secreted apelin as a physical exercise mediator for enhancing hippocampal neuroplasticity and alleviating depressive symptoms.

5.2. Results

5.2.1. Skeletal muscle-specific apelin knockout mice diminished anti-anhedonic and antidepressant effects of voluntary running

To determine whether apelin deficiency in skeletal muscle affects muscle mass and strength, body weight changes were monitored for 4 weeks in APLN-mKO mice, followed by muscle strength tests (Fig. 5.1a). APLN-mKO mice at the age of 10 weeks exhibited a 41.15% reduction in APLN mRNA levels in the gastrocnemius muscle (Fig. 5.1c; $P < 0.05$). In comparison to Flox mice (control), APLN-mKO mice at the age of 8 weeks demonstrated increased body weight gain (Fig. 5.1b; $P < 0.05$), reduced grip strength of all four limbs (Fig. 5.1d; $P < 0.01$, alongside with a shorter latency to fall in the accelerated rotarod test (Fig. 5.1e; $P < 0.05$). These findings suggest that muscle-specific apelin knockout impairs muscle strength and motor coordination.

Interestingly, muscle-specific apelin knockout did not affect the muscle mass-to-body weight ratios of the TA, Gas, EDL, and Sol muscles (Fig. 5.1f-i; $P > 0.05$).

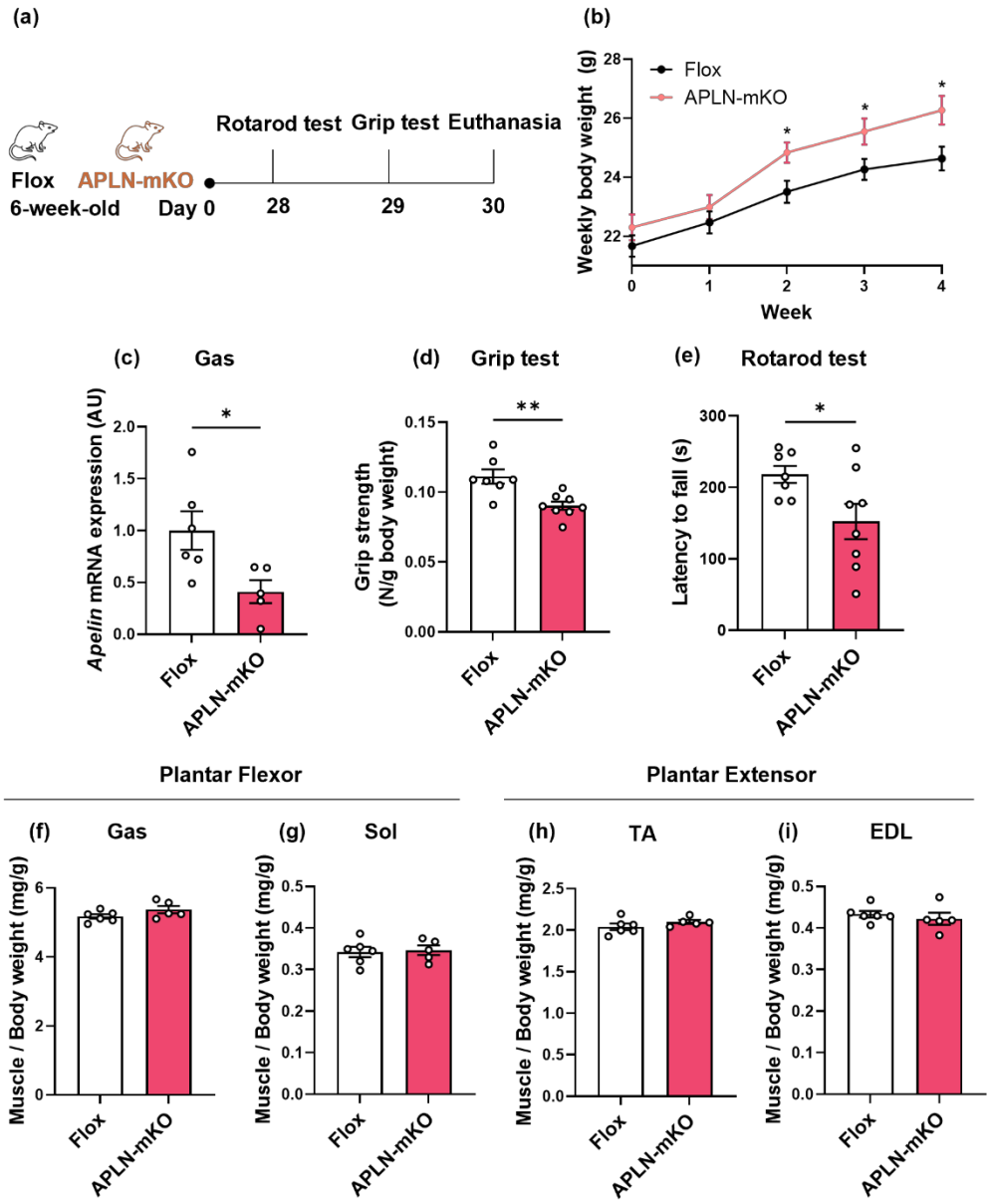

Figure 5.1 Muscle-specific apelin knockout reduced muscle strength but not muscle mass.

(a) Study timeline. (b) Weekly body weight changes. Muscle-specific apelin knockout mice significantly reductions in (c) APLN mRNA expression levels in Gas, (d) four-limb grip strength in the grip test, and (e) latency to fall in the rotarod test. Muscle apelin deficiency has no effects on muscle mass of the (f) Gas, (g) Sol, (h) TA, and (i) EDL. Data are presented as mean ± S.E.M. Statistical significance assessed using unpair T-test: *$p < 0.05$, **$p < 0.01$. Gas: gastrocnemius, Sol: soleus, TA: tibialis anterior, EDL: extensor digitorum longus.

In Chapter 4, we demonstrated that the level of anhedonia-like behavior was in a negative correlation with serum apelin levels. To determine whether myokine apelin plays a critical role in mediating beneficial effects of physical exercise on reducing depressive behaviors and promoting adult hippocampus neurogenesis, we subjected 6-week-old APLN-mKO mice and Flox mice to 4-week voluntary running (Fig. 5.2a). APLN-mKO runners exhibited a comparable daily running activity to Flox (control) runners (Fig. 5.2b-c; Flox: 7627±899 m, APLN-mKO: 6949±1470 m; $P > 0.05$).

Anhedonia-like behaviors were assessed by sucrose preference test (SPT) and the sucrose splash test (SST). In the SPT, two-way ANOVA indicated a significant effect of muscle apelin knockout on sucrose preference (Fig. 5.2d; $F(1, 28) = 26.14$, $P < 0.0001$), no main effects of running (Fig. 5.2d; $F(1, 28) = 1.686$, $P = 0.0687$), but significant interaction effects (Fig. 5.2d; $F(1, 28) = 11.44$, $P < 0.01$). Post-hoc test showed that voluntary running decreased anhedonia-like behavior in Flox mice (Fig. 5.2d; $P < 0.05$ for Flox + Sed vs. Flox + Run), but not in APLN-mKO mice (Fig. 5.2d; $P > 0.05$ for APLN-mKO + Sed vs. APLN-mKO + Run). Similar results were observed in the SST, confirming the apelin is linked to running-reduced anhedonia-like behavior. Two-way ANOVA showed that neither muscle apelin knockout nor their interaction significantly affected grooming time (Fig. 5.2e; $F(1, 28) = 4.146$, $P > 0.05$ for Apelin KO effects; $F(1, 28) = 4.057$, $P > 0.05$ for interaction effects), but there was a significant running effect (Fig. 5.2e; $F(1, 28) = 4.891$, $P < 0.01$). The Mann-Whitney test indicated that voluntary running increased grooming time in Flox (control) mice (Fig. 5.2e; $P < 0.05$, Flox + Sed vs. Flox + Run), whereas muscle apelin knockout significantly diminished the effects of running on enhancing grooming time (Fig. 5.2e; $P < 0.01$, Flox + Run vs. APLN-mKO + Run).

Anxiety-like behavior was assessed using the open field test (OFT). Two-way ANOVA revealed that there were no significant effects of either running or muscle apelin knockout on locomotor activity and \time spent in the center of the OFT (Fig. 5.2f-g; effect of APLN-mKO and voluntary running: $F(1, 28) = 0.5747$, and 0.3584, $P > 0.05$; interaction effect: $F(1, 28) = 3.616$, $P = 0.0676$). These findings indicate that muscle apelin is required for voluntary running to elicit antidepressant effects, but not anxiety-like behaviors.

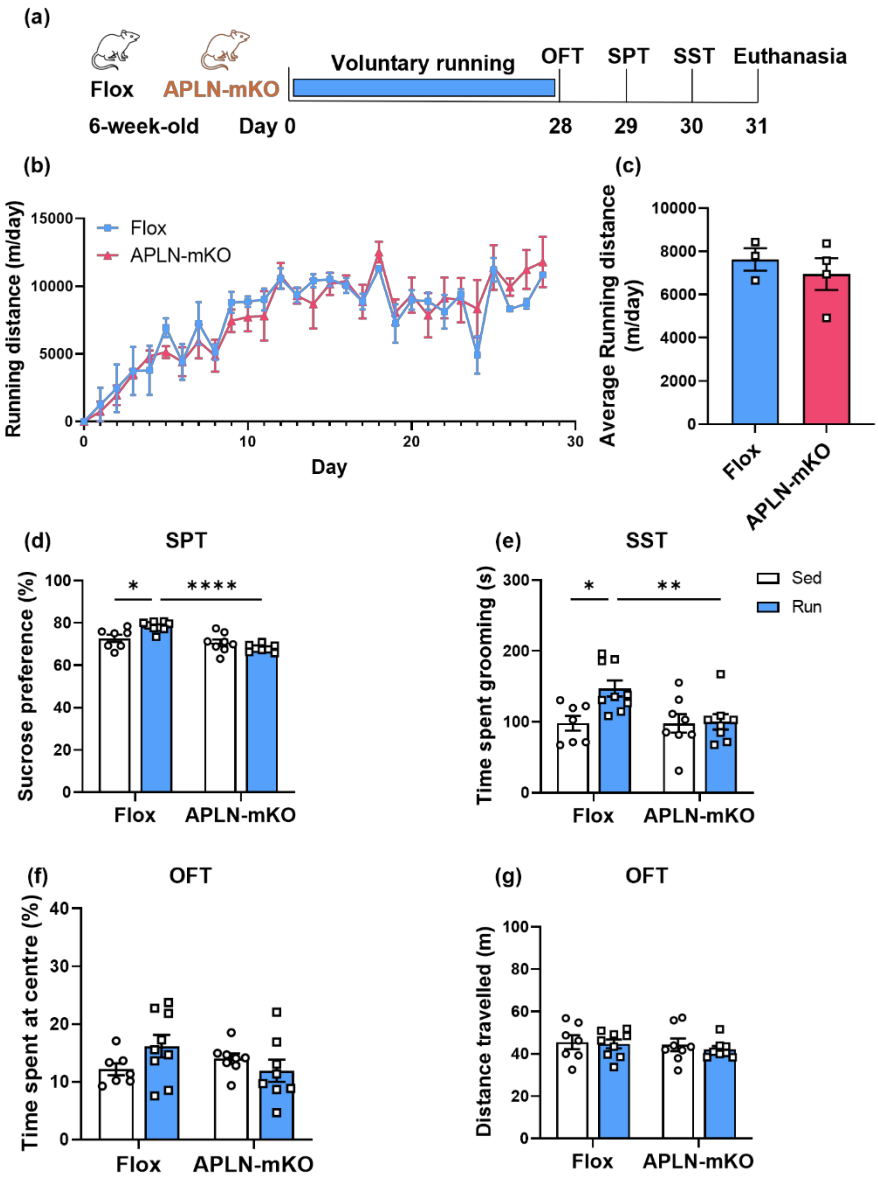

Figure 5.2 Skeletal muscle-specific knockout of apelin diminished anti-anhedonic effects of 4-week voluntary running.

(a) Study timeline. Flox: control mice with APLN-Flox background; APLN-mKO: muscle specific apelin knockout mice; Sed: sedentary mice; Run: runner mice. (b) Daily running distance of 4-week voluntary running. In runner mice, muscle specific apelin knockout did not affect (c) the average daily running distance but abolished the increase in (d) sucrose preference in the SPT, and (e) grooming time in the SST. Muscle specific apelin knockout did not affect (f) time spent in the center, and (g) total distance traveled in the OFT. Data are presented as mean ± S.E.M. Statistical significance assessed using unpair T-test; two-way ANOVA with Tukey's post hoc test: *p < 0.05, **p < 0.01,

****p < 0.0001. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test.

We further examined whether muscle apelin knockout affects serum apelin levels and depressive phenotypes. The two-way ANOVA demonstrated that muscle specific apelin knockout significantly reduced serum apelin levels (Fig. 5.3a; $F(1, 12) = 10.70$, $P < 0.01$), though there were no main effect of running and their interaction (Fig. 5.3a; $F(1, 12) = 1.696$ and 1.226, $P > 0.05$). Post hoc test showed that muscle apelin deficiency decreased serum apelin levels in runner mice (Fig. 5.3a; $P < 0.05$, Flox + Run vs. APLN-mKO + Run). Notably, voluntary running in Flox mice did not alter serum apelin levels (Fig. 5.3a; $P > 0.05$, Flox + Exe vs. APLN-mKO + Exe).

To elucidate the relationship between serum apelin levels and behavioral outcomes, we conducted correlation analyses. Serum apelin levels exhibited a positive correlation with percentage of sucrose preference, indicating a role of apelin in anhedonia-like behaviors as examined in the SPT (Fig. 5.3b; $R^2 = 0.2843$, $P < 0.05$). However, there was no correlation between serum apelin levels and grooming behaviors in the SST (Fig. 5.3c; $R^2 = 0.0722$, $P > 0.05$). These findings suggest that lack of muscle apelin reduces serum apelin levels which could be linked to anhedonia-like behaviors.

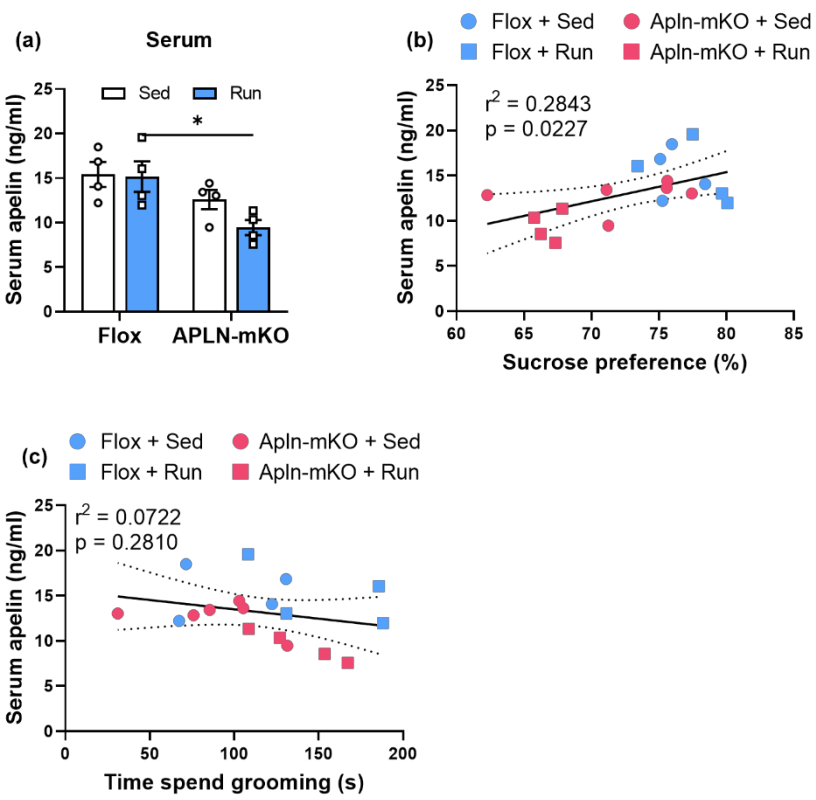

Figure 5.3 Skeletal muscle specific apelin knockout decreased serum apelin levels, which was associated with anhedonia-like behaviors.

(a) Skeletal muscle specific apelin knockout mice with running displayed significantly lower serum apelin levels when compared to Flox control runners. Flox: control mice with APLN-Flox background; APLN-mKO: muscle specific apelin knockout mice; Sed: sedentary mice; Run: runner mice. (b) Linear regression analysis showed a positive correlation between sucrose consumption percentage in sucrose preference test and serum apelin levels. (c) Linear regression analysis showed no correlation between time spent grooming in sucrose splash test and serum apelin levels. Data are presented as mean ± S.E.M. Statistical significance assessed using Two-way ANOVA with Tukey's post hoc test, and linear regression analysis: *$p < 0.05$.

We further elucidated the role of muscle apelin in mediating the positive effects of voluntary running on hippocampal neurogenesis. Immunohistochemical analyses using Ki-67 labeling for proliferating cells and doublecortin (DCX) staining for immature neurons were used. Two-way ANOVA demonstrated significant effects of voluntary running on increasing the number of Ki-67$^+$ cells in the hippocampal DG (Fig. 5.4a-c; $F(1, 24) = 10.74$, $P < 0.01$ in the ventral DG, $F(1, 24) = 21.53$ and 19.76, $P <$

0.001 in dorsal DG and total DG), indicating increased hippocampal cell proliferation by running. Muscle apelin knockout has significant main effect on the number of Ki-67$^+$ cells in the dorsal DG and total number in the DG (Fig. 5.4a-c; $F(1, 24) = 13.47$ and 8.904, $P < 0.01$) but had no significant effect on the ventral DG (Fig. 5.4a-c; $F(1, 24) = 2.858$, $P = 0.1039$). There were no interaction effects on the number of Ki-67$^+$ cells in the DG (Fig. 5.4a-c; $F(1, 24) = 1.335$, $P > 0.05$). Post hoc test showed that running significantly increased the number of Ki-67$^+$ cells in total DG (Fig. 5.4a-c; $P < 0.01$ the dorsal and ventral DGs; $P < 0.001$ total number in the DG). Conversely, muscle apelin knockout runners showed no increase in number of Ki-67$^+$ cells in the dorsal DG (Fig. 5.4a & c; $P < 0.01$).

In results of examining changes of number of immature neurons in the DG, two-way ANOVA revealed main effects of running and muscle apelin deficiency on the number of DCX$^+$ cells in the DG (Fig. 5.4d-f; main effect of running: $F(1, 24) = 21.02$, $P < 0.001$ in all DGs; APLN-mKO effects: $F(1, 24) = 29.11$, $P < 0.01$ in all DG), but not interaction effect (Fig. 5.4d-f; $F(1, 24) = 3.249$, $P > 0.05$). Post hoc analysis indicated that running increased the number of DCX$^+$ cells in both the dorsal and ventral DGs compared to Flox control group (Fig. 5.4d-f; $P < 0.01$ in dorsal and ventral DG; $P < 0.001$ in total DG). Conversely, in runner mice, muscle apelin deficiency significantly decreased the counts of DCX$^+$ cells in the DG (Fig. 5.4d-f; $P < 0.01$ in ventral DG; $P < 0.001$ in total DG; $P < 0.0001$ in dorsal DG). Our results showed that muscle apelin knockout diminished the effects of voluntary running on promoting adult hippocampal neurogenesis, suggesting the critical role of apelin in mediating the effects of voluntary running on promoting adult neurogenesis.

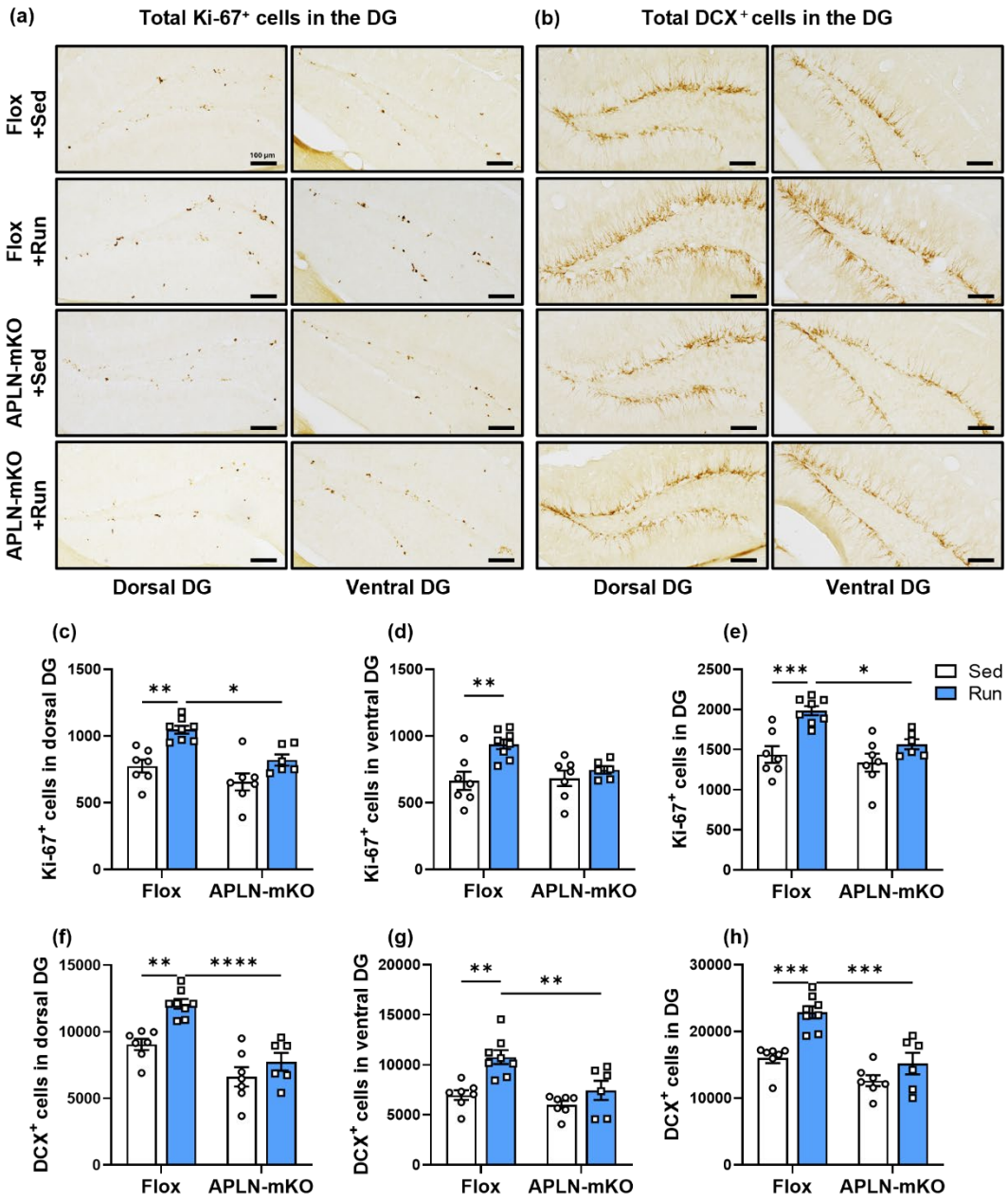

Figure 5.4 Skeletal muscle specific apelin knockout diminished the effects of voluntary running on enhancing adult hippocampal neurogenesis.

(a) and (b) immunohistochemical staining for Ki-67 and DCX in the dentate gyrus. Flox: control mice with APLN-Flox background; APLN-mKO: muscle specific apelin knockout mice; Sed: sedentary mice; Run: runner mice. Skeletal muscle specific apelin knockout diminished the increase of the number of Ki-67^{+} cells in (c) the dorsal DG, (d) the ventral DG, and (e) the entire DG of runner mice (scale bars, 100 μm in 100×). Similarly, skeletal muscle-specific apelin knockout diminished the increase of number of DCX positive cells in these (f-h) DG regions of runner mice. Data are presented as mean ± S.E.M. Statistical significance was determined by Two-way ANOVA with

Tukey's post hoc test, with significance levels of *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. DCX: doublecortin.

5.2.2. Overexpression of apelin in gastrocnemius and tibialis anterior muscles mimics the anti-depressant and pro-neurogenic effects of voluntary running.

In Chapter 4, we confirmed that voluntary running promotes the expression of apelin in muscle, particularly in the gastrocnemius (Gas) and tibialis anterior (TA). To increase muscle specific apelin mimicking the effects of physical exercise in wild type mice, we employed intramuscular injection of an AAV vector driven by MCK promoter to achieve specific muscle expression of apelin (Fig. 5.5a).

Fluorescence signal as reporter gene was observed in the Gas and TA muscles 4-week post-injection (Fig. 5.5b). Western blotting analysis confirmed that AAV-mediated delivery significantly increased apelin levels in the targeted muscles (Fig. 5.5d-e; $P < 0.05$). Notably, apelin overexpression in Gas and TA muscles was associated with elevated serum apelin levels (Fig. 5.5i; $P < 0.01$) and increased apelin and APJ expression in the hippocampus (Fig. 5.5g-h; $P < 0.05$ for apelin, $P < 0.01$ for APJ).

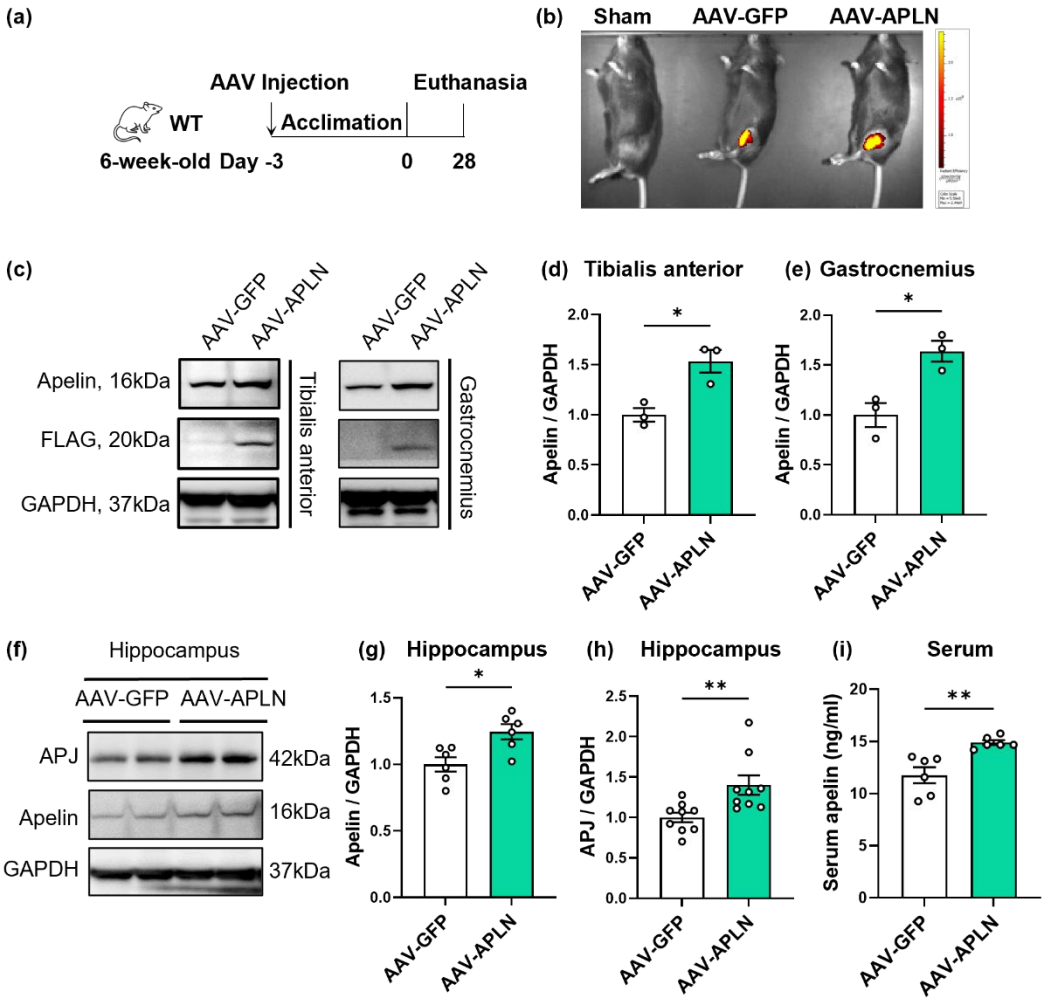

Figure 5.5 Overexpression of apelin in gastrocnemius and tibialis anterior muscles increased protein expression levels of apelin and APJ in the hippocampus.

(a) Experimental timeline. AAV-GFP (Control), and AAV-APLN. (b) Successful intramuscular injection of AAV with reporter gene expression of green fluorescent protein in the TA. (c, f) Western blotting analyses of apelin, FLAG, APJ, tubulin protein levels in the TA, Gas, and hippocampus. Overexpression of apelin in the TA and Gas muscle increased apelin protein expression levels in the (d) TA, (e) Gas, and (g) hippocampus, but had no effect on (h) APJ protein expression in the hippocampus. (i) Overexpression of apelin in the TA and Gas muscles increased serum apelin levels. Data are presented as mean ± S.E.M. Statistical significance assessed using unpaired T-test. *$p < 0.05$, **$p < 0.01$. TA: tibialis anterior, Gas: gastrocnemius.

To determine whether there were antidepressant effects of apelin overexpression in the Gas and TA muscles, we performed behavioral tests four weeks after virus

injection (Fig. 5.6a). Anhedonia-like and depression-like behaviors were evaluated using the SPT, SST, and FST, respectively. Overexpression of apelin in the Gas and TA muscles significantly increased self-grooming time in the SST (Fig. 5.6b; $P < 0.01$) and reduced the time spent immobile in the FST (Fig. 5.6c; $P < 0.05$) but did not affect sucrose consumption ratio in the SPT (Fig. 5.6 d; $P > 0.05$).

Our results showed that apelin overexpression in the muscles decreased locomotor activity (Fig. 5.6e; $P < 0.05$) but did not significantly increase exploration time in the center of the open field (Fig. 5.6f; $P = 0.0764$). The data indicated a decrease in depression-like and anhedonia-like behavior with an increase in muscle apelin expression.

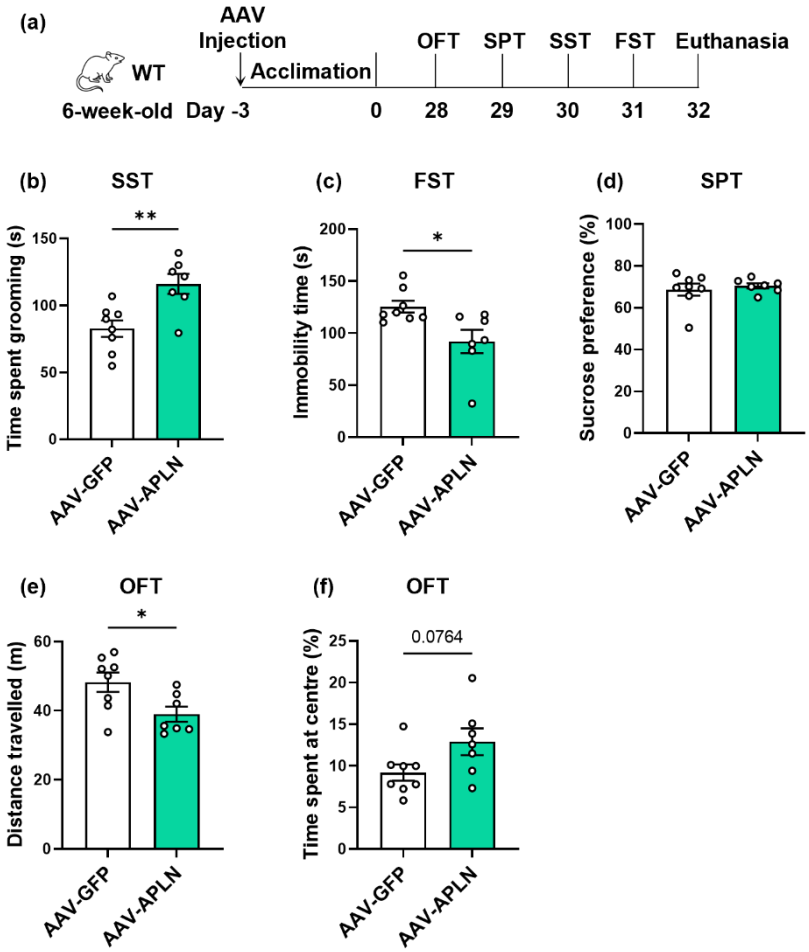

Figure 5.6 Overexpression of apelin in the gastrocnemius and tibialis anterior muscles exerted antidepressant-like behaviors.

(a) Study timeline in mice with intramuscular injection with AAV-GFP or AAV-APLN. Overexpression of apelin in the TA and Gas (b) increased the self-grooming time in SST, (c) decreased the immobility time in FST, (d) but did not affect sucrose

consumption percentage of SPT, indicating the antidepressant effects of muscle apelin. Overexpression of apelin in the TA and Gas decreased (e) the total distance traveled, but (f) did not affect the time spent in the center of OFT. Data are presented as mean ± S.E.M. Unpaired t-test: *p < 0.05, **p < 0.01. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test, FST: forced swim test.

Muscle overexpression of apelin (AV-APLN group) increased number of Ki-67^{+} in both the ventral and dorsal DGs (Fig. 5.7a-c; P < 0.01), and increase number of immature neurons (DCX^{+} cells) , in both the ventral and dorsal hippocampi when compared to AAV-GFP group (Fig. 5.7d-f; P < 0.01 in dorsal DG; P < 0.0001 in ventral DG; P < 0.001 in all DG regions). These findings suggest that overexpression of apelin in the Gas and TA muscles promoted adult hippocampal neurogenesis.

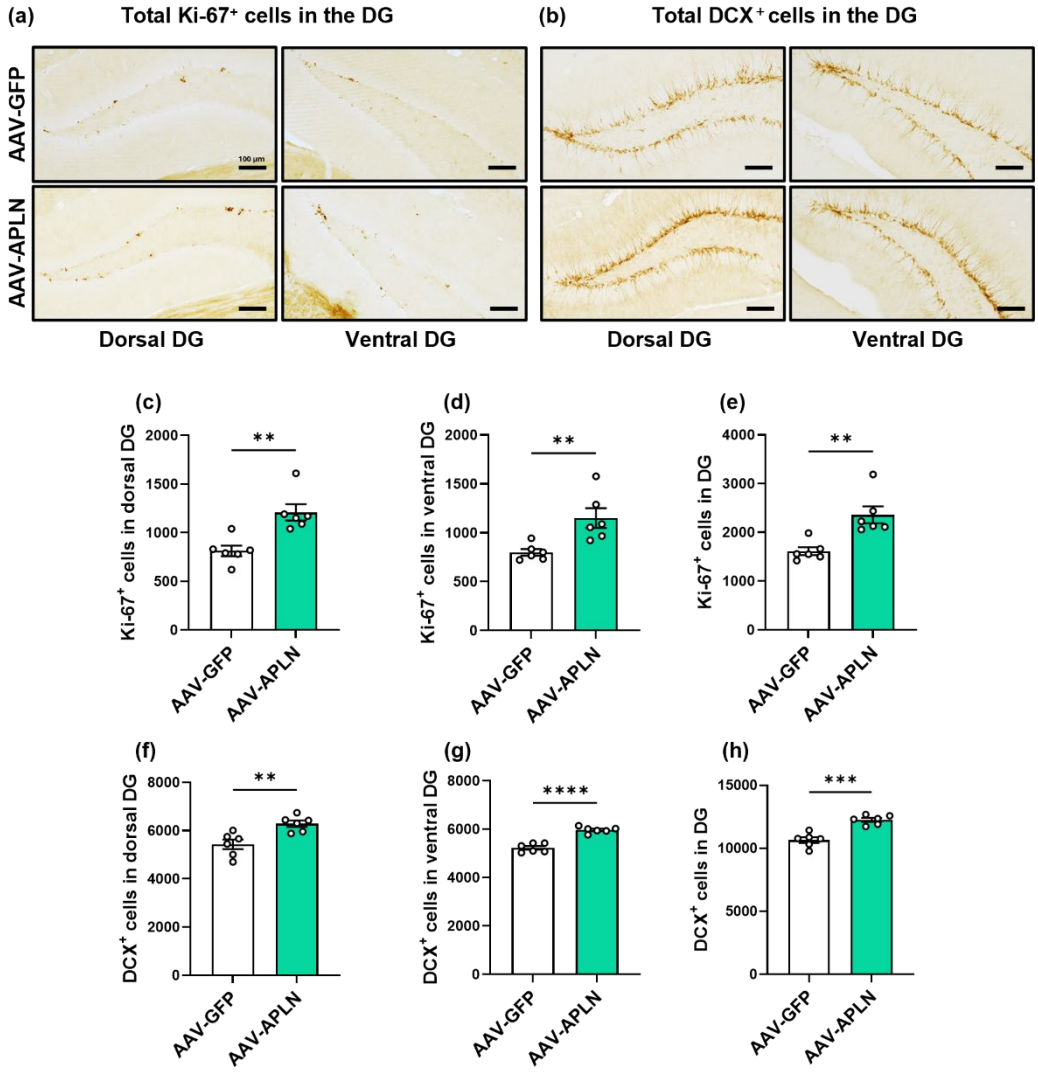

Figure 5.7 Apelin overexpression in the gastrocnemius and tibialis anterior muscles enhanced adult hippocampal neurogenesis.

(a-b) Immunohistochemical staining for Ki-67 and DCX in the DG. Overexpression of apelin in TA and Gas increased the number of Ki-67$^+$ cells in (c) the dorsal DG, (d) the ventral DG, and (e) the entire DG, and (f-h) increased the number of DCX$^+$ cells in these DG regions (scale bars, 100 μm in 100×). Data are presented as mean ± S.E.M. Statistical significance by unpaired Student's t-test: **p < 0.01, ***p < 0.001, ****p < 0.0001. DCX: doublecortin, DG: dentate gyrus.

N-Methyl-D-Aspartate (NMDA) receptor function involves synaptic plasticity, adult neurogenesis, which can impact hippocampal function and are linked to antidepressant effects. However, it remains unclear whether the antidepressant role of apelin is mediated through NMDA receptor function. Therefore, we investigated the impact of apelin overexpression in the Gas and TA muscles on the expression levels of NMDA receptor subunits in the hippocampus. GluN2A and GluN2B are pivotal subunits of the NMDA receptor, a crucial ion channel in the brain modulated by the neurotransmitter [339,340]. Western blotting analysis revealed that overexpression of apelin in the TA and Gas muscles increased phosphorylation of GluN2A at tyrosine 1246 (Y1246) (Fig. 5.8b; P < 0.05) and GluN2B at tyrosine 1472 (Y1472) sites (Fig. 5.8d; P < 0.01) in hippocampal synaptoneurosome extractions, but no effect of protein expression of total GluN2A and GluN2B subunits (Fig. 5.8c & e; P > 0.05). GluN2A phosphorylation at Y1246 and GluN2B phosphorylation at Y1472 site are involved in regulating excitatory synaptic transmission and plasticity [341,342]. Therefore, these findings suggest that apelin may promote synaptic plasticity via enhanced NMDA receptor function. GluA1, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, another crucial ionotropic glutamate receptor for regulating synaptic plasticity in the hippocampus [343,344]. Muscle overexpression of apelin did not alter its expression levels at phosphorylation site S831 (Fig. 5.8f; P = 0.0613), and total AMPA protein expression (Fig. 5.8g; P > 0.05).

Calcium-activated neutral proteases, calpain-1 and calpain-2, that can be modulated by activating glutamate receptors including NMDA and AMPA receptors [345,346]. Calpains (calcium-activated neutral proteases) is reported to be involved in glutamatergic synapses [347]. Our findings demonstrated that apelin overexpression in

muscle significantly increased synaptic calpain-2 expression levels (Fig. 5.8k; $P < 0.01$), but not calpain-1 expression levels (Fig. 5.8j; $P > 0.05$) and postsynaptic density-95 (PSD-95) and synaptophysin (Fig. 5.8l & m; $P > 0.05$).

AMPKα, the catalytic subunit of the 5'AMP-activated protein kinase (AMPK), is crucial for maintaining cellular energy balance and regulating synaptic plasticity [348]. Our data revealed that overexpression of apelin in muscle increased total AMPKα expression levels (Fig. 5.8i; $P < 0.05$), but no effect on AMPKα phosphorylation at T172 (Fig. 5.8h; $P > 0.05$). In summary, our results showed that overexpression of apelin in the Gas and TA muscles could promote phosphorylation of NMDA receptor subunits and increase calpain-2 and AMPKα expression in the hippocampus, suggesting apelin's action on modulating NMDA receptor and downstream signaling calpain-2 in enhancing synaptic function.

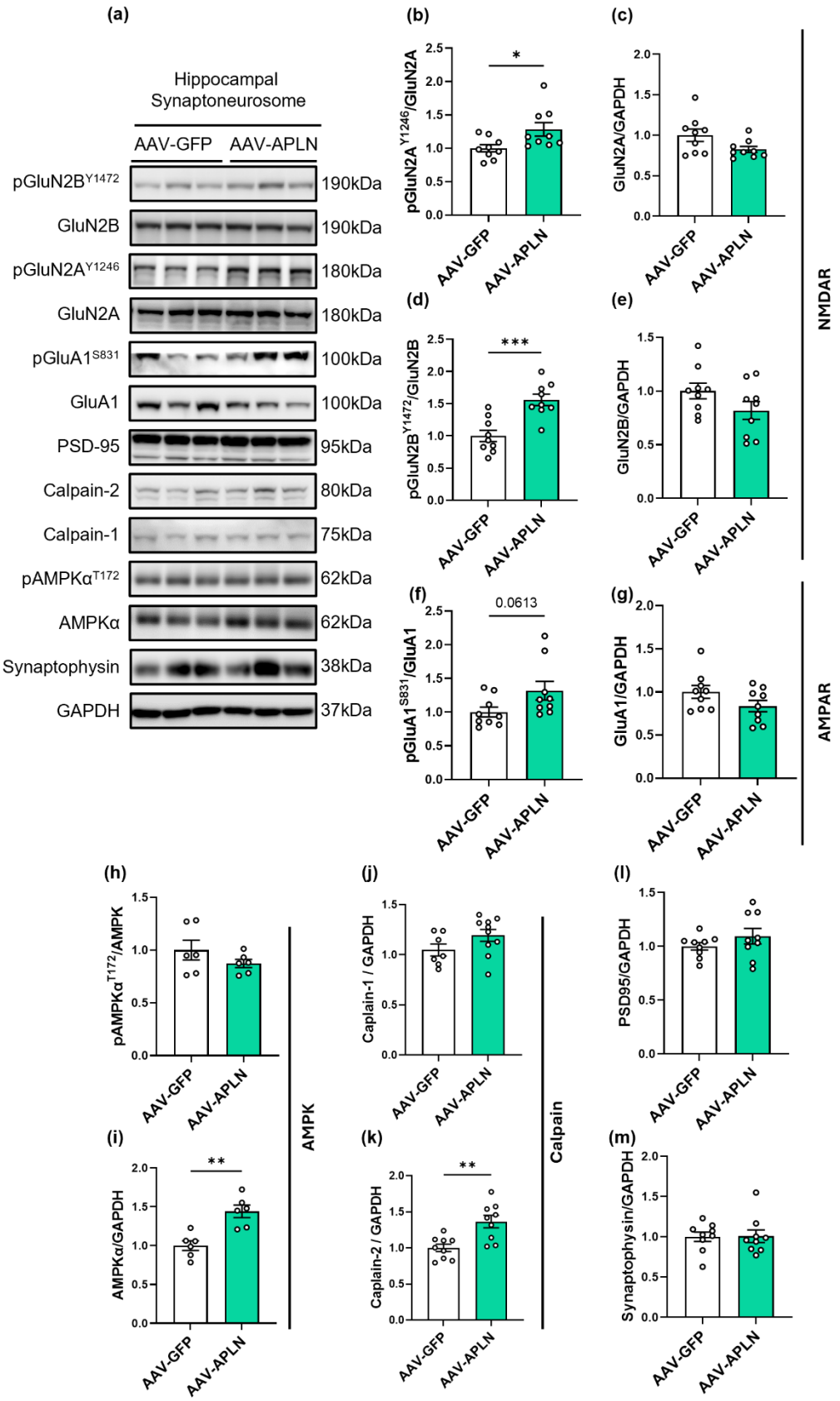

Figure 5.8 Overexpression of apelin in the gastrocnemius and tibialis anterior up-regulated synaptic NMDA receptors and calpain-2 protein expression.

(a) Western blotting analysis of hippocampal synaptoneurosome protein levels. Apelin overexpression increased protein expression levels of (b) phospho-GluN2B (Y1246) and (d) phospho-GluN2A (Y1472) but had no effect on (c) total GluN2B expression levels and (e) total GluN2A. There was no significant effect on (f) phospho-GluA1 (S831) and (g) total GluA1. Apelin muscle overexpression increased (i) total AMPKα expression levels but showed no effect on (h) phospho-AMPKα (T172). Apelin muscle overexpression increased (k) Calpain-2 expression levels but has no effect on (j) Calpain-1, (l) PSD95, and (m) synaptophysin protein expression levels. Data are presented as mean ± S.E.M. Unpaired t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Casein kinase 2 (CK2) is known to mediate synaptic plasticity in the hippocampus [349,350], and the apelin receptor APJ can modulate its activity [351]. However, the relationship between CK2 and apelin-mediated synaptic NMDA receptor functionality remains unclear. Therefore, we first assayed the catalytic subunits CK2α and the stable dimer CK2β in the hippocampal synaptoneurosome (Fig. 5.9a). Western blotting analysis revealed that overexpression of apelin in the TA and Gas muscles resulted in an increase in CK2α (Fig. 5.9b; $P < 0.05$), but had no effect on CK2β (Fig. 5.9c; $P > 0.05$). CK2 is a key factor potentially linking APJ to NMDA receptor phosphorylation [242,245]. Since APJ is not expressed in the synaptoneurosome, we investigated the effect of apelin overexpression on CK2 protein expression in the hippocampal cytosol fraction. We found that apelin overexpression in muscle promoted the expression of CK2α and CK2β in hippocampal cytosol fraction (Fig. 5.9e & f; $P < 0.001$). These data suggest that the overexpression of apelin in the Gas and TA muscles promotes hippocampal CK2 protein expression, potentially mediating NMDA receptor phosphorylation.

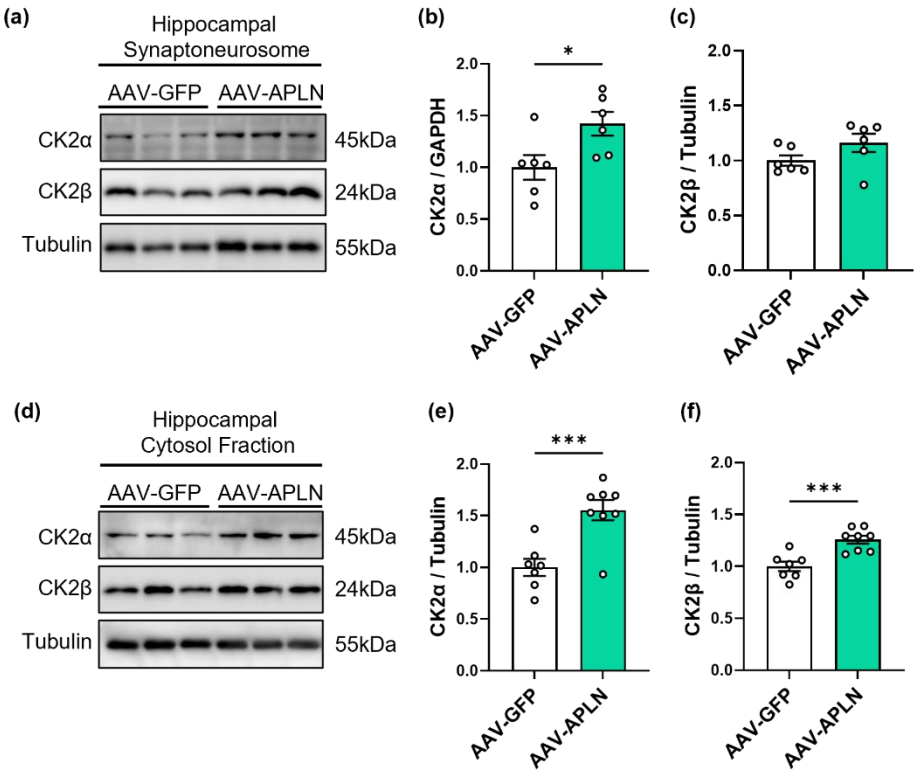

Figure 5.9 Overexpression of apelin in the gastrocnemius and tibialis anterior up-regulated CK2 expression in hippocampus.

(a) Western blotting analysis of hippocampal synaptoneurosome CK2 levels. Muscle apelin overexpression increased protein expression levels of (b) CK2α but has no effect on (d) CK2β in hippocampal synaptoneurosome. (d) Western blotting analysis of CK protein levels in hippocampal cytosol fraction. Apelin overexpression increased protein expression levels of (e) CK2α and (f) CK2β. Data are presented as mean ± S.E.M. Unpaired t-test, *$p < 0.05$, ***$p < 0.001$. CK2: Creatine kinase 2.

To further investigate the effects of overexpression of muscle apelin in TA and Gas muscles on hippocampal synaptic transmission and plasticity, we conducted a detailed analysis of excitatory synaptic currents, the NMDA/AMPA receptor ratio, and paired-pulse facilitation using patch-clamp electrophysiology (Fig. 5.10a-j). Our findings reveal a significant increase in both the frequency (Fig. 5.10c; $P < 0.05$) and amplitude (Fig. 5.10e; $P < 0.01$) of spontaneous excitatory postsynaptic currents (sEPSCs) in the AAV-APLN group compared to the control (AAV-GFP group) indicate that apelin overexpression leads to a general enhancement of excitatory synaptic activity. Muscle apelin overexpression elevated NMDA/AMPA ratio (Fig. 5.10g; P <

0.05), suggesting that apelin overexpression may preferentially affect NMDA receptor-mediated synaptic transmission, which is consistent with the enhanced function of NMDA receptors that we observed (Fig. 5.8b-e). Notably, there is a significant increase in the amplitude of evoked excitatory postsynaptic currents mediated by NMDA receptors (eEPSC-NMDA) at stimulation intensities of 50, 60, 70, and 80 μA (Fig. 5.10i; $P < 0.05$), whereas the amplitude of evoked excitatory postsynaptic currents mediated by AMPA receptors (eEPSC-AMPA) remains largely unaffected (Fig. 5.10h; $P > 0.05$), except at 80 μA ($P < 0.05$). This specificity suggests that muscle apelin overexpression predominantly influences NMDA receptor signaling pathways. Furthermore, the reduction in the paired-pulse ratio at a 50 ms interval (Fig. 5.10k; $P < 0.05$) implies that apelin may enhance short-term synaptic plasticity by increasing the probability of synaptic release. However, muscle apelin overexpression has no effect at longer inter-pulse intervals of 100 and 200 ms (Fig. 5.10l & m; $P > 0.05$), indicating that apelin's modulatory effects are more pronounced in rapid, short-term synaptic dynamics. In summary, overexpression of muscle apelin enhances glutamatergic synaptic transmission and NMDA receptor-mediated responses, while selectively modulating short-term synaptic plasticity at specific temporal scales.

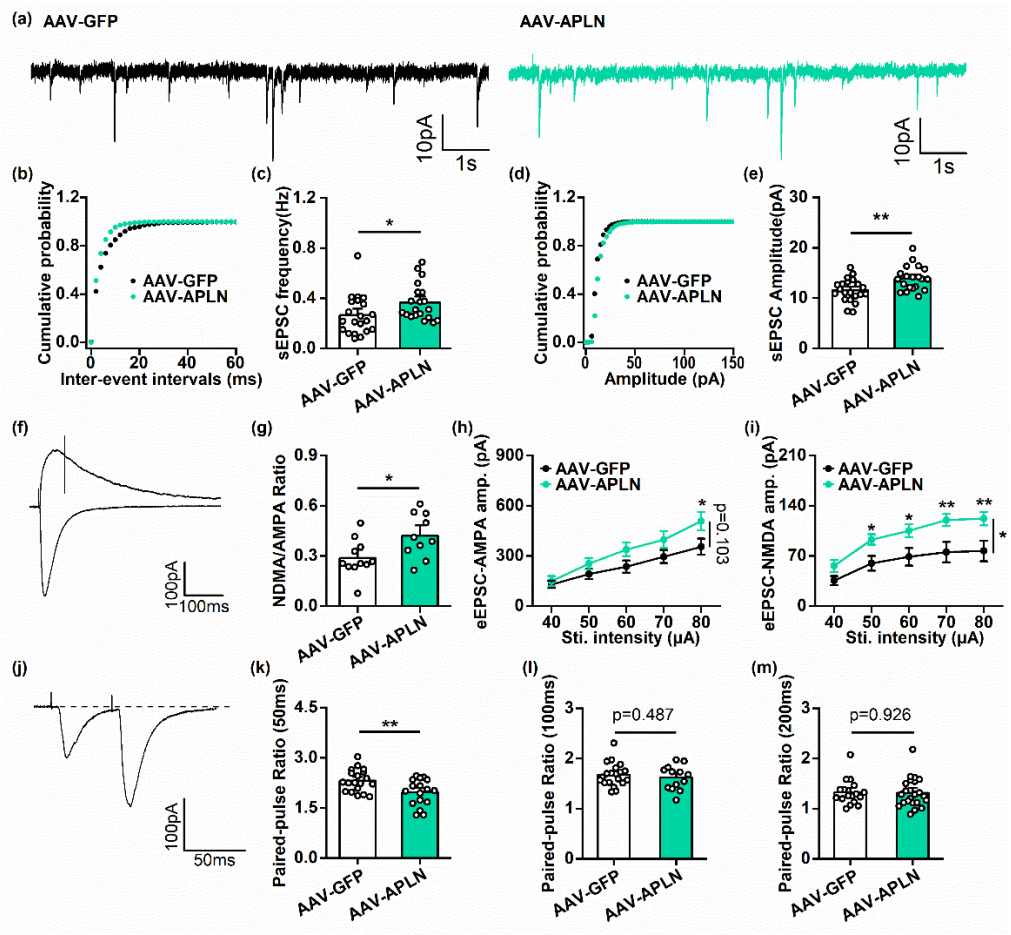

Figure 5.10 Overexpression of apelin in the gastrocnemius and tibialis anterior enhanced glutamatergic synaptic transmission and NMDA receptor-mediated post-synaptic currents.

(a) Whole-cell recording traces of mEPSCs in CA1 pyramidal neurons from AAV-GFP and AAV-APLN mice. (b) Cumulative probability plots for inter-event intervals (ms) and (d) amplitudes (pA) of mEPSCs. Muscle apelin overexpression increased both the (c) frequency and (e) amplitude of sEPSCs. (f) Whole-cell recording traces of maximal AMPA and NMDA receptor currents in the hippocampal CA1 region of AAV-GFP and AAV-APLN mice. Overexpression of muscle apelin led to a higher (g) NMDA/AMPA ratio, with increased (i) NMDA current amplitude and no change in AMPA current amplitude (h). (j) Traces showing whole-cell recordings at a 50 ms inter-stimulus interval. Muscle apelin overexpression decreased the paired-pulse ratio at (k) 50 ms, with no significant effects at (l) 100 ms or (m) 200 ms intervals. Data are shown as mean ± S.E.M. Statistical analysis was performed using an unpaired t-test, with *$p < 0.05$ and **$p < 0.01$.

We next investigated whether overexpression of apelin can mimic antidepressant effects of running in stressed mice induced by chronic unpredictable stress (CUS). 3 days-post intramuscular injection, mice were subjected to 4 weeks of voluntary running and/or CUS (Fig. 5.11a) same as performed in Chapter 4.

One-way ANOVA showed significant effects of CUS, running, and muscle apelin overexpression on sucrose preference in SPT (Fig. 5.11b; $F(3, 37) = 16.76$, $P < 0.0001$), grooming behaviors in SST (Fig. 5.11c; $F(3, 37) = 9.759$, $P < 0.0001$), and immobility time in FST (Fig. 5.11d; $F(3, 37) = 4.116$, $P < 0.05$). Post hoc analysis indicated that running and overexpressed muscle apelin levels increased sucrose preference in stressed mice (Fig. 5.11b; $P < 0.0001$ for Control vs. CUS; CUS vs. CUS + Exe; CUS vs. CUS + AAV-APLN). Moreover, both running and apelin overexpression in muscles enhanced grooming behaviors in SST in the stressed mice (Fig. 5.11c; $P < 0.01$ for CUS vs. CUS + Exe; CUS vs. CUS + AAV-APLN). Chronic stress did not significantly alter grooming time (Fig. 5.11c; $P > 0.05$ for Control vs. CUS). In the FST, apelin overexpression reduced immobility time compared to stressed mice, while neither chronic stress nor running alone affected immobility time significantly (Fig. 5.11d; $P = 0.0605$ for Control vs. CUS; $P > 0.05$ for CUS vs. CUS + Exe).

There were no significant effects of CUS, running, and muscle apelin overexpression on time spent in the center (Fig. 5.11e; $F(3, 37) = 1.549P > 0.05$) or locomotor activity in the OFT (Fig. 5.11f; $F(3, 37) = 0.5614$, $P > 0.05$), indicating no effects of on anxiety-like behaviors and locomotor activity. However, the behavioral findings suggest that muscle apelin overexpression has similar effect to voluntary running-elicit antidepressant and anti-anhedonic effects in stressed mice

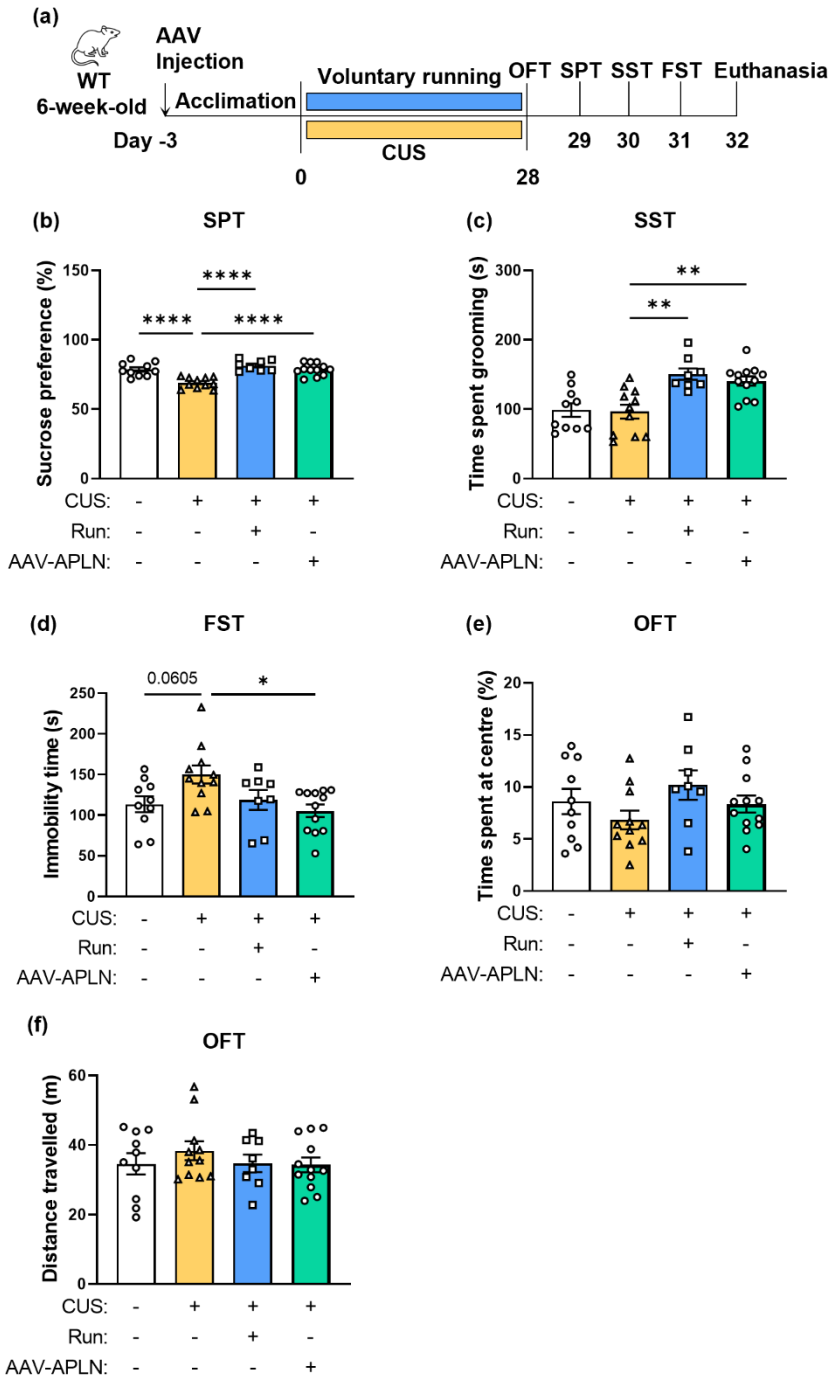

Figure 5.11 Overexpression of apelin in the gastrocnemius and tibialis anterior mimicked antidepressant effects of voluntary running in stressed mice.

(a) Experimental timeline CUS: chronic unpredictable stressed mice; CUS+Run: runner mice with chronic unpredictable stress; CUS+AAV-APLN: stressed mice with apelin overexpression in Gas and TA muscle. Overexpression of apelin mimicked the antidepressant effects of voluntary running in stressed mice, as shown by increased(b) sucrose preference in the SPT, (c) self-grooming time in the SST, and decreased (d) time spent immobile in the FST. Overexpression of apelin did not affect (e) time spent

in the center arena, and (f) distance traveled in OFT in stressed mice. Data are presented as mean ± S.E.M. A one-way ANOVA followed by Tukey's post hoc test was used. *p < 0.05, **p < 0.01, ****p < 0.0001. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test, FST: forced swim test.

5.3. Discussion

In this Chapter, we unveil the role of muscle-specific knockout and overexpression of apelin on depressive-like behaviors in a mouse model with chronic stress. Our findings demonstrate that skeletal muscles specific knockout of apelin diminished some behavioral benefits of voluntary running, particularly anhedonia-like behavior in sucrose splash test. Moreover, overexpression of apelin in muscles mimicked the antidepressant effects of voluntary running. This effect is associated with enhanced adult neurogenesis, NMDA receptor function potentially mediated by APJ/CK2/GluN2B signalling pathway that is related to regulation of synaptic plasticity in the hippocampus. These data have indicated the critical role of apelin in mediating the antidepressant effects of voluntary running and suggested the potential role of apelin in mediating the muscle-brain crosstalk in depression.

5.3.1. Myokine apelin is a key mediator of the antidepressant effects of running

In Chapter 4, it is revealed that voluntary running elicited antidepressant effect and increased apelin expression in the muscle. To further elucidate whether muscle apelin serves as a pivotal factor of the antidepressant effects of running, apelin knocked out specifically in skeletal muscle we observed that muscle apelin deficiency attenuated the anti-anhedonic effect of running though there was a decrease in apelin serum levels. Furthermore, overexpression of apelin in the GAS and TA muscles via intramuscular injection of AAV-APLN mirrored the antidepressant effects observed with running in stressed mice. Evidence from previous studies have suggested the crucial role of apelin in depression. For instance, Xiao et al. [285] demonstrated that intrahippocampal injection of recombinant apelin reversed depression-like behaviors induced by forced swimming stress. Moreover, Li et al. [19] illustrated the antidepressant effects of acute intracerebroventricular injection of apelin-13 with its action on activating ERK and PI3K signaling pathways. However, some studies have indicated that apelin deficiency has antidepressant effects., Bullich et al. [204] reported that 12-month-old apelin global knockout mice being fed a standard diet starting exhibited spontaneous antidepressant-like behavior , while 6-month-old apelin global knockout mice fed with a high-fat diet (HFD) showed increased anxiety like behavior. Lv et al. [271] found that a role for APJ and its opioid receptors in inducing depression-like behaviors, as indicated by increased immobility in stress-related tests. These opposite effects of apelin on symptoms of

depression and anxiety suggest that further in-depth research is necessary to explore this issue.

Changes of muscle mass and muscle strength were examined in muscle-specific knockout model. We found that muscle apelin specific knockout reduced muscle strength in the grip test and impaired coordination ability in the rotarod test. Consistent with our findings, a study reported that muscle-specific apelin knockdown in 12-month-old mice accelerated muscle weakness, suppressed mitochondrial quantities and function [17]. These results suggest that apelin plays a critical role in maintaining muscle strength. The Gas and TA muscles, located in the lower limbs, are primarily responsible for plantarflexion and dorsiflexion of the foot at the ankle joint [352]. Vinel et al. [17] further demonstrated that overexpressing apelin in these muscles for eight weeks improved muscle fiber density, grip strength, and endurance in aged mice with sarcopenia, effectively mimicking the beneficial effects of running. Similarly, we employed apelin overexpression in the Gas and TA muscles to replicate the antidepressant effects of voluntary running in both healthy and stressed mice. Interestingly, our findings revealed that while apelin overexpression reduced locomotor activity in healthy mice, it had no effect on stressed mice. Locomotor activity is influenced by a combination of factors, including muscle fatigue and motivation. However, there is no direct evidence that apelin reduces muscle fatigue and decreases motivation, recent studies suggest that apelin may alleviate muscle fatigue by enhancing glucose uptake and mitochondrial function [353,354]. In summary, the relationship between apelin and locomotor activity is complex and likely depends on the interplay of physiological, psychological, and environmental factors. Further research is necessary to elucidate these interactions and confirm our findings.

Permeability of passing through the blood-brain barrier (BBB) is critical myokines to elicit neuroprotective effects in the central nervous system (CNS). Sinen and Bülbül [355] demonstrated that intraperitoneal injection with apelin-13 increased its levels in cerebrospinal fluid (CSF). Consistently, we found that overexpression of apelin in Gas and TA muscles elevated the levels of apelin and its receptor APJ expression in the hippocampus, it is possible that apelin may directly act on the brain by crossing the BBB. Additionally, studies have shown that apelin-13 preserves BBB permeability following ischemic stroke [356,357]. However, whether apelin can cross the BBB requires further investigation

Increased number of adult hippocampus neurogenesis is associate with the antidepressant effects of exercise [29,30,290,291,358]. We further demonstrated that skeletal muscle specific apelin knockout diminished voluntary running to enhance adult hippocampal neurogenesis. In contrast, increasing muscle apelin levels increased the number of proliferating cells and immature neurons in the hippocampal DG. A recent study indicated that apelin enhances neural stem cell (NSC) proliferation and differentiation, while APJ antagonist ML221 administration has the opposite effect [330]. This finding highlights apelin's potential to promote adult neurogenesis and influence NSC fate towards neuronal lineage. Apelin also inhibits neuronal death, thereby improves neuronal survival. Additionally, a study found that intracerebroventricular (i.c.v.) administration of apelin-13 for one week significantly reduced neuronal death induced by chronic stress in animals [287]. Emerging data have showed that apelin could have neuroprotective effects in preventing neuronal loss.

5.3.2. Myokine apelin enhanced synaptic plasticity by increasing synaptic NMDA receptor function

NMDA and AMPA receptor-mediated glutamatergic transmission play an important role in synaptic plasticity in the hippocampus. Our research showed that muscle apelin overexpression increased NMDA/AMPA receptor ratio, suggesting a preferential enhancement of NMDA receptor-mediated synaptic transmission. This is consistent with our immunoblotting findings that muscle apelin overexpression promotes phosphorylation of NMDA receptor subunits GluN2A at the Y1246 site and GluN2B at the Y1472 site, without affecting AMPA receptors. These phosphorylation events are crucial for NMDA receptor localization and function, as well as for mood regulation and memory enhancement. The significant increase in the amplitude of evoked excitatory postsynaptic currents mediated by NMDA receptors (eEPSC-NMDA), while AMPA receptor-mediated currents (eEPSC-AMPA) remain largely unaffected, further highlights the specificity of apelin's action on NMDA receptor pathways.

Consistent with our findings, a recent study highlighted apelin's role in mitigating excitotoxicity by promoting the phosphorylation of the GluN2B subunit at the S1480 site as well as regulated NMDA receptor–mediated currents [246], suggesting apelin could interact with NMDA receptors by modulating phosphorylation of subunits. Additionally,

apelin-36 has been shown to inhibit neurotoxicity by activating GluN2B phosphorylation at S1480 site, attenuating calpain activation, and reducing Ca^{2+} accumulation in cerebrocortical neuron cultures [242]. However, this study reported that apelin attenuated NMDA-induced neurotoxicity independently of GluN2B phosphorylation at the Y1472 site. Phosphorylation at the Y1472 site on GluN2B is critical for NMDA receptor localization, mood regulation, and memory enhancement [341]. Studies have shown that disrupting Y1472 phosphorylation on GluN2B can confer resilience to depression-like behaviors in mice [359]. Additionally, increased levels of phosphorylated Y1472 on GluN2B subunits is associated with spatial memory improvement in aging via maintaining NMDA receptors on the synaptic membrane [360].

Similarly, GluN2A phosphorylation at Y1246 site is involved in regulating excitatory synaptic transmission and plasticity [342]. Recent findings suggest that the suppression of GluN2A, rather than GluN2B, mediates the rapid antidepressant-like effects of ketamine and MK-801 (dizocilpine), NMDA receptor blockers, to increase excitability of hippocampal neurons [361]. However, the role of apelin in phosphorylating GluN2B at Y1472 and GluN2A at Y1246 has not been reported. Our current results showed that muscle apelin increased phosphorylation of GluN2B at the Y1472 site. This suggests that apelin could enhance phosphorylation of GluN2A and GluN2B to enhance hippocampal neuroplasticity and exert antidepressant effects. However, the mechanisms of how apelin interact with NMDA receptor subunit warrant further investigation.

Phosphorylation at Y1472 site of GluN2B subunits is associated with its binding to PSD-95, which anchors NMDA receptors on synaptic membranes and enhances LTP [360,362]. Our recent experiments reveal that overexpression of muscle apelin significantly enhances excitatory synaptic activity, as indicated by increased frequency and amplitude of sEPSCs. This suggests a general enhancement of excitatory synaptic transmission, potentially linked to increased synaptic synaptophysin and PSD95 expression, although our protein expression analysis did not show changes in these markers. Luo et al. [238] reported similar results in which treatment with apelin-13 did not change synaptophysin expression in the hippocampus, but significantly increased its levels in a streptozotocin-induced AD rats displaying cognitive impairment. In lesion-induced parkinsonism rats, apelin administration up-regulated PSD-95 expression levels in the hippocampus [236,239]. These findings support the notion that apelin could

modulate synaptic function and can be potentially targeted treating neurodegenerative diseases.

Additionally, the reduction in the paired-pulse ratio at a 50 ms interval implies that apelin enhances short-term synaptic plasticity by increasing the probability of synaptic release. Paired-pulse ratio reflects presynaptic release changes, where a lower PPR suggests heightened neurotransmitter release, potentially driven by residual presynaptic calcium [363,364]. Previous studies have reported that the mechanism by which apelin enhances synaptic release probability may involve the modulation of presynaptic calcium dynamics or interactions with specific synaptic proteins [365,366]. Furthermore, apelin also contribute to synaptic health and resilience, offering potential therapeutic value in neurodegenerative diseases characterized by synaptic dysfunction [367]. Overall, this rapid modulation of synaptic dynamics suggests potential therapeutic applications in conditions with impaired synaptic plasticity.

Our findings demonstrate that apelin overexpression in muscle significantly enhances the expression of CK2α and CK2β in the hippocampal cytosol fraction, as well as increases CK2α and the phosphorylation of GluN2B and GluN2A in the hippocampal synaptoneurosome. These results align with previous studies which identified CK2 as a critical modulator of NMDA receptor-dependent synaptic plasticity, particularly in the context of LTP [368]. Our data suggest that apelin may exert its effects through CK2-mediated phosphorylation of NMDA receptor subunits, thereby enhancing synaptic plasticity. This is consistent with the report from Cook [242] that apelin can modulate NMDA receptor signaling to promote neuronal survival. Furthermore, our findings extend the work which highlighted the role of APJ in modulating the expression of CK2α by activating Gαi/Gαq of APJ subunit [351]. The observed increase in CK2α and GluN2B phosphorylation supports the hypothesis that CK2 selectively modulates synaptic NMDA receptors and may contribute to antidepressant effects by prompting synaptic plasticity. Future research should explore the specific pathways through which apelin and CK2 interact to regulate NMDA receptor activity and investigate the therapeutic potential of modulating this pathway in depression.

In addition, our studies found that muscle apelin up-regulated expression of calpain-2 but not calpain-1 expression levels in the hippocampus. Calpain-1 and -2 are two isoforms of calpain, a family of calcium-dependent cysteine proteases that play distinct roles in regulating glutamate receptors [205]. Upon activated by synaptic NMDA

receptor, calpain-1 degrades the pH domain and leucine rich repeat protein phosphatase 1 (PHLPP1-α/β), leading to the activation of pro-survival Akt and ERK pathways to exerts neuroprotective effect [369]. On the other hand, calpain-2, which can be triggered by extrasynaptic NMDAR stimulation, degrades the striatal-enriched protein phosphatase (STEP), resulting in neurotoxicity [370]. Additionally, calpain-2 can be activated by BDNF through ERK-mediated phosphorylation, enhancing local protein synthesis via mTOR activation, consequently contributes to the consolidation phase of LTP [371]. Unlike calpain-1, which is involved in the induction phase of LTP, activation of calpain-2 following theta burst stimulation (Theta-BS) leads to a biphasic effect on ERK activation, and affecting the magnitude of LTP [372]. A recent study showed that intraperitoneal injection of APJ antagonist ML233 maintained function of retinal ganglion cells by inhibiting calpain-mediated breakdown of neuronal structures and anti-apoptotic pathways, suggesting that blocking apelin signaling could protect retinal neurons from NMDA-induced excitotoxicity [245]. With NMDA receptor activation, we observed an increase in calpain-2 expression levels in the hippocampus suggesting the signaling cascade apelin/APJ-NMDAR-calpain signaling pathway in mediating physical exercise induced synaptic plasticity in the hippocampus. More experiments will be needed to confirm this speculation.

In hippocampus, the activation AMPKα can influence neuronal energy balance, which is vital for maintaining synaptic plasticity and cognitive functions [348,373]. Our findings demonstrate that muscle apelin increased the levels of AMPKα in hippocampal synaptoneurosome extractions but did not affect the phosphorylation of AMPKα at T172 site, indicating that AMPK is not activated by muscle apelin in the hippocampus. Previous studies showed that apelin-13 exerted neuroprotective effects in ischemic stroke, suggesting a therapeutic potential through mechanisms involving AMPKα activation [262,374]. Additionally, a decrease in hippocampal neurogenesis has been associated with a reduced response of AMPKα in stressed mice. Exercise increases adult neurogenesis via activation of AMPKα [375]. Although our results do not demonstrate a direct link between AMPKα activation and apelin-mediated hippocampal neurogenesis, they provide a foundation for further exploration of apelin's role in hippocampal plasticity.

In conclusion, our results reveal that muscle apelin deficiency diminished running-induced antidepressant effects and hippocampal neurogenesis. Conversely,

overexpression of apelin in muscle mimicked antidepressant and pro-neurogenic effects of running. Taking the data together, it is possible that muscle apelin could potentiate NMDA receptors and increase synaptic plasticity via calpain-2 signaling pathway. These findings indicate the potential role of muscle apelin as a mediator for the antidepressant effects, highlighting the crucial role of apelin in the muscle-brain axis in depressive symptoms.

Chapter 6. APJ Is Required for the Antidepressant Effects of Physical Exercise via Regulating Hippocampal Plasticity

6.1. Introduction

In previous chapters, we demonstrated that muscle apelin knockout inhibited the antidepressant effects of running, while muscle apelin overexpression mimicked these effects, both of which are associated with hippocampal plasticity. This plasticity involves adult neurogenesis, synaptic plasticity, and dendritic complexity, all crucial for emotion regulation and cognitive function [376,377]. Depression disrupts these processes, leading to structural and functional changes in the hippocampus, such as reduced neurogenesis and synaptic loss [378,379]. Exercise has emerged as a promising intervention for depression due to its ability to stimulate neurogenesis, increase BDNF levels [290,358], and enhance synaptic plasticity in the hippocampus [114,380].

Apelin, a myokine responsive to exercise [17], exerts antidepressant effects on the hippocampus [24,283]. It has been reported that apelin-13 suppresses neuronal apoptosis by activating the APJ/PI3K/Akt and MAPK/ERK1/2 pathways [202,261], promoting mitochondrial integrity and reducing oxidative stress [254,255], thus enhancing cell survival. Additionally, apelin-13 modulates neuroinflammation, offering potential therapeutic benefits for neurodegenerative diseases and mood disorders involving hippocampal dysfunction [274,275]. However, the role of apelin in hippocampal plasticity remains unclear. While apelin can promote neurogenesis by inhibiting cell death through the AMPK/PGC-1α signaling pathway [287], its effects on cell proliferation and differentiation are unknown. In terms of synaptic plasticity, apelin preserves synaptic integrity and modulates key signaling pathways like BDNF/TrkB, which may prevent synaptic dysfunction and cognitive deficits in neurodegenerative models [237,239,252]. However, its role in synaptic plasticity related to depression is yet to be determined.

Furthermore, NMDA receptor mediated neuronal transmission is crucial for hippocampal synaptic plasticity [48], with its dysregulation linked to impaired neuroplasticity and depression [44,359,361]. Exercise enhances NMDA receptor function, supporting synaptic plasticity and neurogenesis, thereby alleviating depressive symptoms [381,382]. Notably, apelin has been reported to promote the phosphorylation of synaptic NMDA receptors, inhibiting excessive NMDA-induced neurotoxicity [242]. Our previous findings showed that muscle apelin overexpression enhances synaptic NMDA

receptor phosphorylation in the hippocampus, suggesting a potential interaction between apelin and synaptic NMDA receptors in hippocampal plasticity. Whether this mechanism contributes to the antidepressant effects of exercise requires further investigation.

Therefore, this chapter aims to investigate whether hippocampal apelin receptor, APJ, is crucial for exercise-enhanced hippocampal plasticity and depression alleviation. Understanding the molecular pathways of apelin-APJ involved in exercise-induced hippocampal plasticity could pave the way for targeted therapies that harness the benefits of physical activity to combat depression.

6.2. Results

It is confirmed that apelin receptor, APJ, is expressed in neurons and oligodendrocytes, but not in microglia or monocytes, suggesting a role for APJ in central nervous system signaling [383,384]. To determine whether APJ expression is limited to specific neuron types, we evaluated coronal sections from the dentate gyrus (DG) of wild type (WT) mice. Our analysis revealed that most APJ-expressing cells were co-labeled with the glutamatergic neuron marker CaMK2a, whereas a minor portion were co-labeled with GABAergic marker GAD67 within the DG (Fig 6.1a-b). These findings indicate a predominance of APJ expression in hippocampal glutamatergic neurons compared to GABAergic neurons.

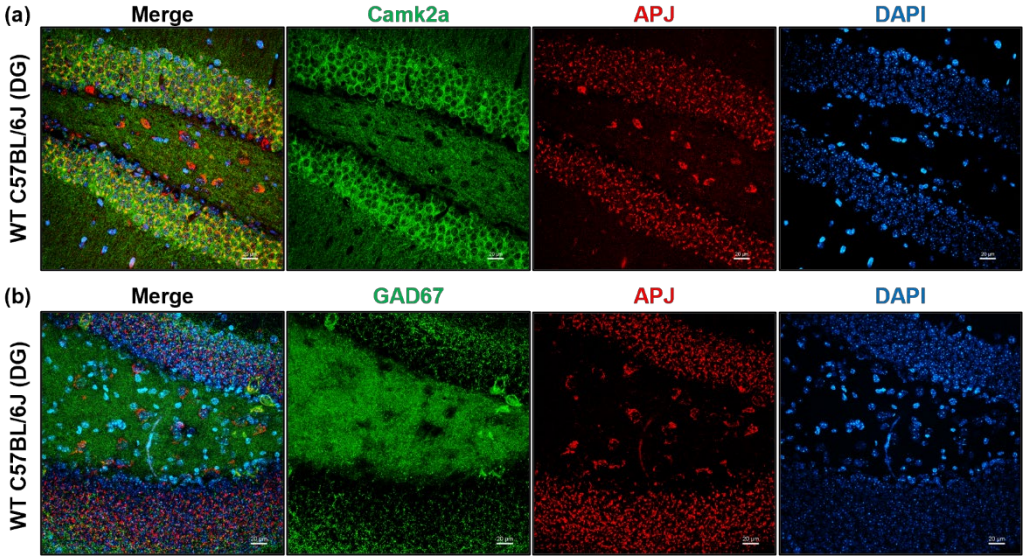

Figure 6.1 APJ is localized in hippocampal glutamatergic neurons.
(a) The expression of APJ and co-labeled with Camk2a (glutamatergic neurons), and with (b) GAD67 (GABAergic neurons) in hippocampal DG from wild type C57BL/6J mice (scale bars, 20 μm in 400×).

The ventral hippocampus is a key brain region involved in depression [385,386]. To investigate the role of APJ in ventral hippocampal glutamatergic neurons in the antidepressant effects of running, we specifically knocked down APJ by intra-ventral hippocampus injection of AAV-DIO-shAPLNR-RFP (APJ Gene symbol: APLNR, ID: 23796) in Camk2a-Cre mice, while Camk2a-Cre mice receiving AAV-RFP injection served as the control group (Fig. 6.2a). We found increased RFP-positive cells and decreased APJ (APLNR) mRNA in the ventral hippocampus (Fig. 6.2b-c; $P < 0.05$).

This indicates successful knockdown of APJ expression in hippocampal glutamatergic neurons.

To investigate whether APJ plays a critical role in mediating the antidepressant effects of physical exercise, we subjected ventral hippocampal APJ knockdown mice and control mice to 4 weeks of voluntary running following a 7-day postoperative acclimatization period (Fig. 6.2d). Anhedonia-like and depression-like behaviors were assessed using the sucrose preference test (SPT), sucrose splash test (SST), and forced swim test (FST), respectively.

In the FST, two-way ANOVA indicated a significant effect of APJ knockdown and their interaction on immobility time, but no main effect of running (Fig. 6.2e; $F(1, 43) = 4.100$, $P < 0.05$ for APJ knockdown; $F(1, 43) = 2.724$, $P > 0.05$ for running; $F(1, 43) = 7.873$, $P < 0.01$ for interaction). Post-hoc Tukey test revealed that voluntary running decreased immobility time in control mice (Fig. 6.2e; $P < 0.05$ for AAV-RFP + Sed vs. AAV-RFP + Run), but this effect was absent in APJ knockdown mice (Fig. 6.2e; $P > 0.05$ for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run). APJ knockdown runners exhibited higher immobility time compared to control runners (Fig. 6.2e; $P < 0.05$ for AAV-RFP + Run vs. AAV-shAPLNR + Run), suggesting that APJ knockdown diminishes the antidepressant effect of running.

In the SPT, two-way ANOVA showed a significant effect of running and their interaction on sucrose preference, but no main effect of APJ knockdown (Fig. 6.2f; $F(1, 43) = 2.014$, $P > 0.05$ for APJ knockdown; $F(1, 43) = 7.256$, $P < 0.05$ for running; $F(1, 43) = 7.453$, $P < 0.01$ for interaction). Post-hoc Tukey test indicated that running increased sucrose preference in control mice (Fig. 6.2f; $P < 0.01$ for AAV-RFP + Sed vs. AAV-RFP + Run), whereas APJ knockdown runners showed lower sucrose preference compared to control runners (Fig. 6.2f; $P < 0.05$ for AAV-RFP + Run vs. AAV-shAPLNR + Run).

In the SST, two-way ANOVA revealed a significant effect of running on grooming time, but no main effect of APJ knockdown or their interaction (Fig. 6.2g; $F(1, 43) = 0.2745$, $P > 0.05$ for APJ knockdown; $F(1, 43) = 10.17$, $P < 0.01$ for running; $F(1, 43) = 2.327$, $P > 0.05$ for interaction). Post-hoc Tukey test showed that voluntary running increased grooming time in control mice (Fig. 6.2g; $P < 0.05$ for AAV-RFP + Sed vs. AAV-RFP + Run), but this effect was not observed in APJ knockdown mice (Fig. 6.2g; $P > 0.05$ for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run). These results

suggest that knockdown of APJ in hippocampal glutamatergic neurons diminish the antidepressant effect of running.

Anxiety-like behavior was assessed using the open field test (OFT). Two-way ANOVA indicated no significant effects of either running or APJ knockdown on time spent in the center area and distance traveled in the open field (Fig. 6.2h-i; For time spent in the center, $F(1, 43) = 0.6970$, $P > 0.05$ for APJ knockdown; $F(1, 43) = 4.482$, $P > 0.05$ for running; $F(1, 43) = 2.064$, $P > 0.05$ for interaction; For distance traveled, $F(1, 43) = 0.5001$, $P > 0.05$ for APJ knockdown; $F(1, 43) = 0.7097$, $P > 0.05$ for running; $F(1, 43) = 0.02392$, $P > 0.05$ for interaction). These findings suggest that neither running nor hippocampal APJ knockdown significantly affects anxiety-like behaviors.

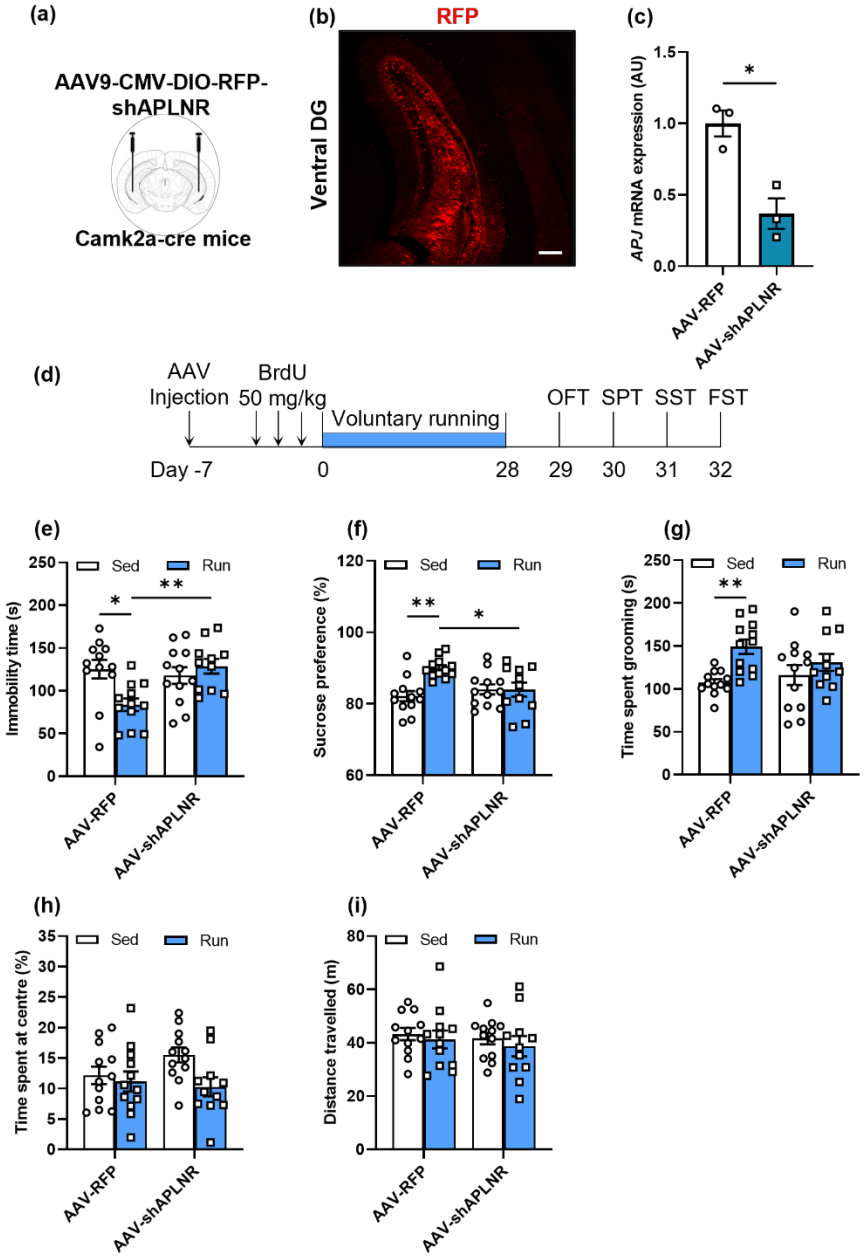

Figure 6.2 Knockdown of APJ in hippocampal glutamatergic neurons diminished the antidepressant effect of running.

(a) Injection of pAV-CMV-DIO-RFP-mir30shAPLNR into the ventral hippocampus of Camk2a-Cre mice. RFP: Red fluorescent protein. (b) Successful injection of AAV with reporter gene expression of red fluorescent protein (scale bars, 100 μm in 100×), and decreased (c) the APJ mRNA expression in ventral hippocampus. (d) Study timeline. AAV-RFP: Camk2a-Cre mice with AAV-DIO-RFP injection; AAV-shAPLNR: Camk2a-Cre mice with AAV-DIO-shAPLNR injection; Sed: sedentary mice; Run: runner mice. In runner mice, ventral hippocampal APJ knockdown in glutamatergic neurons diminished the decreased in (e) immobility time in FST, abolished the increase in (f) sucrose preference in the SPT, and (g) grooming time in the SST. Ventral hippocampal APJ knockdown did not affect (h) time spent in the center, and (i) total distance traveled in the OFT. Data are presented as mean ± S.E.M. Statistical significance assessed using unpair T-test; two-way ANOVA with Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$. OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test.

Glutamatergic neurons in DG are crucial for adult hippocampal neurogenesis, including the regulation of progenitor cell proliferation [387,388], as well as the modulation of neuronal maturation and differentiation [389]. However, whether APJ is involved in this beneficial effect remains unknown. We investigated the role of APJ in mediating the positive effects of voluntary running on hippocampal neurogenesis.

Immunohistochemical analyses using Ki-67 labeling for proliferating cells were conducted (Fig. 6.3a). Two-way ANOVA demonstrated significant effects of voluntary running on increasing the number of Ki-67$^+$ cells in the ventral and total DG (Fig. 6.3b-d; $F(1, 23) = 41.41$, $P < 0.0001$ in ventral DG; $F(1, 23) = 17.79$, $P < 0.001$ in total DG), but not in dorsal DG ($F(1, 23) = 3.030$, $P > 0.05$), indicating increased hippocampal cell proliferation due to running. Although APJ knockdown had no main effect on the number of Ki-67$^+$ cells in all regions of the DG (Fig. 6.3b-d; $F(1, 23) = 0.3082$ in dorsal, 0.05368 in ventral, and 0.2468 in total, $P > 0.05$), there were significant interaction effects on the number of Ki-67$^+$ cells in the DG (Fig. 6.3b-d; $F(1, 23) = 12.76$, $P < 0.01$ for dorsal DG; $F(1, 23) = 26.29$, $P < 0.0001$ in ventral DG; $F(1, 23) = 23.93$, $P < 0.0001$ in total DG). Post-hoc Tukey test showed that running significantly increased the

number of Ki-67$^+$ cells in the DG (Fig. 6.3b-d; $P < 0.01$ for AAV-RFP + Sed vs. AAV-RFP + Run in dorsal and ventral DGs; $P < 0.001$ in total DG). However, this positive effect of running was absent in APJ knockdown mice (Fig. 6.3b-d; $P > 0.05$ for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run in all DGs), whereas APJ knockdown runners showed a lower number of Ki-67$^+$ cells compared to control runners (Fig. 6.3b-d; $P < 0.05$ AAV-RFP + Run vs. AAV-shAPLNR + Run in dorsal DG; $P < 0.01$ in ventral and total DG). These results suggest that knockdown of APJ in hippocampal glutamatergic neurons diminishes the effect of running on proliferating cell numbers. Interestingly, APJ knockdown increased the number of Ki-67$^+$ cells in the ventral and total DG (Fig. 6.3c-d; $P < 0.01$ for AAV-RFP + Sed vs. AAV-shAPLNR + Sed in ventral DG; $P < 0.05$ in total DG), indicating that the loss of APJ might trigger compensatory mechanisms that promote cell proliferation. However, the detail mechanism requires further investigation.

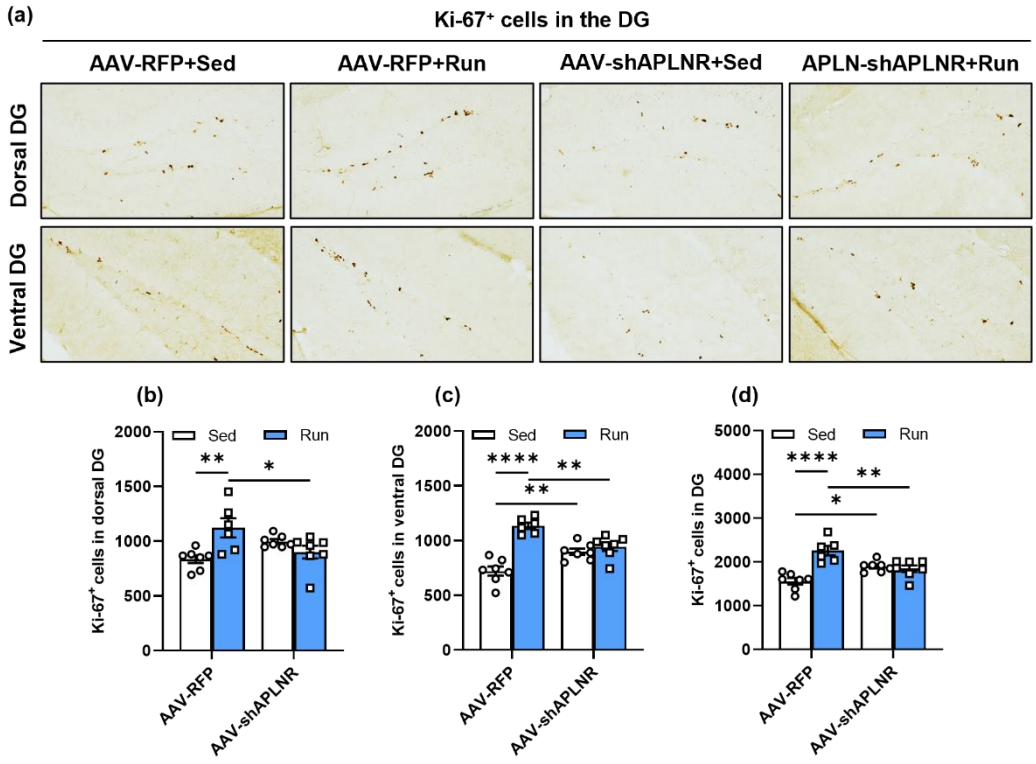

Figure 6.3 Knockdown of APJ in hippocampal glutamatergic neurons diminished the effects of voluntary running on the augmentation of proliferating cell numbers in hippocampal DG.

(a) Immunohistochemical staining for Ki-67 in the dentate gyrus. AAV-RFP: Camk2a-Cre mice with AAV-DIO-RFP injection; AAV-shAPLNR: Camk2a-Cre mice with AAV-DIO-shAPLNR injection; Sed: sedentary mice; Run: runner mice. Knockdown of APJ

in hippocampal glutamatergic neurons diminished the increase of the number of Ki-67$^+$ cells in (b) the dorsal DG, (c) the ventral DG, and (d) the entire DG of runner mice (scale bars, 100 μm in 100×). Data are presented as mean ± S.E.M. Statistical significance was assessed by Two-way ANOVA followed by Tukey's post hoc test, with significance levels of *p < 0.05, **p < 0.01, ****p < 0.0001.

It has been reported that glutamatergic neurons influence the differentiation and survival of new neurons in the hippocampal DG, but whether APJ plays a role in these effects is unknown. Before running, mice received intraperitoneal injections of BrdU for three days to label proliferating cells (Fig. 6.2d). After tissue collection, we labeled immature neurons using doublecortin (DCX) staining and detected neuronal survival by BrdU co-labeling (Fig. 6.4a).

Two-way ANOVA indicated significant effects of voluntary running, APJ knockdown, and their interaction on the number of DCX$^+$ cells in the ventral and total DG (Fig. 6.4b-d; In ventral DG: $F(1, 20) = 17.09$, $P < 0.001$ for running effects, $F(1, 20) = 7.881$, $P < 0.05$ for APJ knockdown effects, $F(1, 20) = 13.92$, $P < 0.01$ for interaction effects; In total DG: $F(1, 20) = 9.650$, $P < 0.01$ for running effects, $F(1, 20) = 7.881$, $P < 0.05$ for APJ knockdown effects, $F(1, 20) = 9.807$, $P < 0.01$ for interaction effects), but in the dorsal DG, only their interaction had a significant effect (Fig. 6.4b-d; $F(1, 20) = 5.828$, $P < 0.05$ in dorsal DG). Post-hoc Tukey analysis showed that voluntary running increased the number of DCX$^+$ cells in the DG (Fig. 6.4b-d; $P < 0.05$ for AAV-RFP + Sed vs. AAV-RFP + Run in dorsal, $P < 0.001$ in ventral, and $P < 0.01$ in total DG). However, this effect was absent in APJ knockdown mice, where running did not increase the number of DCX$^+$ cells (Fig. 6.4b-d; $P > 0.05$). In runners, APJ knockdown reduced the number of DCX$^+$ cells compared to control runners (Fig. 6.4b-d; $P < 0.05$ for AAV-RFP + Run vs. AAV-shAPLNR + Run in dorsal, $P < 0.001$ in ventral, and $P < 0.01$ in total DG), suggesting that APJ knockdown in ventral hippocampal glutamatergic neurons diminishes the positive effect of running on the number of immature neurons.

Two-way ANOVA showed significant effects of running and its interaction with APJ knockdown on the number of BrdU-labeled surviving neurons in the DG, although APJ knockdown alone had no significant effect (Fig. 6.4e-g; In total DG: $F(1, 20) = 19.33$, $P < 0.001$ for running effects; $F(1, 20) = 0.5885$, $P < 0.01$ for interaction effects;

F (1, 20) = 9.093, P > 0.05 for APJ knockdown effects). Post-hoc Tukey analysis indicated that voluntary running increased the number of $BrdU^+$ cells in the DG of control mice (Fig. 6.4e-g; AAV-RFP + Sed vs. AAV-RFP + Run in P < 0.001 in dorsal DG, P < 0.0001 in ventral and total DGs), but this effect was not observed in APJ knockdown mice (Fig. 6.4e-g; P > 0.05 for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run in all DG). In runners, APJ knockdown reduced the number of $BrdU^+$ cells in the ventral and total DG compared to control runners (Fig. 6.4e-g; P < 0.01 for AAV-RFP + Run vs. AAV-shAPLNR + Run in ventral DG; P < 0.05 in total DG), suggesting that APJ knockdown in ventral hippocampal glutamatergic neurons diminishes the beneficial effects of running on neuronal survival.

We then assessed the effect of running and APJ knockdown on neuronal differentiation by calculating the ratio of $BrdU^+DCX^+$ co-labeled cells to the total number of DCX^+ cells in the subgranular zone of the DG. Two-way ANOVA showed a significant effect of running on this ratio in the ventral and total DGs, but no significant effects of APJ knockdown or their interaction (Fig. 6.4h-j; Running effects: F (1, 20) = 7.047, P < 0.05 in ventral DG; F (1, 20) = 7.959, P < 0.05 in total DG). Post-hoc Tukey analysis indicated that voluntary running increased the ratio of $BrdU^+DCX^+$ cells in ventral and total DGs of control mice (Fig. 6.4i-j; P < 0.05 AAV-RFP + Sed vs. AAV-RFP + Run in in ventral and total DG), but this effect was absent in APJ knockdown mice (Fig. 6.4h-j; P > 0.05 for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run in DG), suggesting that APJ knockdown in ventral hippocampal glutamatergic neurons diminish the promotion of neuronal differentiation by running.

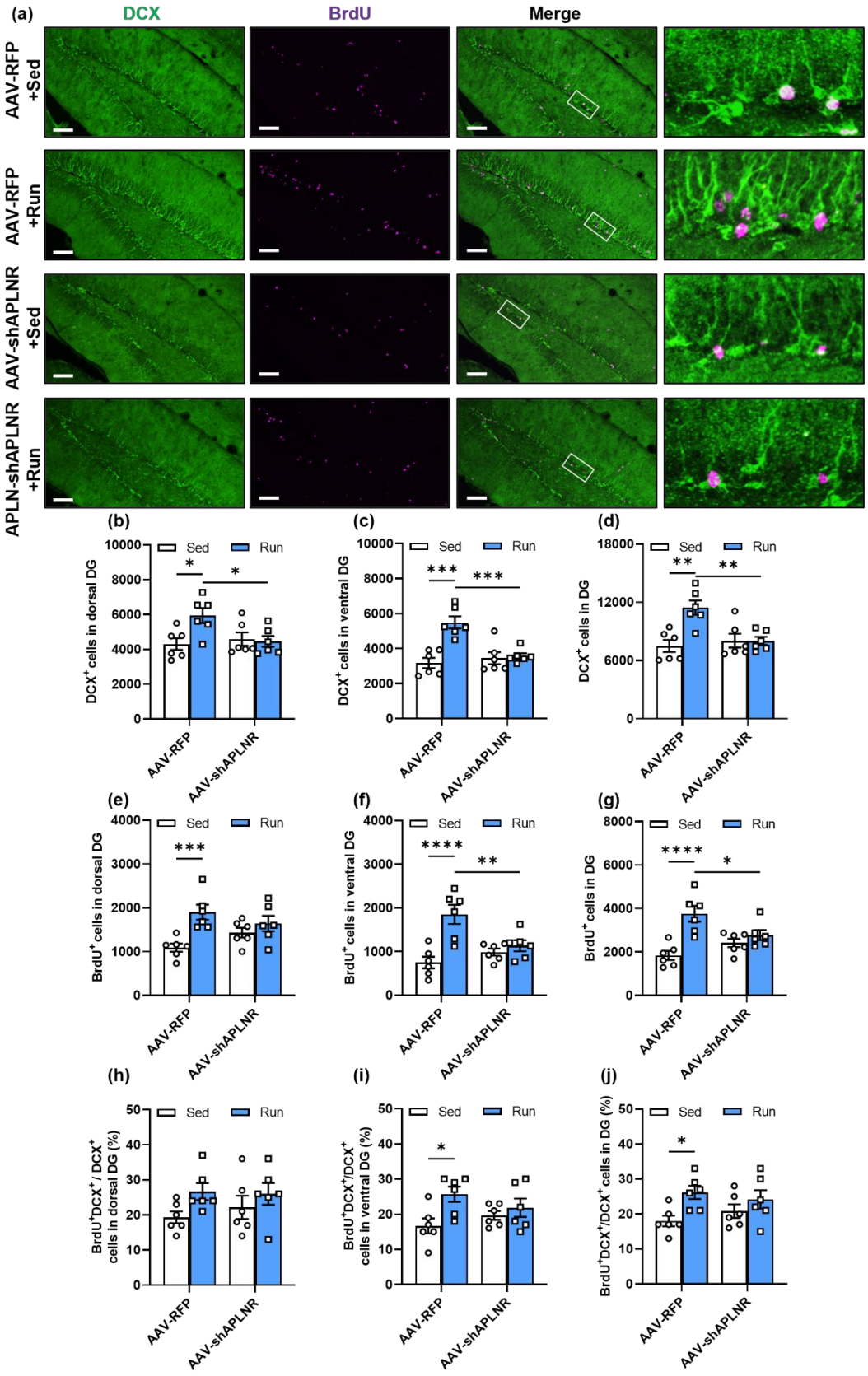
(a)
DCX
BrdU
Merge
AAV-RFP +Sed
AAV-RFP +Run
AAV-shAPLNR +Sed
APLN-shAPLNR +Run
(b)
DCX+ cells in dorsal DG
(c)
DCX+ cells in ventral DG
(d)
DCX+ cells in DG
(e)
BrdU+ cells in dorsal DG
(f)
BrdU+ cells in ventral DG
(g)
BrdU+ cells in DG
(h)
BrdU+DCX+ / DCX+ cells in dorsal DG (%)
(i)
BrdU+DCX+/DCX+ cells in ventral DG (%)
(j)
BrdU+DCX+/DCX+ cells in DG (%)
Sed
Run
AAV-RFP
AAV-shAPLNR

Figure 6.4 Knockdown of APJ in hippocampal glutamatergic neurons diminished the beneficial effects of voluntary running on the neuronal survival and differentiation in hippocampal DG.

(a) Immunofluorescent staining for DCX co-labeled with BrdU in the DG. AAV-RFP: Camk2a-Cre mice with AAV-DIO-RFP injection; AAV-shAPLNR: Camk2a-Cre mice with AAV-DIO-shAPLNR injection; Sed: sedentary mice; Run: runner mice. Knockdown of APJ in hippocampal glutamatergic neurons diminished the increase of the number of DCX cells in (b) the dorsal DG, (c) the ventral DG, and (d) the entire DG of runner mice. Similarly, the knockdown of APJ in hippocampal glutamatergic neurons diminished the increase of (f-h) number in BrdU positive cells, and ratio of (h-j) $BrdU^+DCX^+$ of DCX^+ cells in these DG regions from runner mice (scale bars, 100 μm in 100×). Data are presented as mean ± S.E.M. Statistical significance was assessed by Two-way ANOVA followed by Tukey's post hoc test, with significance levels of *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. DG: dentate gyrus; DCX: doublecortin.

It has been established that running enhances the function of NMDA receptors at hippocampal synapses. In Chapter 5, we demonstrated that muscle-secreted apelin also promotes NMDA receptor function at hippocampal synaptoneurosome extraction. However, whether APJ is involved in the beneficial effects of running on NMDA receptor function remains unknown. In ventral hippocampal synaptoneurosome extractions (Fig. 6.5a), two-way ANOVA indicated significant effects of running on the phosphorylation of NMDA receptor subunits GluN2B, GluN2A, and AMPA receptor subunit GluA1 (Fig. 6.5b, d, f; $F(1, 26) = 17.48$, $P < 0.001$ in phospho-GluN2B; $F(1, 26) = 20.38$, $P < 0.001$ in phospho-GluN2A; $F(1, 26) = 5.100$, $P < 0.05$ in pGluA1). APJ knockdown had a significant effect only on the phosphorylation of GluN2B (Fig. 6.5d; $F(1, 26) = 5.809$, $P < 0.01$ in phospho-GluN2B). There were no main effects of running or APJ knockdown on the total expression of NMDA and AMPA receptors in the ventral hippocampus (Fig. 6.5c, e, g; $P > 0.05$).

Post-hoc Tukey test showed that running increased the phosphorylation of GluN2B at the Y1472 site (Fig. 6.5c; $P < 0.01$ for AAV-RFP + Sed vs. AAV-RFP + Run), while APJ knockdown in ventral hippocampus reduced this promotion in runner mice (Fig. 6.5c; $P < 0.05$ for AAV-RFP + Run vs. AAV-shAPLNR + Run). Running also

promoted the phosphorylation of GluN2A at the Y1246 site (Fig. 6.5d; $P < 0.01$ for AAV-RFP + Sed vs. AAV-RFP + Run) and GluA1 at the S831 site (Fig. 6.5f; $P < 0.05$ for AAV-RFP + Sed vs. AAV-RFP + Run) in control mice. However, in APJ knockdown mice, running slightly increased this phosphorylation, but not significantly ($P > 0.05$ for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run). We further examined the postsynaptic density protein PSD-95 and the presynaptic protein synaptophysin. Two-way ANOVA showed no significant effects of running or APJ knockdown in hippocampal glutamatergic neurons on PSD-95 and synaptophysin expression levels (Fig. 6.5h-i; $P > 0.05$). These results suggest that running enhances the phosphorylation of NMDA and AMPA receptors in the ventral hippocampus, while APJ knockdown in glutamatergic neurons inhibits the running-induced phosphorylation of the GluN2B receptor.

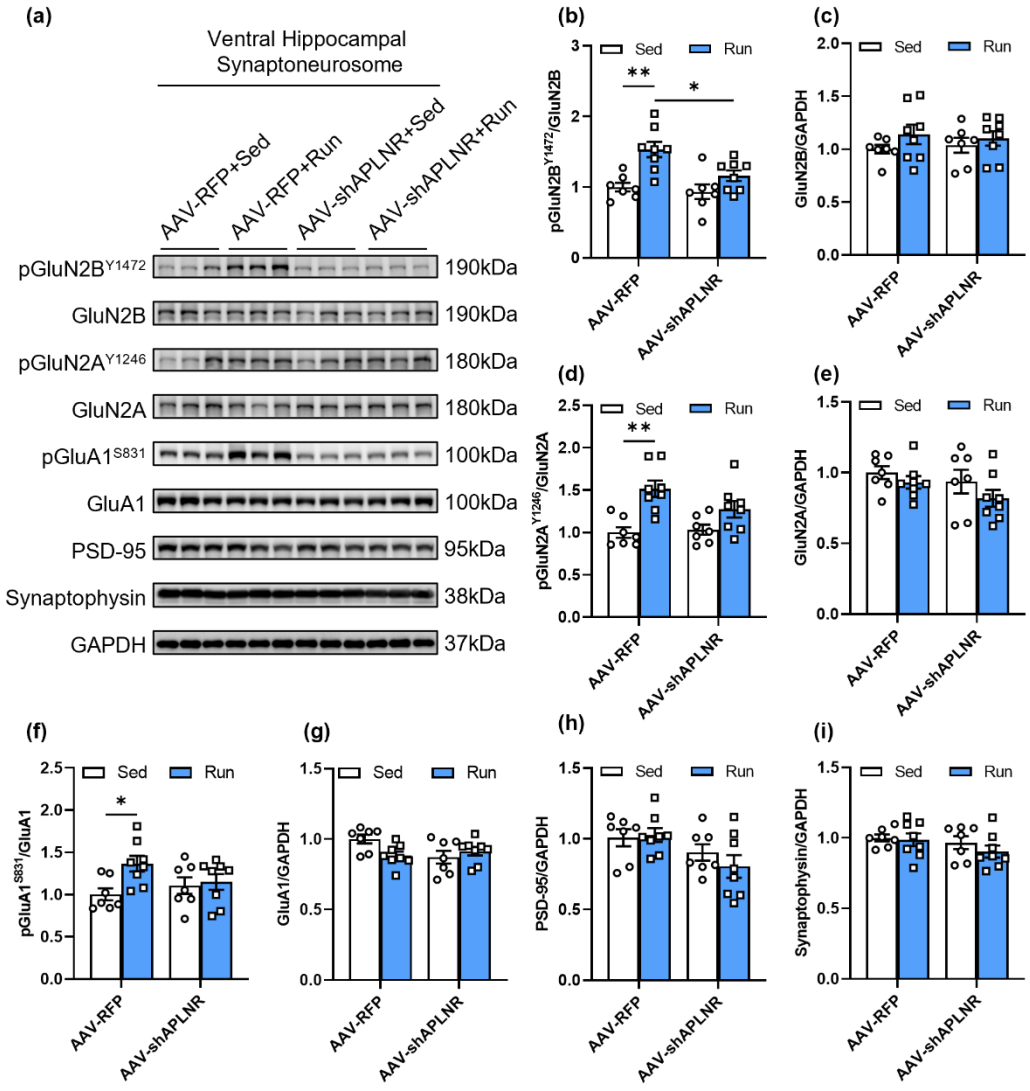

Figure 6.5 Knockdown of APJ in hippocampal glutamatergic neurons diminished the effects of voluntary running on enhancing synaptic NMDA receptor phosphorylation in ventral hippocampus.

(a) Western blotting analysis of ventral hippocampal synaptoneurosome protein levels. AAV-RFP: Camk2a-Cre mice with AAV-DIO-RFP injection; AAV-shAPLNR: Camk2a-Cre mice with AAV-DIO-shAPLNR injection; Sed: sedentary mice; Run: runner mice. Knockdown of APJ in hippocampal glutamatergic neurons diminished the increased of running effects on protein expression levels of (b) phospho-GluN2B (Y1246), (d) phospho-GluN2A (Y1472), and (f) phospho-GluA1 (S831). But neither running nor APJ knockdown affected total (c) GluN2B, (e) GluN2A, (g) GluA1, (h) PSD-95, and (i) synaptophysin protein expression levels. Data are presented as mean ± S.E.M. Statistical significance was analyzed by Two-way ANOVA followed by Tukey's post hoc test, with significance levels of *$p < 0.05$, **$p < 0.01$.

In Chapter 5, we found that overexpression of muscle apelin can enhance the phosphorylation of NMDA receptors at hippocampal synaptoneurosome extraction, potentially related to the expression of CK2α in the cytosol fraction. To verify whether this signaling cascade is involved in running-induced phosphorylation of NMDA receptors, we examined the expression of CK2α and CK2β in the ventral hippocampus (Fig. 6.6a). Two-way ANOVA indicated a significant effect of APJ knockdown on CK2α expression (Fig. 6.6b; $F(1, 20) = 5.989$, $P < 0.05$), but no significant effects of running or their interaction (Fig. 6.6b; $P > 0.05$). Post-hoc Tukey analysis showed that running increased CK2α expression in the cytosol fraction of the ventral hippocampus in AAV-RFP mice (Fig. 6.6b; $P < 0.05$ for AAV-RFP + Sed vs. AAV-RFP + Run). In running mice, APJ knockdown in glutamatergic neurons reduced the exercise-induced increase in CK2α expression (Fig. 6.6b; $P < 0.01$ for AAV-shAPLNR + Sed vs. AAV-shAPLNR + Run). However, two-way ANOVA showed no significant effects of running, APJ knockdown, or their interaction on CK2β expression in the cytosol fraction of the ventral hippocampus (Fig. 6.6c; $P > 0.05$). These results suggest that APJ knockdown in ventral hippocampal glutamatergic neurons abolishes the exercise-induced increase in CK2α expression.

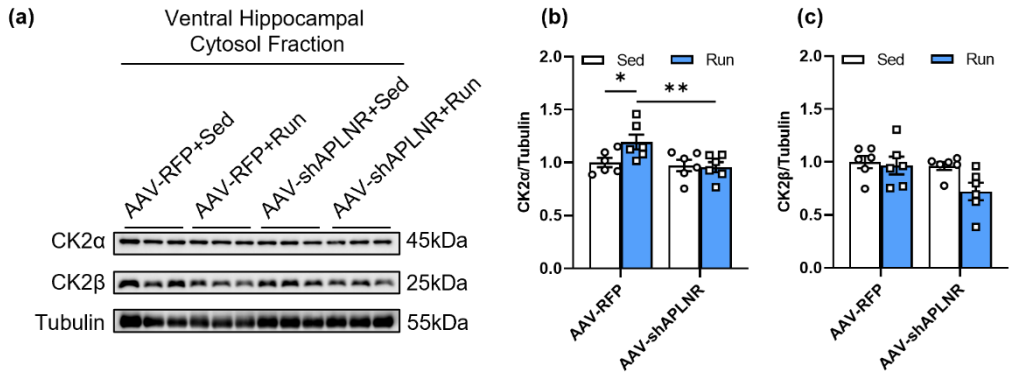

Figure 6.6 Knockdown of APJ in hippocampal glutamatergic neurons diminished the effects of voluntary running on enhancing CK2α expression in ventral hippocampus.

(a) Western blotting analysis of CK protein levels in ventral hippocampal cytosol fraction. AAV-RFP: Camk2a-Cre mice with AAV-DIO-RFP injection; AAV-shAPLNR: Camk2a-Cre mice with AAV-DIO-shAPLNR injection; Sed: sedentary mice; Run: runner mice. Knockdown of APJ in hippocampal glutamatergic neurons diminished the increased protein expression levels of (b) CK2α in runner mice. However, neither running nor APJ knockdown affected (c) CK2β expression levels. Data are presented as mean ± S.E.M. Statistical significance was assessed by Two-way ANOVA followed by Tukey's post hoc test, with significance levels of *$p < 0.05$, **$p < 0.01$. CK2: Creatine kinase 2.

6.3. Discussion

In this chapter, we investigated the antidepressant effects of apelin receptors (APJ) in glutamatergic neurons of the ventral hippocampus, particularly in the context of voluntary running. Knockdown of APJ in these neurons diminished the beneficial effects of running on adult hippocampal neurogenesis, specifically impairing neuronal maturation and survival. Furthermore, we demonstrated that APJ in glutamatergic neurons is crucial for the running-induced enhancement of NMDA receptor GluN2B function in ventral hippocampal synaptoneurosome, potentially linked to the expression of CK2α, a downstream signaling factor of APJ. These findings highlight the essential role of APJ in mediating the antidepressant effects of voluntary running, suggesting that apelin-APJ-mediated hippocampal plasticity is pivotal for the antidepressant benefits of exercise.

In previous chapters, we demonstrated that running increased APJ expression in the hippocampus and was associated with antidepressant effects. Overexpression of peripheral apelin via intramuscular injection of AAV exhibited antidepressant effects and increased APJ expression in the hippocampus, emphasizing the role of APJ in this brain region. The hippocampus, particularly the ventral region, is key for emotional regulation [390,391], where apelin exerts its antidepressant effects [275,285]. Our study showed that APJ is predominantly expressed in glutamatergic neurons rather than GABAergic neurons in hippocampus. The antidepressant effects of running are closely linked to the modulation of glutamatergic neurotransmission, particularly within the hippocampus [392,393]. This suggests that APJ in ventral hippocampal glutamatergic neurons is potentially related to depression. Our results provide direct evidence that the knockdown of APJ in these neurons diminishes the antidepressant effects of running and suggest that targeting APJ may shed new light on the development of antidepressant strategies.

We observed that the antidepressant effect of running increased APJ expression in the glutamatergic neurons of the hippocampus. This upregulation was associated with increased hippocampal plasticity, including cell proliferation, the increased number of immature neurons, and neuronal survival. Interestingly, running increased neuronal differentiation only in the ventral hippocampus, suggesting a sustained effect on the differentiation of neural progenitor cells (NPCs) in this region. Notably, knockdown of APJ in glutamatergic neurons diminished these beneficial effects of running. A recent

study indicated that apelin enhances cell proliferation and differentiation, while APJ antagonist ML221 administration has the opposite effect [330]. Furthermore, apelin-13 was found to increase hippocampal neurogenesis by inhibiting neuronal death [287]. Therefore, our findings support the view that apelin and APJ are involved in hippocampal neurogenesis, highlighting their potential as targets for developing novel antidepressant therapies.

NMDA receptor-mediated glutamatergic transmission is crucial for synaptic plasticity in the hippocampus. Interestingly, the knockdown of APJ in glutamatergic neurons of the ventral hippocampus reduced the running-promoted phosphorylation of the NMDA receptor subunit GluN2B, while having no significant effect on GluN2A or the AMPA receptor subunit GluA1. This reduction highlights the specific role of GluN2B in both antidepressant effects and hippocampal plasticity, as running-induced synaptic plasticity depends on GluN2B subunits [380]. In Chapter 5, we found that overexpression of muscle apelin promoted phosphorylation of both GluN2B and GluN2A at hippocampal synapses and increased the expression of CK2α in the cytosol. Our current results suggest that the effects of APJ on GluN2B phosphorylation in glutamatergic neurons may be related to CK2α expression. Cook et al. [242] reported that apelin binding to APJ can mitigate excessive NMDA-induced neurotoxicity by controlling GluN2B phosphorylation through CK2 protein activity. As a G protein-coupled receptor, APJ can activate CK2α expression via the Gαi/αq subunit [351]. These findings support our results and highlight the APJ/CK2α/GluN2B pathway as a potential mechanism involved in exercise-mediated hippocampal functional plasticity. Therefore, further experiments are needed to verify whether CK2α is key to the APJ-GluN2B-mediated signaling pathway, which could have implications for developing therapeutic strategies targeting synaptic plasticity.

Furthermore, our results show that four weeks of voluntary running had no effect on the postsynaptic protein PSD-95 and the presynaptic protein synaptophysin in the ventral hippocampus. This finding challenges the prevailing concept that exercise promotes hippocampal plasticity and exerts antidepressant effects by increasing synaptic protein expression [394,395]. Several factors may account for these inconsistencies: type of exercise and brain regions. Different forms of exercise may lead to varying effects on synaptic proteins and may also be brain region-specific [396]. For instance, a recent study by Sadri et al. [397] reported that eight weeks of aerobic exercise training

improved PSD-95 expression in the prefrontal cortex (PFC) and the hippocampus, but had no effect on synaptophysin expression. In contrast, Liu et al. [398] found that short-term resistance exercise increased the expression of presynaptic proteins such as synaptotagmin 1 and PSD-95 in the PFC, but did not affect PSD-95 expression in the hippocampus. In addition, the choice of disease models and the age of subjects can further influence outcomes. Wang et al. [399] reported that long-term voluntary running increased PSD-95 expression in the hippocampus of an Alzheimer's disease mouse model (APP/PS1 mice) but had no effect on WT mice. Similarly, Siette et al. [400] found that voluntary running restored hippocampal synaptophysin and PSD-95 in aged rats (20-month-old), but did not affect these proteins in adult rats (7-month-old), suggesting age-specific effects. Overall, these studies highlight the complexity of exercise-induced synaptic changes, suggesting that factors such as exercise type, brain region, and individual differences play crucial roles. Therefore, further studies are needed to verify the association of synaptic proteins with exercise-mediated hippocampal plasticity and the antidepressant effects of this link.

Notably, our results showed that the knockdown of APJ alone in glutamatergic neurons of the ventral hippocampus did not induce depressive-like behavior, aligning with Bullich et al.'s report [204] that systemic knockout of APJ did not lead to depression- or anxiety-related effects. This also suggests that the impact of APJ deficiency on depression is independent of brain region differences. Interestingly, APJ knockdown did not affect hippocampal plasticity in our study, as evidenced by the unchanged NMDA and AMPA receptor subunits and adult neurogenesis in the ventral hippocampus. However, we observed an elevation in the number of Ki-67-positive cells, possibly indicating compensatory facilitation of postoperative trauma repair due to APJ deletion in glutamatergic neurons. As there are no further studies on the role of hippocampal APJ deletion in depressive-like behavior, our findings provide favorable evidence that ventral hippocampal glutamatergic APJ is involved in the antidepressant effect of exercise, potentially through mechanisms related to neurogenesis and mood regulation.

Chapter 7. The Loss of Muscle Apelin Accelerated Age-associated Muscle Atrophy without Affecting Cognitive Impairments

7.1. Introduction

Systemic aging encompasses gradual deterioration in physiological functions and the heightened susceptibility to age-related diseases across the entire body [401]. This includes various systems and tissues, such as the musculoskeletal system and the nervous system. Sarcopenia, an age-related condition marked by a progressive muscle weakness, significantly impacts the independence of older adults [402,403], whereas cognitive deficits involves a reduction in cognitive abilities affecting one or more domains including loss of memory and language [404].

Emerging clinical evidence suggests that sarcopenia may play a contributory role in the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) [405]. A cross-sectional study has demonstrated a notable correlation between muscle strength and cognitive function in older adults [406]. Additional research has indicated that older women with pre-sarcopenia or sarcopenia (201 and 544 participants, respectively) were twice as likely to develop cognitive impairment compared to their non-sarcopenic counterparts [407,408]. Another study indicated that muscle strength or physical function, rather than leanness, is a more reliable predictor of cognitive performance [409]. These studies suggest that age-related muscle weakness or even sarcopenia-like syndrome are more likely to induce neurodegenerative diseases during the aging process. However, the mechanisms behind the link between muscle aging and neurodegenerative diseases are still unknown.

Systemic factors such as hormones and cytokines can influence both muscle and brain health. Apelin, a myokine released by muscle cells, plays an essential role in muscle metabolism and function. Its production is mediated by exercise but decreased with age, and correlated with a decline in muscle function [221,354,410]. Notably, exercise-induced elevation in serum apelin levels protects against sarcopenia [21]. In addition, apelin has a protective role in cognitive impairment in neurodegenerative diseases. Apelin-13 is involved in repairing neuronal damage in the hippocampus and mitigating cognitive impairment and AD pathology via the PGC-1α/PPARγ signaling pathway [23].

Notably, intranasal administration of apelin-13 has proven to be effective in ameliorating cognitive deficits in AD animal models by enhancing synaptic plasticity, indicating a promising therapeutic target [411]. Furthermore, apelin-13 exhibits anti-apoptotic, antioxidant, and anti-inflammatory properties, which are vital for maintaining neuronal integrity and function [205]. Collectively, these findings underscore the potential of apelin as a therapeutic agent for treating cognitive impairment in neurodegenerative disorders like AD. However, it remains unknown whether apelin deficiency exacerbates aging-related cognitive impairment.

In previous chapters, we confirmed the muscle apelin as a key factor in exercise-induced antidepressant effects and hippocampal plasticity. Muscle-specific knockout of apelin in young mice (10-week-old) decreased muscle strength and motor coordination but showed no effects on muscle mass. Given that systemic aging induces muscle weakness and cognitive decline, this chapter aims to investigate whether the loss of skeletal muscle apelin exacerbates age-associated muscle aging and cognitive impairment.

7.2. Results

7.2.1. Skeletal muscle apelin deficiency exacerbated age-related muscle strength decline and motor coordination impairment.

We examined the impact of apelin deficiency on age-associated muscle function loss by assessing muscle strength and motor coordination in adult (6-month-old), middle-aged (12-month-old), and aged (18-month-old) APLN-mKO mice, using Flox background mice as controls (Fig. 7.1a).

We first compared the weight gain of mice at different ages. Two-way ANOVA indicated that aging showed main effects on body weight gain (Fig 7.1b; $F(2, 40) = 13.29$, $P < 0.0001$), while there were no significant main effects of muscle-specific knockout apelin ($F(1, 40) = 2.846$, $P > 0.05$) and interaction ($F(2, 40) = 0.3977$, $P > 0.05$). Mann-Whitney post hoc test indicated that muscle-specific apelin knockout significantly increased age-associated body weight gain in Flox control mice (Fig. 7.1b; $P < 0.05$ for 6-month Flox vs. 12-month Flox, and 6-month APLN-mKO vs. 12-month APLN-mKO; $P < 0.01$ for 6-month APLN-mKO vs. 18-month APLN-mKO).

We then measured four-limb muscle strength using the grip strength test. Two-way ANOVA showed there are significant effects of apelin knockout in skeletal muscle (Fig. 7.1c; $F(1, 40) = 30.25$, $P < 0.0001$) and aging ($F(2, 40) = 33.17$, $P < 0.0001$) on muscle strength, with no significant interaction ($F(2, 40) = 0.3395$, $P > 0.05$). Post hoc analysis presented a decline in muscle strength in middle-aged and aged Flox (control) mice compared to adult Flox mice ($P < 0.001$ for 6-month Flox vs. 12-month Flox; $P < 0.0001$ for 6-month Flox vs. 18-month Flox). Additionally, the absence of apelin in the muscle accelerated decrease in muscle strength in comparison between control and APLN-mKO ($P < 0.05$ for 6-month Flox vs. 6-month APLN-mKO; $P < 0.001$ for 12-month Flox vs. 12-month APLN-mKO).

Motor coordination was assessed using the accelerated rotarod test. Two-way ANOVA demonstrated that both apelin knockout in muscle (Fig. 7.1d; $F(1, 40) = 15.64$, $P < 0.001$), aging ($F(2, 40) = 25.74$, $P < 0.001$), and their interaction ($F(2, 40) = 8.296$, $P < 0.01$) significantly affected motor coordination. Post hoc analysis revealed that 12- and 18-month Flox control mice were more prone to fall from the rotarod compared to 6-month adult Flox control mice (Fig. 7.1d; $P < 0.0001$ for 6-month Flox vs. 12-month Flox; $P < 0.0001$ for 6-month Flox vs. 18-month Flox). Interestingly, muscle apelin deficiency significantly reduced latency to fall in 6-month Flox mice (Fig. 7.1d; $P <$

0.0001 for 6-month Flox vs. 6-month APLN-mKO) but had no effect on motor coordination weakness in middle-aged (12-month) or aged mice (18-month) (Fig. 7.1d; $P > 0.05$), indicating that muscle apelin is crucial in mitigating motor coordination decline during aging. In conclusion, these results indicate that muscle apelin deficiency induces loss of muscle strength and exacerbates age-associated muscle function decline.

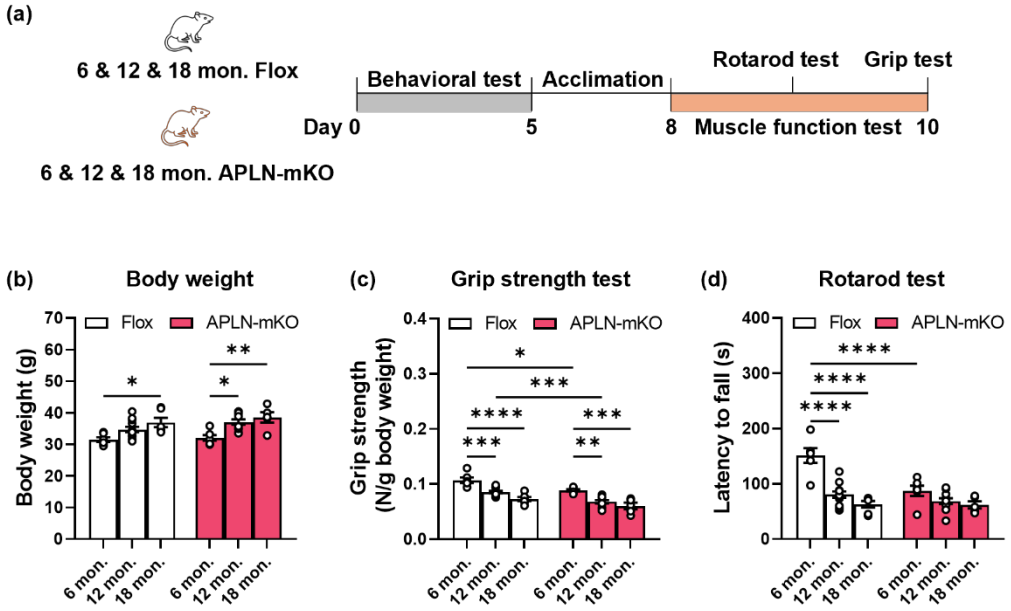

Figure 7.1 Skeletal muscle-specific apelin knockout accelerated age-related decline in muscle strength and muscle strength.

(a) Study timeline of muscle function test and behavioral test. Flox: control mice with APLN-Flox background mice; APLN-mKO: muscle specific apelin knockout mice. Both aging and muscle-specific knockout of apelin (b) increased the body weight gain, (c) decreased the four-limb grip strength normalized to the body weight, and (d) weakened the motor coordination in rotarod test, assessed by latency to fall. Data are presented as mean ± S.E.M. Statistical significance was assessed using a two-way ANOVA with Fisher's LSD post hoc analysis, with significance levels of *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

7.2.2. Skeletal muscle apelin deficiency did not affect age-related cognitive impairment and depression-like behavior.

To confirm whether muscle-specific knockout of apelin impairs cognitive function during aging, we measured spatial recognition and object discrimination using the Y-maze task and novel object recognition test (NORT), respectively. The two-way

ANOVA illustrated no significant effects of aging (Fig. 7.2b & c; $F(2, 56) = 1.163$, $P > 0.05$ for exploration ratio and discrimination index), muscle apelin deficiency ($F(1, 56) = 0.006783$, $P > 0.05$ for exploration ratio and discrimination index). There was significant interaction ($F(2, 56) = 2.038$, $P > 0.05$ for exploration ratio and discrimination index) in the exploration ratio and index in NORT. Post hoc analysis showed that 12- month, but not 18-month, Flox mice exhibited lower ratios of novel object exploration in NORT compared to 6-month mice, indicating that aging induced cognitive impairment (Fig. 7.2b & c; $P < 0.05$ for 6-month Flox vs. 12-month Flox; $P > 0.05$ for 6-month Flox vs. 18-month Flox). However, muscle-specific apelin knockout had no effect on novel object discrimination at different ages (Fig. 7.2b & c; $P > 0.05$).

Additionally, two-way ANOVA showed that aging (Fig. 7.2d & e; $F(2, 62) = 5.151$, $P < 0.01$ for exploration ratio and index), but not muscle-specific apelin knockout ($F(1, 62) = 0.1516$, $P > 0.05$ for exploration ratio and index) and their interaction ($F(2, 62) = 1.542$, $P > 0.05$ for exploration ratio and index), had significant effects on spatial memory in the Y-maze task. Post hoc analysis found that 18-month Flox and 6-month mice had lower ratios and indexes of exploration in the novel arm than 12- and 18-month mice (Fig. 7.2d & e; $P < 0.05$ for 6-month Flox vs. 18-month Flox; $P < 0.01$ for 12-month Flox vs. 18-month Flox), whereas APLN-mKO mice displayed no significant difference (Fig. 7.2d & e; $P > 0.05$). These results suggest that aging, but no muscle specific apelin knockout, reduces object discrimination and spatial memory.

We then investigated whether muscle-specific apelin knockout affects depression- or anxiety-like behavior in aging mice. As in the previous two chapters, we tested anhedonia-like behavior and anxiety-like behaviors. Two-way ANOVA showed main effect of aging (Fig. 7.2f; $F(2, 63) = 8.349$, $P < 0.001$), but no main effect of muscle specific apelin knockout ($F(1, 63) = 0.7350$, $P > 0.05$), and interaction ($F(2, 63) = 1.893$, $P > 0.05$) on immobility time in FST. Post hoc analysis found that aged mice developed depressive-like behaviors indicated by elevated immobility time in FST compared to adult and middle-aged mice (Fig. 7.2f; $P < 0.05$ for 6-month Flox vs. 18-month Flox; $P < 0.01$ for 12-month Flox vs. 18-month Flox), but this effect was absent when apelin was knocked out in muscle (Fig. 7.2f; $P > 0.05$). In SST, Two-way ANOVA showed that ageing (Fig. 7.2g; $F(2, 62) = 3.678$, $P < 0.05$), but not muscle apelin deficiency ($F(1, 62) = 0.0007868$, $P > 0.05$) and their interaction ($F(2, 62) = 0.6568$, $P > 0.05$), has effects on grooming behavior. Post hoc analysis indicated that aged mice

exhibited shorter grooming duration in SST compared to adult and middle-aged mice (Fig. 7.2f; $P < 0.05$ for 6-month Flox vs. 18-month Flox; $P < 0.05$ for 12-month Flox vs. 18-month Flox). In SPT, Two-way ANOVA showed that neither muscle apelin deficiency (Fig. 7.2h; $F (1, 62) = 1.323$, $P > 0.05$), nor aging ($F (2, 62) = 1.266$, $P > 0.05$) had significant effects on the sucrose preference in SPT, indicating that muscle apelin knockout affected anhedonia-like behaviors.

For anxiety-like behaviors, two-way ANOVA showed that neither muscle-specific apelin knockout (Fig. 7.2i; $F (1, 63) = 0.2329$, $P > 0.05$) nor aging ($F (2, 63) = 4.418$, $P > 0.05$) had significant effects on time spent in the central zone of the OFT. These results indicate that muscle apelin knockout has no effect on exaggerating ageing-induced anhedonia- and anxiety-like behaviors.

Interestingly, we found a significant effect of aging (Fig. 7.2j; $F (2, 63) = 21.56$, $P<0.0001$), but not muscle specific apelin knockout ($F (1, 63) = 0.4327$, $P > 0.05$) or their interaction ($F (2, 63) = 0.1658$, $P > 0.05$), on locomotor activity in the OFT. Post hoc analysis revealed that aged mice had significant reduction in traveling distance in OFT compared to adult and middle-aged mice, regardless of whether apelin was knocked out in muscle (Fig. 7.2j; $P < 0.05$ for 6-month Flox vs. 12-month Flox; 6-month APLN-mKO vs. 12-month APLN-mKO; 12-month APLN-mKO vs. 18-month APLN-mKO; $P < 0.0001$ for 6-month Flox vs. 18-month Flox; 6-month APLN-mKO vs. 18-month APLN-mKO), indicating that aging reduces locomotor activity.

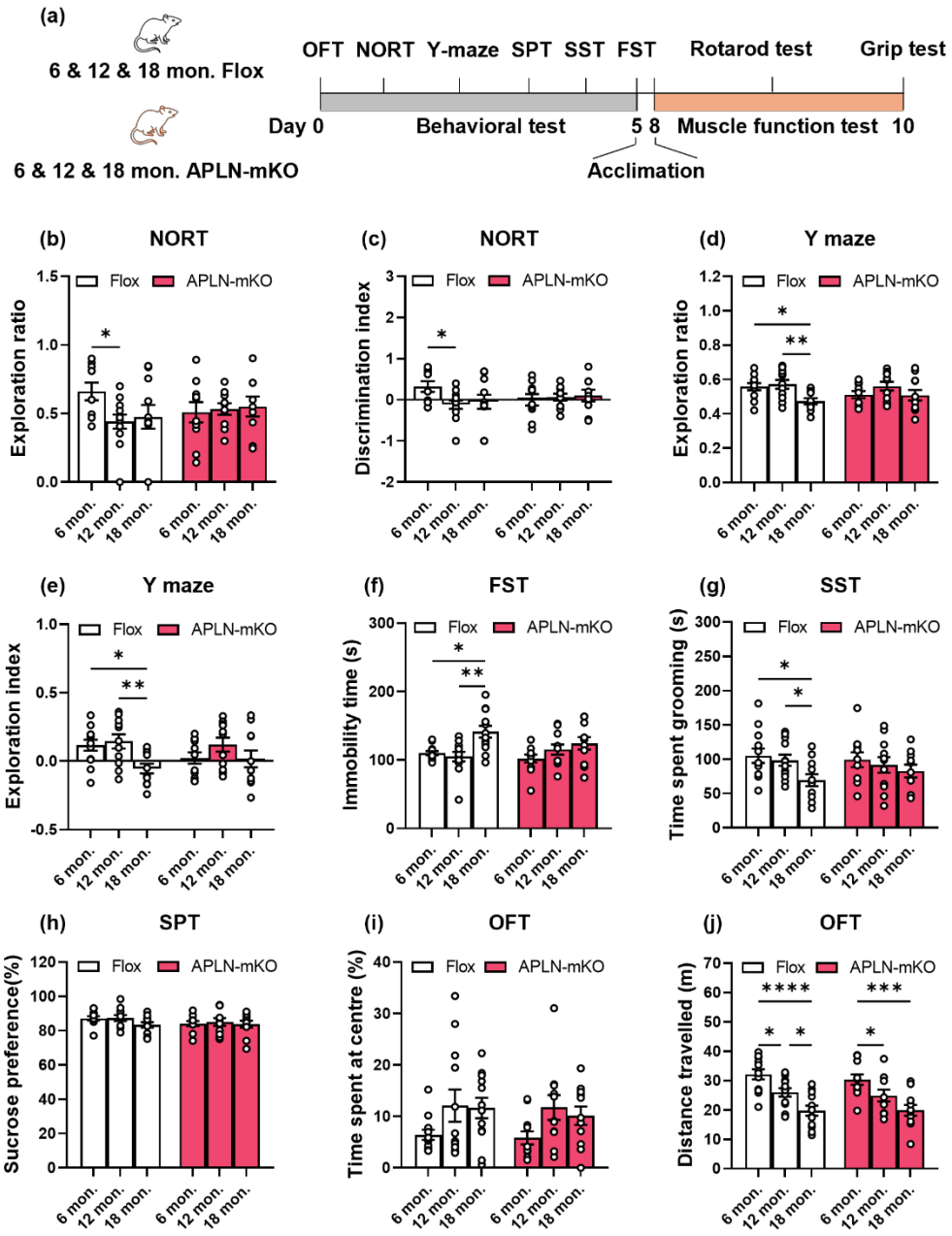

Figure 7.2 Skeletal muscle-specific apelin knockout has no effect on age-related cognitive deficiency and emotional dysregulation

(a) Study timeline; Flox: control mice with APLN-Flox background mice; APLN-mKO: muscle-specific apelin knockout mice. Muscle-specific knockout of apelin had no effect, but aging decreased cognitive deficit as shown by (b) reduced the exploration ratio, and (c) the discrimination index of novel objects in NORT and decreased (d) the exploration ratio and (e) index of novel arm in Y-maze. Muscle-specific knockout of apelin had no effect, but aging exhibited the depression-like behavior indicated by (f) increased immobility time in FST, (g) reduced the time spent grooming in SST, and (h) suppressed the sucrose preference in the SPT. Both aging and muscle apelin deficiency had no

effect on anxiety-like behavior indicated by (i) the duration in the center of OFT. Age decreased (j) the total distance traveled in OFT in both Flox control and APLN-mKO mice. Data are presented as mean ± S.E.M. A two-way ANOVA with Fisher's LSD post hoc test was conducted, with significance levels of *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. NORT: novel object recognition test, OFT: open field test, SPT: sucrose preference test, SST: sucrose splash test, FST: forced swim test.

7.3. Discussion

In this chapter, we found that skeletal muscle-specific apelin knockout exacerbates age-related muscle strength loss, motor coordination deficit. However, apelin deficiency in muscle had no effect on age-related cognitive impairment or depression-like behavior.

The apelin-APJ signaling pathway has been implicated in the aging process. Knockout of APLN and the APJ gene accelerates systemic aging in mice, while upregulation of apelin restores muscle function [232]. As aging progresses, natural declines in muscle mass and strength, known as sarcopenia, contribute to reduced mobility, increased fall risk, and decreased well-being in the elderly [8]. In Chapter 5, we demonstrated that skeletal muscle apelin deficiency in young mice (10-week-old) impairs muscle strength and motor coordination. Here, we found that aging (at the age of 6-, 12-, and 18-month-old) and muscle-specific apelin knockout both weaken muscle strength and coordination. Consistent with our findings, Vinel et al., [17] found that apelin production declines with age, and absence of skeletal muscle apelin accelerates muscle weakness. Notably, apelin overexpressed reverses age-associated sarcopenia in aged mice, suggesting that apelin protects muscle tissue by promoting growth, enhancing repairment, and improving metabolic functions. Furthermore, exercise-induced apelin improves body composition, muscle strength, and insulin resistance, and is associated with increased muscle fiber size and capillarization [354]. Recent clinical trials have demonstrated encouraging outcomes for apelin agonists. Azelaprag (AMG-986, or BGE-105), a small-molecule apelin receptor agonist, significantly improves muscle size, quality, and protein synthesis in older adults subjected to bed rest conditions [412–414]. This is particularly relevant as periods of inactivity, such as hospitalization, can accelerate muscle atrophy in older individuals. These findings highlight the contributory role of apelin agonists as treatments for muscle atrophy and underscore the importance of the apelin pathway in maintaining muscle health during aging.

In addition to sarcopenia, aging diminishes cognitive function [415]. Our findings show that aged mice developed short-term learning and memory deficits. However, skeletal muscle-specific apelin knockout did not affect spatial cognition and working memory in all age groups, although it induced muscular function deficit. Although clinical studies in recent years have confirmed that sarcopenia is accompanied by cognitive decline in elders, the relationship between apelin and cognition is

controversial. Previous studies reported that apelin-13 administration impairs cognitive functions by decreased short-term' formation (delay of 30 minutes), but did not affect long-term memory's consolidation (delay of 24 hours) [416,417]. This suggests an inhibitory role in certain memory processes in apelin, but the detailed mechanisms remain unknown. Nonetheless, one previous study reported that knockout of APJ (apelin receptor) heightens fear responses, particularly in males, suggesting that the regulatory role of apelin in fear conditioning potentially different from the therapeutic role in cognitive deficits of neurodegenerative diseases [229].

It is worth noting that recent studies suggest apelin enhances memory consolidation in mice including spatial recognition and working memory. Apelin-13 ameliorates cognitive deficits in neurodegenerative diseases model such as AD and PD. Intranasal administration of apelin alleviates cognitive impairment in a streptozotocin-induced AD model by reducing oxidative stress [411]. In a PD model, apelin-13 significantly reduces cognitive impairments induced by 6-hydroxydopamine, improving performance in memory and spatial navigation tasks [418]. In contrast, our study found that skeletal muscle-specific apelin knockout did not affect cognitive functions across different age groups. This was unexpected, given that apelin's neuroprotective effects, including reducing oxidative stress and inflammation in the age-relative model via serial signal pathway. For instance, apelin provides neuroprotection effects following subarachnoid hemorrhage by reducing inflammation and oxidative stress through the AMPK/Thioredoxin-interacting protein (TXNIP)/NLRP3 signaling pathway, enhancing long-term spatial memory [26,248]. In chronic stress models, apelin-13 improves recognition memory via activation of PI3K and ERK1/2 pathways and mitigates memory deficits and depressive symptoms [19,286]. It enhances hippocampal BDNF expression, suggesting its therapeutic potential for stress-related cognitive impairments [419]. Additionally, apelin-13 alleviates cognitive impairments by increasing BDNF levels and reducing hippocampal inflammation, improving spatial memory and reducing neuronal and glial cell damage [420]. However, the lack of impact on age-related cognitive decline in our study may indicate that the mechanisms by which apelin influences cognition are distinct from those affecting muscle health. It is possible that apelin's cognitive effects are more pronounced in pathological conditions rather than in normal aging.

Moreover, skeletal muscle-specific apelin knockout did not affect depression- or anxiety-like behaviors in adult, middle-aged, aged, and young mice (10-week-old mice in Chapter 5), despite aging itself inducing depression-like behavior. This finding contrasts with preclinical studies where apelin administration demonstrated antidepressant and anxiolytic effects [18,22,285]. One plausible explanation for this discrepancy is that the absence of apelin in muscle may not directly impact the brain's emotional regulation pathways. This suggests that apelin's role in mood regulation might be mediated through systemic or central mechanisms rather than localized muscle effects. In addition, previous research has highlighted the biphasic effects of apelin, where global knockout in 12-month-old mice resulted in antidepressant-like behavior, while younger mice (6 months old) exhibited anxiety-like behavior[204]. This indicates that apelin's influence on mood may vary with age and physiological context, reflecting a complex interaction between metabolic and emotional pathways. The lack of significant research on age-related mood disorders associated with apelin underscores the need for further investigation into its antidepressant mechanisms. Understanding these pathways could reveal novel therapeutic targets for mood disorders, particularly in the context of aging.

A limitation of our study is the absence of a systemic knockout of apelin, which prevents us from ruling out the possibility of compensatory secretion of apelin from other tissues. While Rai et al. [221] showed that apelin knockout is associated with accelerated aging in cardiovascular, renal, and reproductive systems, further studies are needed to reveal the relationship between apelin and aging-associated cognitive and emotional deficits. In conclusion, skeletal muscle apelin deficiency aggravates age-associated muscle weakness but does not affect cognitive function or emotional regulation.

Chapter 8. Conclusion and Future Directions

8.1. Conclusion

Physical exercise is well-established as a potent antidepressant, yet the molecular mechanisms that underpin its therapeutic benefits are still not fully understood. This study investigates the molecular basis of exercise-induced antidepressant effects, focusing on apelin, a novel myokine associated with sarcopenia, and its potential role in modulating hippocampal neuroplasticity. Utilizing a stressed mouse model induced by chronic unpredictable stress (CUS), we observed a significant reduction in the expression of apelin and receptor APJ in the hippocampus. Remarkably, a 4-week voluntary running ameliorated depression-like behaviors, correlating with an upregulation of the apelin and APJ expression and enhanced adult neurogenesis in the hippocampus. In the mice with running, we confirmed that skeletal muscles, particularly the tibialis anterior (TA) and gastrocnemius (Gas), are crucial organs for apelin secretion.

Our findings reveal that muscle-specific apelin knockout abolished the antidepressant effects of voluntary running, underscoring the indispensable role of apelin in the muscle-brain axis. Furthermore, overexpression of apelin in TA and Gas muscles replicated the antidepressant effects of running, enhanced hippocampal neurogenesis and increased synaptic NMDA receptor-mediated synaptic transmission with promoting phosphorylation levels. This suggests a novel mechanistic pathway in which muscle-derived apelin influences brain function.

In addition, we further confirmed the role of receptors APJ in glutamatergic neurons of the ventral hippocampus in mediating the antidepressant effects of voluntary running. We found that knocking down APJ in these neurons impaired the running-induced benefits on adult hippocampal neurogenesis, synaptic NMDA receptor function, potentially through APJ/CK2α/GluN2B signaling pathways. These findings position apelin as a crucial factor of the antidepressant effects of physical exercise, highlighting its potential as a therapeutic target for depression (Fig. 8.1).

Since apelin is involved in sarcopenia, our data also suggests a potential role of apelin in linking sarcopenia and increased onset of depression in the elderly. To explore apelin's role in aging, we found that muscle apelin deficiency accelerated age-related muscle atrophy and functional decline. However, it had no effect on age-associated cognitive deficits or depression-like behavior. This discrepancy indicates that apelin secreted by muscles selectively influences pathways related to physical performance, without directly affecting cognitive deterioration during aging. In conclusion, this research offers a foundational theoretical framework for future investigations into the muscle-brain axis and developing novel strategies for treating depression in elderly with muscle atrophy.

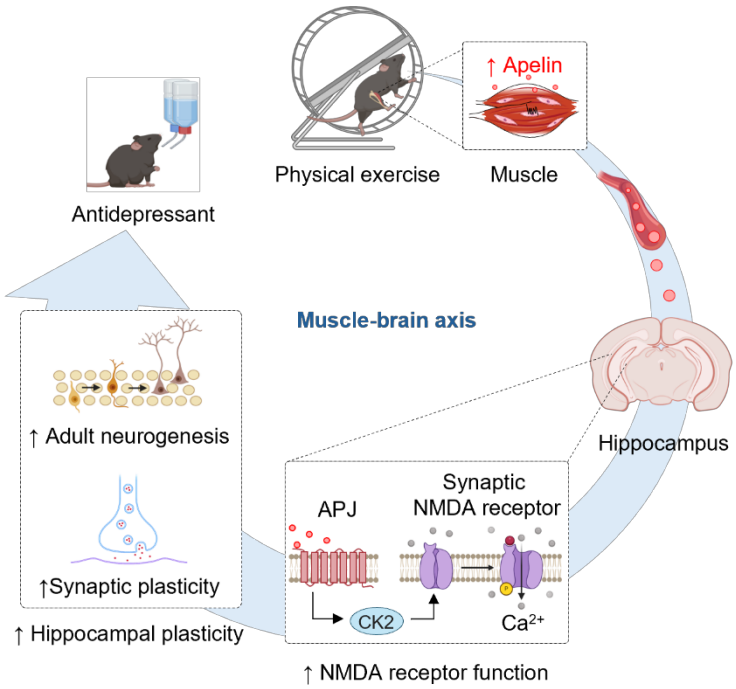

Figure 8.1 Antidepressant effects of the muscle apelin mediated by physical exercise on enhanced hippocampal plasticity via muscle-brain axis.

Physical exercise exerts an antidepressant effect by promoting the secretion of apelin from the skeletal muscles, which is secreted into the bloodstream and transported to the hippocampus. In the hippocampus, apelin binds to the APJ receptor, activating CK2 signaling and enhancing synaptic NMDA receptor function, leading to increased calcium influx. These mechanisms collectively improve synaptic plasticity and adult neurogenesis, contributing to the antidepressant and neuroplasticity-promoting effects of exercise. These processes highlights the mediating role of muscle-secreted apelin in modulating brain function and mood following exercise.

8.2. Future Directions

In the forthcoming investigation, we aim to elucidate the mechanisms in which apelin modulates hippocampal plasticity and neurotransmission, contributing to its antidepressant effects. Our findings in Chapter 5 indicate that muscle apelin overexpression promotes hippocampal APJ levels, enhancing the phosphorylation of postsynaptic NMDA receptors and subsequent expression of Calpain 2—a protein pivotal for calcium regulation and synaptic plasticity which is associated with the hippocampal CK α and β protein expression. In Chapter 6, we observed that knocking down APJ in glutamatergic neurons of the ventral hippocampus reduced the voluntary running-induced enhancement of NMDA receptor phosphorylation, potentially mediated by CK2α expression. Other *in vitro* studies have demonstrated the capacity of the apelin and APJ pathway to attenuate NMDA-mediated Ca^{2+} influx and neurotoxicity via CK2 activation [242] (Fig. 8.2). Despite these insights, the role of apelin-enhanced synaptic NMDA receptor function in mediating antidepressant effects remains to be clarified. Therefore, future investigation aims to validate the role of apelin in synaptic plasticity and calcium homeostasis employing electrophysiological techniques, such as patch-clamp recordings, alongside calcium imaging modalities. We will further investigate the APJ/CK2 pathway's involvement by silencing APJ and inhibiting CK2 in the hippocampi of runner mice, assessing function of synaptic NMDA receptor functionality. Finally, we will use behavioral tests to verify whether the antidepressant benefits of physical exercise involving apelin are indeed facilitated through the modulation of synaptic plasticity depending on the APJ/CK2 pathway.

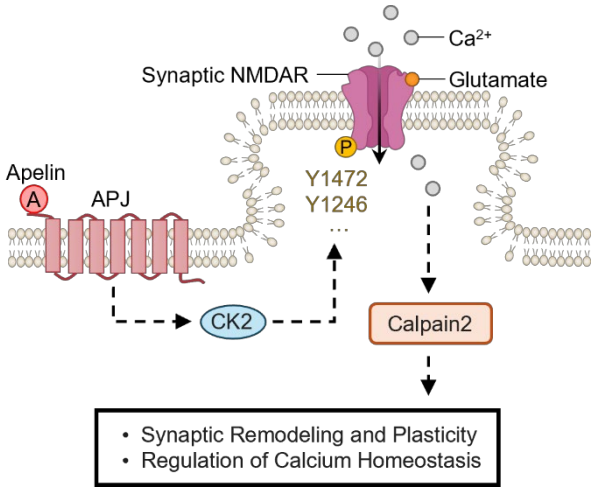

Figure 8.2 Proposed mechanism of apelin and NMDA interaction via activating CK2.

APJ triggers CK2 activation upon binding with apelin. This activation subsequently mediates the phosphorylation of synaptic NMDA receptors at the Y1472 site in GluN2B and the Y1246 site in GluN2A. Upon binding glutamate, the NMDA receptors are activated to allow permeability of calcium into the cells and promote calpain 2 expressions. This increase in calpain 2, along with calcium homeostasis, leads to increased synaptic plasticity remodeling.

To further elucidate the mechanisms underlying apelin-induced adult hippocampal neurogenesis, we propose a comprehensive study exploring the molecular and cellular processes involved. Previous findings in Chapter 4 found that voluntary running enhances hippocampal neurogenesis through the upregulation of hippocampal apelin and APJ expression. Muscle-specific knockout of apelin negated these effects, while overexpression of apelin in muscle promoted hippocampal neurogenesis mimicking pro-neurogenic effects of running. Consistent to our results, intracerebroventricular (i.c.v.) injection of exogenous apelin has been shown to promote hippocampal neurogenesis by reducing neuronal apoptosis and enhancing dendritic complexity [287]. Nevertheless, the specific mechanism by which apelin enhances hippocampal neurogenesis remains unknown. Therefore, our future studies will focus on examining the therapeutic effects of apelin in neural progenitor cells (NPCs) proliferation, and differentiation, as well as its key signaling pathways. Understanding the mechanism by which apelin promotes adult hippocampal neurogenesis could provide valuable insights into potential therapeutic strategies for neurodegenerative diseases and mood disorders.

In Chapter 7, we observed that while muscle-specific apelin deficiency accelerates age-associated muscle function loss, it does not affect age-related cognitive deficits. This divergence suggests that muscle-secreted apelin selectively targets pathways related to physical function without directly influencing cognitive decline in aging. Therefore, another important future direction is to validate the neuroprotective effects of apelin in aging, especially sarcopenia-related cognitive and mood disorders. We propose to conduct studies to elucidate the neuroprotective mechanisms of apelin in aging. Firstly, we will genotype aging muscle-specific apelin knockout (APLN-mKO) mice to confirm the targeted deletion and to unveil the systemic effects of this deletion in neuronal function and cognitive health. We will assess the role of apelin in neuronal

degeneration, synaptic function, and neuroinflammation in the hippocampus and other relevant brain regions.

Furthermore, we will explore the potential role of apelin to mitigate aging-related cognitive and mood disorders by analyzing behavioral outcomes in these APLN-mKO mice, including assessments of learning, memory, and affective behaviors. This will involve a combination of behavioral testing, histological analysis, and molecular profiling to determine how the absence of muscle-derived apelin influences brain function and structure. By integrating these approaches, we aim to uncover novel anti-aging mechanisms mediated by apelin, providing a deeper understanding of myokines in mental health. These findings could provide evidence showing apelin as a potential therapeutic target for treating depression and cognitive decline.

Finally, our data suggests that apelin facilitates the muscle-brain crosstalk, mitigating sarcopenia-associated cognitive deficits, and highlights its potential as a therapeutic agent in geriatric depression. This research underscores the significance of apelin in the intersection of physical and mental health, paving the way for future research on its therapeutic applications in depression and neuroplasticity.

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