THE NEURAL MECHANISM OF RAPID AND SUSTAINED ANTIDEPRESSANT EFFECTS OF SINGLE-BOUT PHYSICAL EXERCISE:

THE INVOLVEMENT OF NUCLEAR TRANSLOCATION OF APPL1 PROTEIN IN ACC-GLUTAMATERGIC NEURONS

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The Neural Mechanism of Rapid and Sustained Antidepressant Effects of Single-bout Physical Exercise: the Involvement of Nuclear Translocation of APPL1 protein in ACC-glutamatergic Neurons

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CERTIFICATE OF ORIGINALITY

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Abstract

Physical exercise is a safe and effective non-pharmacological intervention for depression. Clinical studies indicate that single-bout physical exercise can rapidly improve mood in healthy individuals and depressed patients. However, the neural mechanisms responsible for this rapid onset of antidepressant effects remain unexplored. Adiponectin, an exerkine secreted by adipocytes, binds to its receptor adiponectin receptor (AdipoR) 1 or 2 and interacts with the adapter protein APPL1 to trigger downstream signaling pathways. Previous studies showed that adiponectin is crucial for the antidepressant effects of chronic physical exercise, and an acute increase in brain adiponectin can elicit rapid antidepressant effects within hours. Based on that, we hypothesized that adiponectin-AdipoR-APPL1 signaling may mediate the rapid antidepressant effects of single-bout physical exercise. Here we confirmed the rapid antidepressant effect of single-bout treadmill running in participants with/without depressive symptoms, which was replicated in control and stressed mice. Using the mouse model, our further investigation into neural basis through the whole-brain c-Fos mapping identified the anterior cingulate cortex (ACC) subregion of the medial prefrontal cortex (mPFC) as a pivotal region involved in the exercise-elicited rapid antidepressant effects. ACC-glutamatergic neurons underwent rapid and persistent activation after exercise, which was essential for the antidepressant response elicited by exercise. In addition, increased adiponectin levels in the medial prefrontal cortex were negatively correlated with a decrease in depressive behavior 30 min-post exercise. Adiponectin global knockout by KO mice or AdipoR1 knockdown in ACC-glutamatergic neurons significantly abolished neural activation and the rapid antidepressant response elicited by single-bout physical exercise. Furthermore, the adapter protein APPL1 in the ACC-glutamatergic neurons rapidly translocated into the nuclear after exercise, a process contingent upon adiponectin-AdipoR1 signaling. Nuclear APPL1 facilitated histone acetylation and promoted synaptic gene transcription and synaptic protein expression. Inhibiting APPL1 translocation negated changes in synaptic protein expression, spinogenesis, and the antidepressant effects of single-bout physical exercise, whereas re-introduction of APPL1 to the ACC-glutamatergic neurons of APPL1 knockout mice restored the effects of single-bout physical exercise. Together, the adiponectin-

AdipoR1-APPL1 pathway plays a significant role in mediating the rapid antidepressant effect of single-bout physical exercise via promoting histone acetylation to enhance synaptic protein expression and spinogenesis in the mPFC. The study highlights the clinical significance of a rapid onset of antidepressant effect of single-bout physical exercise, in addition to well-recognized antidepressant effects elicited by chronic exercise training.

Publications and Presentations

Publications in preparation

Tong Cheng, Douglas Affonso Formolo, Yunxiao Zhong, Zonghao Ma, Jiasui Yu, Hussain Akhlaq, Natalia Paludo Silveira Formolo, Li Zhang, Kenneth King-yip Cheng, Georg S. Kranz, Kangguang Lin, Parco Ming-Fai Siu, Jung Sun Yoo, Cora Sau Wan Lai, Aimin Xu, Kwok-Fai So, Suk-Yu Yau. "Adiponectin-induced APPL1 nuclear translocation mediates rapid antidepressant effects of single-bout physical exercise." ***Nature Metabolism*** (in preparation)

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"Mechanisms underlying single-bout exercise-induced rapid antidepressant effects in stressed mice."

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Tong Cheng, Douglas Formolo, Suk-Yu Yau.

"Investigation of neural mechanism underlying antidepressant effects of single bout of physical exercise."

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Table 1. Antibodies used in western blotting and immunofluorescence staining.

Items	Antibody	Host	Company	Dilution	Catalog #
Western blotting - Primary antibody	APPL1	Rabbit	CST	1:1000	3858S
	GluR1	Rabbit	CST	1:1000	13185S
	PSD95	Rabbit	CST	1:1000	2507S
	Synapsin I	Rabbit	CST	1:1000	5297T
	Synaptophysin	Rabbit	CST	1:1000	5461S
	Histone 4	Mouse	CST	1:1000	2935
	Acetyl-Histone 4 (Lys12)	Rabbit	CST	1:1000	13944S
	Actin	Rabbit	CST	1:5000	4967S
	Tubulin	Rabbit	CST	1:5000	2144S
	GAPDH	Rabbit	CST	1:5000	5174
Western blotting - Second antibody	Goat anti-rabbit IgG	-	CST	1:2000	7074
	Goat anti-mouse IgG	-	CST	1:2000	7076
Immunofluorescence - Primary antibody	GABA	Rabbit	Sigma	1:200	A2052
	CAMKII	Rabbit	Abcam	1: 200	ab5683
	c-Fos	Mouse	Abcam	1:200	ab208942
	c-Fos	Rabbit	Abcam	1: 200	ab214672
	APPL1	Mouse	Santa Cruz	1:300	sc-271909
Immunofluorescence - Second antibody	Goat anti-mouse, 488	-	Abcam	1:200	ab150113
	Goat anti-mouse, 568	-	Abcam	1:200	ab175473

	Goat anti-mouse, 568	-	Thermofisher	1:200	A-11004
	Goat anti-rabbit, 568		Thermofisher	1:200	A-11011
	Goat anti-rabbit, 488	-	Thermofisher	1:200	A-11008

Table 2. Virus information

Name	Viral titers (vg/ml)	Supplier and Catalog
AAV2/9-CaMKIIa -GCaMP6s	2.7×10^{12}	Brain VTA
AAV2-Syn-DIO-hM3Dq-mCherry	2.7×10^{13} vg/ ml	Addgene plasmid #44361
AAV2-Syn-DIO-hM4Di-mCherry	2.5×10^{13} vg/ml	Addgene plasmid #44362
AAV2-hSyn-DIO-mCherry	1.5×10^{13} vg/ml	Addgene plasmid #50459
AAV9-CMV-DIO-RFP-mir30shRNA（AdipoR1）	$>1.0 \times 10^{13}$ vg/ml	WZ Biosciences Inc
AAV2- CaMKIIa -Cre	1.5×10^{13} vg/ml	Addgene plasmid #105558
AAV9-CMV-DIO-RFP-mir30shRNA（APPL1）	$>1.0 \times 10^{13}$ vg/ml	WZ Biosciences Inc
AAV9-CMV-DIO-RFP-mir30shRNA (Control)	$>1.0 \times 10^{13}$ vg/ml	WZ Biosciences Inc
AAV9- CaMKII a -APPL1	1.0×10^{13} vg/ml	WZ Biosciences Inc

Table 3. Primer Sequences for RT-PCR

Name	Sequence (5' to 3')
APPL1-F	GGTAGCCAGTGACCCTTTAT
APPL1-R	CTCCTGCCACATCTCCAC
Bdnf-F	ACGACATCACTGGCTGACACT
Bdnf-R	GAAAGAGTAGAGGAGGCTCCAA
c-Fos-F	CACACAGGACTTTTGCGC
c-Fos-R	GACACGGTCTTCACCATTCC
cpg15-F	GCGAGATTTCGTTGAGATCG
cpg15-R	GGGATGACACGGATTGATTTT
GAPDH-F	CCCATCACCATCTTCCAGGAGC
GAPDH-R	CCAGTGAGCTTCCCGTTCAGC

Abbreviations

AAV	Adeno-Associated Viruses
ACC	Anterior Cingulate Cortex
AdipoR1	Adiponectin Receptor 1
AdipoR2	Adiponectin Receptor 2
AMPK	AMP-activated protein kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Akt	Ak Strain Transforming
APPL1	A protein containing PH (pleckstrin homology) domain, PTB (phosphotyrosine binding) domain and leucine zipper motif
BDI	Beck Depression Inventory
BDNF	Brain-Derived Neurotrophic Factor
BAR	Bin–Amphiphysin–Rvs
CUS	Chronic Unpredictable Stress
CA1	Cornu Ammonis 1
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
CREB	Cyclic AMP Response Element-Binding
Cpg 15	Candidate Plasticity Gene 15
CNO	Clozapine-N-Oxide
DG	Dentate Gyrus
DARS	Dimensional Anhedonia Rating Scale
DMSO	Dimethyl sulfoxide
DRN	Dorsal Raphe Nucleus
ELISA	Enzyme-Linked Immunosorbent Assay
FS	Feeling Scale
FAS	Felt Arousal Scale
FAD	Food and Drug Administration
FST	Forced Swim Test
GCs	Glucocorticoids

GABA	Gamma-Aminobutyric Acid
GDNF	Glial cell line-derived neurotrophic factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-Pituitary-Adrenal
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylases
HDACis	Histone Deacetylase inhibitors
HR	Heart Rate
HADS	Hospital Anxiety and Depression Scale
HIIT	High intensity interval training
IGF-1	Insulin-like Growth Factor-1
IL-6	Interleukin 6
IR	Insulin receptor
IGF-1	Insulin-like Growth Factor-1
LTP	Long-term potentiation
LTD	Long-term depression
mPFC	medial Prefrontal Cortex
mTORC1	mechanistic Target Of Rapamycin Complex 1
MeCP1	Methyl-CpG-binding Protein 1
MSS	Mood Survey Scale
MDD	Major depressive disorder
MAOI	Monoamine Oxidase Inhibitors
MDD	Major depressive disorder
MeCP1	Methyl-CpG-binding Protein 1
MHR	Maximum Heart Rate
NMDA	N-Methyl-D-Aspartate
NuRD	Nucleosome remodeling and Deacetylase
NPC	Nuclear Pore Complex

NLS	Nuclear Localization Signal
OFT	Open field test
PPAR	Peroxisome Proliferator-Activated Receptor
PI3K	Phosphatidylinositol 3-Kinase
PANAS	Positive and Negative Affect Scales
POMS	Profile of Mood State
PSD95	Postsynaptic Density protein 95
PH	Pleckstrin Homology
PTB	Phosphotyrosine-binding
PFA	Paraformaldehyde
RT-qPCR	Reverse Transcription-Quantitative polymerase chain reaction
Rab5	Ras-related protein-5
RPE	Rating of Perceived Exertion
ROI	Regions of Interest
SSRIs	Selective Serotonin Reuptake Inhibitors
STAI	State-Trait Anxiety Index
SEES	Subjective Exercise Experiences Scale
SST	Sucrose Splash Test
TMD	Total Mood Disturbance
TCA	Tricyclic Antidepressants
TrkB	Tropomyosin receptor kinase B
TGF	Transforming Growth Factor
TST	Tail Suspension Test
Thy1-YFP	Thy1-yellow fluorescent protein
VAS	Anhedonia Visual Analog Scale

Chapter 1: Introduction

Major depressive disorder is a debilitating illness with severe social and economic impacts, representing the foremost cause of disability worldwide [1]. Individuals suffering from depression exhibit significantly higher rates of suicidal ideation compared to the general population [2]. Increasing evidence indicates that the glutamatergic system plays a critical role in the pathophysiology and treatment of depression [3, 4]. Notably, the dissociative anesthetic ketamine at a low dose rapidly and persistently exerts rapid antidepressant effects for up to 7 to 10 days, with its action by enhancing neuroplasticity through blocking the activity of N-Methyl-D-Aspartate (NMDA) receptors in the inhibitory GABAgeric neurons, further activating the mammalian targets of rapamycin (mTOR) signaling pathway to induce synaptic protein expression and spinogenesis in excitatory neurons in the medial prefrontal cortex [5, 6]. However, ketamine use carries risks of misuse and dissociative symptoms. Therefore, there is an urgent need to develop safer and more effective treatment options for depression.

Physical exercise has been recognized as a promising intervention for alleviating depressive symptoms [7, 8]. It served as a superior alternative to depression compared with antidepressants, given its safety, efficacy, and potential synergistic effects when combined with pharmacological treatments [9]. Emerging clinical evidence suggests that a single-bout physical exercise can elicit rapid effects in elevating mood with its significant enhancements in positive mood and reductions in negative mood [10, 11]. However, the neural mechanisms of these rapid antidepressant-like effects are not yet fully understood. Further research utilizing animal models could elucidate neural mechanisms underlying the antidepressant effects of a single-bout physical exercise.

The therapeutic efficacy of physical exercise in treating depression is attributed to its capacity to enhance neuroplasticity [12], including enhancements in adult neurogenesis [13], synaptogenesis [14], and synaptic plasticity [15]. Central and peripheral secretory factors triggered by physical exercise, known as ‘exerkines’, are key to mediating the antidepressant effects by enhancing neuroplasticity [16]. Adiponectin, an adipocyte-secreted exerkine, can mimic a lot of the metabolic effects of physical exercise [13].

Notably, its trimeric and low-molecular-weight forms, can cross the blood-brain barrier and enter the central nervous system [13]. Adiponectin's biological effects are mainly mediated through two receptors, AdipoR1 and AdipoR2, which are located in different brain regions involved in emotional regulation, including the medial prefrontal cortex (mPFC) [17], hippocampus [18], ventral tegmental area [19] and dorsal raphe nucleus [20], etc. Clinical and pre-clinical studies have linked functions of adiponectin signaling with depressive states [17, 21] and antidepressant therapeutics [17, 22, 23]. Central overexpressing recombinant adiponectin through intracerebroventricular infusion elicited rapid antidepressant responses within hours [17, 24]. Yau et al. have shown that adiponectin is a crucial mediator of the antidepressant effects elicited by chronic physical exercise through promoting neurogenesis in the hippocampus [13, 25]. Furthermore, recent clinical research has demonstrated that a single-bout physical exercise can significantly elevate adiponectin levels in the blood of healthy individuals [26]. Such evidence naturally raised the question of whether adiponectin could serve as a potential mediator for explaining the rapid antidepressant-like effect elicited by single-bout physical exercise.

APPL1, a protein containing PH (pleckstrin homology) domain, PTB (phosphotyrosine binding) domain and leucine zipper motif, is known to directly interact with AdipoRs [27] to facilitate adiponectin signaling across multiple downstream targets, including AMP-activated protein kinase (AMPK) [28], peroxisome proliferator-activated receptor alpha (PPARα) [29], and the phosphatidylinositol 3-kinase (PI3K) / ak strain transforming (Akt) signaling pathway [30]. APPL1 not only orchestrates adiponectin downstream signaling transduction events in the cytoplasm but also can translocate to the nuclear in response to adiponectin stimulus to participate in gene transcription [31, 32]. Studies have demonstrated that physical exercise exerts antidepressant effects by activating the adiponectin/AdipoR1/AMPK signaling pathway in the hippocampus of healthy mice [13] and mice exposed to chronic unpredictable stress (CUS) [33]. However, the detailed mechanisms by which APPL1 contributes to the adiponectin-AdipoR signaling to mediate the antidepressant effects of physical exercise have yet to be fully elucidated.

Building on the research background outlined above, this study aimed to:

1. Determine the specific brain region and cellular target of the rapid antidepressant effects elicited by single-bout physical exercise.

2. Explore the potential role of adiponectin signaling and its adaptor protein APPL1 in the rapid antidepressant effects mediated by single-bout physical exercise.

We conducted a series of experiments to investigate the neural mechanisms underlying the rapid antidepressant effect of single-bout physical exercise. Chapter 2 offers a comprehensive literature review to introduce the theoretical and empirical groundwork for this study. The key experiments were organized into five different chapters (Chapters 3 to 7). Chapter 3 starts with confirming the rapid antidepressant effects of single-bout physical exercise using human and animal experiments. Chapter 4 focuses on the investigation of the neurobiological basis: the activation of the ACC-glutamatergic neurons underlies the rapid antidepressant effects elicited by single-bout physical exercise. Chapter 5 provides evidence showing the involvement of adiponectin-AdipoR1 signaling within ACC-glutamatergic neurons mediating the rapid antidepressant effects of single-bout physical exercise. Chapters 6 focus on the adiponectin-AdipoR1-APPL1 translocation process. Chapter 7 points out the critical role of nuclear APPL1 in triggering molecular changes and antidepressant effects elicited by single-bout physical exercise. Chapter 8 is a summary chapter to summarize the main findings of all experiments.

Chapter 2: Literature Review

2.1 Potential of single-bout physical exercise for treating depression

2.1.1 Neurobiology of depression

The continuously rising prevalence of major depressive disorder (MDD) has become an urgent public health issue globally. Recent epidemiological surveys reported that approximately 300 million people worldwide are affected by MDD [34]. According to data from the World Health Organization, MDD ranked third in terms of disease burden in 2018 and will rise to first place by 2030 [35]. Symptoms of MDD persist and affect various aspects of life, including mood, cognitive functions, and physical health [36]. Depression can be recurrent, occurring even during remission with the most serious outcome leading to suicide [37].

The etiology of depression is highly complex. Depression is not only related to psychological factors but is also influenced by hereditary factors, environmental stress, and other comorbidity factors [38]. Genetic factors play a crucial role in the development of depression with heritability estimated to be between 30-50% [39]. However, no single genetic variation significantly increases the risk for MDD, indicating that the interplay of genetic risk with other factors is more critical in the disease's development. Environmental stressors occurring after traumatic life events such as unemployment or divorce have a significant impact on the development of MDD [40]. Chronic stress can lead to changes in neuronal structure and function [41], hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis [42], and damage to microglia and astrocytes [43]. All these changes are intricately associated with the development and exacerbation of depression, highlighting the complex interplay between chronic stress and depressive disorders. Additionally, MDD frequently co-occurs with comorbidity factors [44], including neurodegenerative diseases, cardiovascular diseases, metabolic disorders, endocrine disorders, and autoimmune diseases. The presence of comorbidities complicates the impact of MDD, increasing the social and economic burden [45] and reducing patients' life expectancy [44].

Numerous hypotheses have been proposed to explain the pathogenesis of MDD including the HPA dysfunction hypothesis [46], the monoamine hypothesis [47], the inflammatory hypothesis [38], and the neuroplasticity hypothesis [48]. The HPA axis is a critical part of the neuroendocrine system. One of the common neurobiological changes observed in patients with depression is an increased activity of the HPA axis, which is accompanied by elevated levels of corticotropin-releasing hormone, adrenocorticotropic hormone, and glucocorticoids (GCs) released by the adrenal gland. GCs exert a negative feedback control on the HPA axis. However, this negative feedback regulation is disrupted in MDD, resulting in persistently high levels of GCs [49]. In addition to the HPA axis dysfunction, another widely accepted hypothesis is the monoamine hypothesis. The traditional monoamine theory suggests that deficiencies in neurotransmitters such as serotonin, norepinephrine, and dopamine are the fundamental cause of clinical depression [50]. For instance, selective serotonin reuptake inhibitors have been proven to successfully treat clinical depression by increasing the concentration of serotonin in the synaptic cleft [51]. However, the monoamine hypothesis cannot fully explain all the pathological changes in depression. In recent years, the inflammatory hypothesis has also received increasing attention. Neuroinflammation is often associated with changes in levels of pro-inflammatory cytokines including interleukins and tumor necrosis factor-alpha [38]. Emerging data suggest that elevated levels of inflammation may increase the risk of developing depression [52, 53]. In addition, the neuroplasticity hypothesis also provides an important perspective to understand the pathogenesis of depression. The onset of depression is attributed to the impairment of homeostatic dysfunction in synaptic plasticity, leading to instability and loss of synaptic connections in the neural circuits involved in emotional regulation [48]. The rapid-acting antidepressant action of low-dose ketamine rapidly induces synaptogenesis to reverse synaptic deficits caused by chronic stress [54]. This discovery provides empirical evidence for the neuroplasticity hypothesis in the treatment response of depression.

Although various hypotheses, including the dysregulation of the HPA axis, monoamine hypothesis, inflammation hypothesis, and the synaptogenic hypothesis have

been proposed to explain the pathogenesis of MDD, not any single hypothesis is insufficient to explain the pathophysiology of MDD.

2.1.2 Pharmacological treatments for depression and its limitations

Scientists have been developing antidepressant drugs since the 1950s. There are three generations of antidepressant drugs till now. The first generation of antidepressants is monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs) [55], etc. These drugs reduce the reuptake of monoamines [56]. The first-generation drugs helped us look at depression from a biochemical perspective and suggested that problems with the monoamine system might cause depression. However, MAOIs have potentially life-threatening side effects, such as nephrotoxicity, hepatic necrosis, and tyramine-induced intracranial hemorrhage [57]. To reduce treatment side effects, second-generation antidepressant drugs, including serotonin-norepinephrine reuptake inhibitors and selective serotonin reuptake inhibitors were developed. Since then, monoamine-based drug therapy has remained the recommended treatment for patients with moderate to severe depression [58]. To date, there are currently over 40 antidepressant drugs available, with most of them targeting the monoaminergic system [59]. However, such pharmacological drug treatment increases the monoaminergic system activity within hours of drug administration, whereas therapeutic response requires weeks to take place [60], and 30% of patients have treatment resistance issues being not responders to the monoaminergic antidepressant treatment [59].

Furthermore, the discovery of the fast and long-lasting antidepressant drug- low-dose ketamine has given hope for solving the delayed response and treatment resistance issues. A single injection of low-dose ketamine can rapidly alleviate depressive symptoms within a few hours and last for at least 7 days in clinical patients [5]. The research indicated that 71% of such patients showed a positive response after receiving ketamine infusion [61]. The initial discovery of low-dose ketamine as a rapid-acting antidepressant has embarked extensive research in both clinical trials and pre-clinical studies. The recent approval by the U.S. Food and Drug Administration (FAD) of esketamine, a nasal spray derivative of ketamine, marks a significant advancement in the treatment of treatment-resistant depression [62]. Nonetheless, ketamine's potential for abuse and its dissociative effect constrain its use as an antidepressant [63]. Considering the adverse effects of these three

generations of antidepressant drugs, it is imperative to explore safer and more efficacious non-pharmacological interventions for depression.

2.1.3 Rapid antidepressant effects of single-bout physical exercise.

A summary of the effect of one single bout of exercise on mood changes based on findings from 15 clinical studies (Table 1) and several animal studies. For the clinical studies, mood changes are measured exclusively affective changes as the primary outcome, including several indexes of mood state (e.g., depression, anger, joy, energy, etc.), anhedonia, affective valence, anxiety state, cooping emotions, and well-being. For animal studies, a series of behavioral tests, such as the open field test and forced swimming tests, were used to measure anxiety-like or depression-like behaviors in rodent models.

2.1.3.1 Clinical studies

In this sub-section, the exercise protocols of these 15 clinical studies are discussed to identify the exercise modality and exercise intensity that can elicit positive mood changes in depressed patients. The sample evaluated in the clinical studies included depressed inpatients, outpatients, and participants with depression symptoms.

Exercise modalities are classified as aerobic (e.g., walking, running, cycling, etc.), anaerobic (e.g., resistance training), or mixed, which include components of both aerobic and anaerobic exercise, such as climbing and martial arts. For aerobic exercise, most studies investigated the pre- and post-effect of exercise and reported positive affective changes after a single bout of exercise. Only two studies have evaluated the effectiveness of various exercise modalities [64, 65]. Thaller and colleagues [65] compared the effects of a single bout of climbing with Nordic walking in in-patients with depression, anxiety, or obsessive-compulsive disorder. Climbing improved positive affect and perceived activation while Nordic walk improved affective valence and anxiety when compared to sedentary control condition. Bodin and colleagues [64] compared the effects of two different exercise modalities, cycling or martial arts, on the depressed patient's self-efficacy construct in terms of the participants' belief in their capacity to complete three bouts of 15-minute exercise at increased difficulty. Martial arts was superior to cycling not only in terms of affective changes but also in improving self-efficacy in depressed patients

[64]. There is only one study, done by Nosrat and colleagues [66], investigated the effects of a single session of anaerobic exercise (resistance training) on affective changes in patients with human immunodeficiency virus (HIV) infection and depressive symptoms [66]. Exercise increased affective valence immediately and 10 min-post exercise, and also increased perceived activation during, immediately, and 10 min-post exercise [66]. This study showed that single-bout anaerobic exercise effectively elevates mood in HIV patients with depressive symptoms.

Table 1 summarized 15 studies with varied exercise intensities, including self-selected, light, moderate, and high intensities, and graded intensity carried over until exhaustion. The intensity of the exercise was measured using different techniques, including Borg's scale of perceived exertion, and percentage of maximum heart rate reserve or oxygen consumption. Of note, three studies specifically investigated the impacts of different exercise intensities on affective response after a single bout of exercise [67-69]. Knapen and colleagues [67] investigated the effects of three different exercise intensities of cycling (at prescribed intensity, self-selected intensity with heart rate feedback, or self-selected intensity without heart rate feedback) on state anxiety and subjective well-being in inpatients clinically diagnosed with depression and/or anxiety. Anxiety state decreased in all three conditions. Positive well-being increased only for the two self-selected conditions. This improved response with self-selected intensity might correlate with the participants' increased willingness to exercise when they choose the intensity [67]. Meyer and colleagues [68, 69] investigated the effects of four different exercise intensities (light, moderate, high, and self-selected intensity, with a rest condition as control) on depression scores in 24 depressed women. Exercising at all three fixed exercise intensities resulted in improvement in depression scores 10 and 30 min post-exercise when compared to the baseline [69]. Of note, in a secondary analysis, the improvements in depressed mood by physical exercise were not affected by antidepressant intake. Therefore, according to this study, physical exercise is an equally effective treatment in patients regardless of antidepressants [69]. Moreover, in a follow-up publication where the results after the patients' preferred exercise intensity (self-selected) were compared with those of the most similar prescribed exercise intensity, the authors

observed that the improvements in depressed mood were higher with the exercise intensity being prescribed than the ones with the patient's will in choosing exercise intensity [68].

In summary, the above evidence suggests that a single-bout exercise in different modalities (aerobic, anaerobic, or mixed) improves the affective response of clinically depressed patients and individuals with depression symptoms. In addition, depressed patients could benefit from exercising at light to high intensity. Furthermore, the depressed patient benefits more from a prescribed-intensity exercise compared with those of self-selected exercise intensity. However, self-selected intensity might increase the participants' increased willingness to exercise.

Table 2.1 Effects of single-bout physical exercise in mood changes in clinical studies

Author (year)	Sample	Exercise Protocol	Outcomes	Results
(Meyer et al. 2022) [70]	Depressed patients (n=30)	Moderate intensity cycling (30 min, Borg's scale)	Depression (POMS)	↔Dimensional anhedonia ↓ Depression ↓ State anhedonia
		Quite rest (control)	State anhedonia (VAS)	
			Dimensional anhedonia (DARS)	
(Thaller et al. 2022) [65]	In-patients with depression, anxiety, or OCD (n= 21)	Climbing (100 min)	Affective valence (FS)	Climbing ↑ Positive affect ↑Perceived activation ↔ Negative affect ↔ Affective valence ↔ State anxiety
		Nordic walking (75 min)	Perceived activation (FAS)	
			Affective response (PANAS)	Nordic walk ↑ Affective valence ↔ Positive affect ↔ Negative affect ↔Perceived activation ↓ State anxiety
		Sedentary control. *in a within subject design	State anxiety (STAI)	
(Niedermeier et al. 2021) [71]	Depressed in-patients (n=23)	Brisk outdoor walking (30 min, light to moderate-Borgs' scale)	Affective valence (FS)	↑Perceived activation (immediately) ↑ Activity ↔ Affective valence ↔ Elation ↔ Calmness ↔ Depression ↔ Contemplation ↓ Fatigue ↓ Anger ↓ Excitement
		Sitting and reading (control) *in a within subjects' design	Perceived activation (FAS)	
			Mood (MSS)	

(Legrand, et al. 2018) [72]	Participants with depressive symptoms (n=18)	Moderate outdoor running (20 min, ~70% Max HR)	Mood state – energy and fatigue (POMS)	Outdoor running ↑ Energy ↔ Fatigue
		Moderate indoor running (20 min, ~70% Max HR)		Indoor running ↑ Energy ↔ Fatigue
		Video educational session (control) *in a within subjects' design		
(Kleinstäuber et al. 2017) [73]	Depressed in-patients (n=40)	Climbing (2.5 hours, n=20)	Affective response (PANAS)	↑ Positive affect ↑ Cooping emotions ↓ Negative affect
		Muscle relaxation (control, 30 min, n=20)	Cooping emotions	
(Nosrat et al. 2017) [66]	Participants with HIV and depressive symptoms (n=10)	Self-selected intensity resistance training (20 min)	Affective valence (FS)	↑ Affective valence ↑ Perceived activation
		Video education (control)	Perceived activation (FAS)	
(Frühauf et al. 2016) [74]	Depressed in-patients (n=14)	Self-selected intensity outdoor walking (60 min)	Affective valence (FS)	Outdoor condition (walking) ↑ Affective valence* ↑Perceived activation ↔ Elation ↔ Calmness ↔Contemplativeness ↓ Excitation ↓ Depression# ↓ Fatigue#

				*Higher magnitude compared to indoor condition #Non-significant trend
		Indoor cycling (60 min)	Perceived activation (FAS)	Indoor condition (cycling) ↑ Affective valence ↔ Perceived activation ↔ Excitement ↔ Elation ↔ Calmness ↔Contemplativeness ↔ Fatigue ↔ Depression ↓ Anger# #Non-significant trend
		Resting (60 min)	Mood (MSS)	
(Stanton, et al. 2016) [75]	In-patients with depression (n=14)	Self-selected intensity, mixed aerobic (20 min)	Affective valence (FS)	Depression ↑ Affective valence ↔Perceived activation
	Anxiety (n=12)	Resistance training exercise (20 min)	Perceived activation (FAS)	Anxiety ↔ Affective valence ↔Perceived activation
	Bipolar disorder (n=14)		Perceived exertion (RPE)	Bipolar ↑ Affective valence ↔Perceived activation
(Meyer, et al. 2016) [69]	Depressed women (n=24)	Cycling at light, moderate, or high intensity (Borg's RPE sale) (30 min)	Depression (POMS)	Light intensity ↓ Depression Moderate intensity ↓ Depression High intensity

		Quite rest *in a within subjects' design		↓ Depression
(Heggelund et al. 2014) [76]	Schizophrenic patients (n=20)	High intensity aerobic interval training (4x/4 min at 89-95% of Max HR)	State anxiety (STAI)	Schizophrenia subjects ↑ Positive affect ↑ Well-being ↑ Fatigue ↔ Negative affect ↓Psychological distress ↓ State anxiety
	Depressed patients (n=13)		Affective response (PANAS)	Depression subjects ↑ Positive affect ↑ Well-being ↑ Fatigue ↔ Negative affect ↓Psychological distress ↓ State anxiety
	Healthy participants (n=20)		Subjective well-being (SEES)	Healthy subjects ↑ Positive affect ↑ Well-being ↑ Fatigue ↔ Negative affect ↔Psychological distress ↓ State anxiety
(Berman et al. 2012) [77]	Depressed participants (n=20)	Subjects randomly assigned to nature or urban walking (2.8 miles, 50-55 min, intensity not measured)	Affective response (PANAS)	Nature walking ↑ Positive affect* ↑ Working memory ↓ Negative affect *Increased with a higher magnitude compared to urban walk

			Short-term working memory (BDS)	Urban walking ↑ Positive affect ↔ Working memory ↓ Negative affect
(Weinstein et al. 2010) [78]	Depressed participants (n=14)	Graded treadmill exercise (25 min, including 10 min with 75% and 5 min with 85 of Max VO2)	Mood state (POMS)	Depressed subjects immediate: ↓ Depression 30-min post-exercise ↑ Depression ↑ Fatigue
	Healthy participants (n=16)		Depression symptoms' severity (BDI)	Healthy subjects ↔ Depression ↔ Fatigue ↔ Vigor
(Knapen et al. 2009) [67]	In-patients with depression and/or anxiety disorder (n=48)	Cycling at self-selected (20 min)	State anxiety (SAI)	Prescribed intensity (50% Max HR) ↓ State anxiety ↓ Negative affect ↓ Fatigue
		Prescribed intensity (50% Max HR)	Subjective well-being (SEES)	Self-selected intensity ↓ State anxiety ↓ Negative affect ↑ Positive well-being ↑ Fatigue

(Bartholomew, et al. 2005) [79]	Recently depressed participants (within 2 weeks) (n=40)	Treadmill exercise (30 min, 60-70% of Max HR)	Mood state (POMS)	↑ Vigor ↑ Subjective well-being ↔ Anger ↔ Confusion ↔ Depression ↔ Tension ↔ Subjective distress ↔ Fatigue
		Quite rest	Subjective well-being (SEES)	
(Bodin, et al. 2004) [64]	Depressed patients (n=12)	Cycling (> 60% of Max HR, 45 min)	Affective response (PANAS)	Cycling ↔ Positive affect ↔ Negative affect ↔ State anxiety
		Martial arts (45 min) *in a within subjects' design	State anxiety (STAI)	Martial arts ↑ Positive affect ↔ Negative affect ↓ State anxiety

Abbreviation:

Positive and Negative Affect Scales (PANAS); Profile of Mood State (POMS); State-Trait Anxiety Index (STAI); Subjective Exercise Experiences Scale (SEES); Beck Depression Inventory (BDI); Backward Digit Span (BDS); Feeling Scale (FS); Felt Arousal Scale (FAS); Mood Survey Scale (MSS); Rating of Perceived Exertion (RPE); Anhedonia Visual Analog Scale (VAS); Dimensional Anhedonia Rating Scale (DARS)

2.1.3.2 Animal studies

Limited animal studies [80-83] have indicated that single-bout physical exercise effectively modulates anxiety-like and depression-like behaviors by regulating neuronal activation in brain regions related to emotion regulation and by increasing Brain-Derived Neurotrophic Factor (BDNF) expression. These animal studies not only highlight the significant role of single-bout exercise in mood regulation but also provide valuable insights into underlying neurobiological mechanisms.

Low-intensity acute treadmill exercise can effectively induce neuronal activity associated with anti-depressant and anti-anxiety effects in rats [80, 83]. Specifically, low-

speed running significantly increased the activity of serotonin neurons in the dorsal raphe nucleus (DRN) and reduced anxiety and depressive behaviors [80]. Further analysis of running speeds and durations revealed that low-speed running significantly activated serotonin neurons in the DRN regardless of the duration, whereas high-speed running sustained for 60 minutes significantly activated DRN neurons [83].

In addition, acute exercise may produce positive effects on emotion through the modulation of BDNF. A review summarized that single-bout physical exercise could regulate the acquisition, consolidation, and extinction of fear memory by increasing BDNF levels [81]. An animal study further explored the effects of acute exercise on BDNF expression and behavior in mice. The study found that high-intensity running significantly increased the mRNA expression of BDNF IV in the hippocampus but simultaneously reduced exploratory behavior [82].

These findings collectively highlight the multifaceted impact of single-bout physical exercise on neuronal activity and emotional regulation, emphasizing the importance of exercise intensity and duration in achieving optimal mental health benefits. Future research should continue to explore these variables to better understand the underlying neural mechanisms.

2.2 Insights from fast-acting antidepressants with promoting effects on neuroplasticity

2.2.1 Neuroplasticity in depression

Until the 1960s, it was commonly believed that the adult nervous system had limited capacity for change [84]. However, subsequent research has demonstrated that the adult mammalian brain is continuously shaped by environmental stimuli. This phenomenon, known as "neuroplasticity [85], is a fundamental contributor to adaptive functioning. Neuroplasticity has been studied at various levels, including ion channels, synapses, circuits, and behavioral changes. All these levels are highly interdependent and interlinked [86]. Neuroplasticity endows individuals with the ability to adapt to constantly changing demands and environments. This leads to positive behavioral changes, such as enhanced

learning and memory capabilities. However, abnormalities in neuroplasticity may lead to maladaptive behavioral outcomes, such as the symptoms of dementia and depression seen in neurodegenerative and psychiatric disorders.

Dysregulation of neuroplasticity includes impaired synaptic connections, neuronal atrophy, and reduced neural circuit connectivity within brain regions, which in turn induce depressive behaviors [87]. Chronic stress exposure dysregulates neuroplasticity in the mPFC (Fig 2.1). Repeated stress can reduce the expression of BDNF, which weakens the signaling of mTOR complex 1, and consequently disrupts synaptic protein expression and synapse formation [5, 54]. Moreover, chronic stress activates microglia, the resident immune cells of the brain, which cause neuronal atrophy by regulating synaptic pruning on neurons [88]. Synapses reduction in the mPFC leads to depression-like behavior in rodents [54], suggesting a causal relationship between synaptic function and behavior. Neuroplasticity dysregulation associated with depression not only affects a single brain region (like mPFC) but also impacts neural circuit connectivity between multiple brain areas including the prefrontal cortex, hippocampus, amygdala, ventral tegmental area, and nucleus accumbens [89]. The mPFC and its circuitry are among the most extensively studied brain connections. This includes its links to the amygdala, which regulates fear and anxiety; to the dorsal raphe nucleus, associated with helplessness behavior; to the lateral habenula, related to anhedonia and aversion; and to the bed nucleus of the stria terminalis, connected with anxiety and negative emotions [90].

In summary, neuroplasticity plays a crucial role in adaptive functions, and its dysregulation is closely linked to the pathophysiology of depression. Chronic stress can lead to neuroplasticity dysregulation in key brain areas such as the prefrontal cortex and affect the neural circuitry connections between related brain regions. The following sub-sections will elaborate on how antidepressant drugs exert their rapid antidepressant effects by targeting neuroplasticity, providing insights into the treatment of depression and the development of new antidepressant interventions.

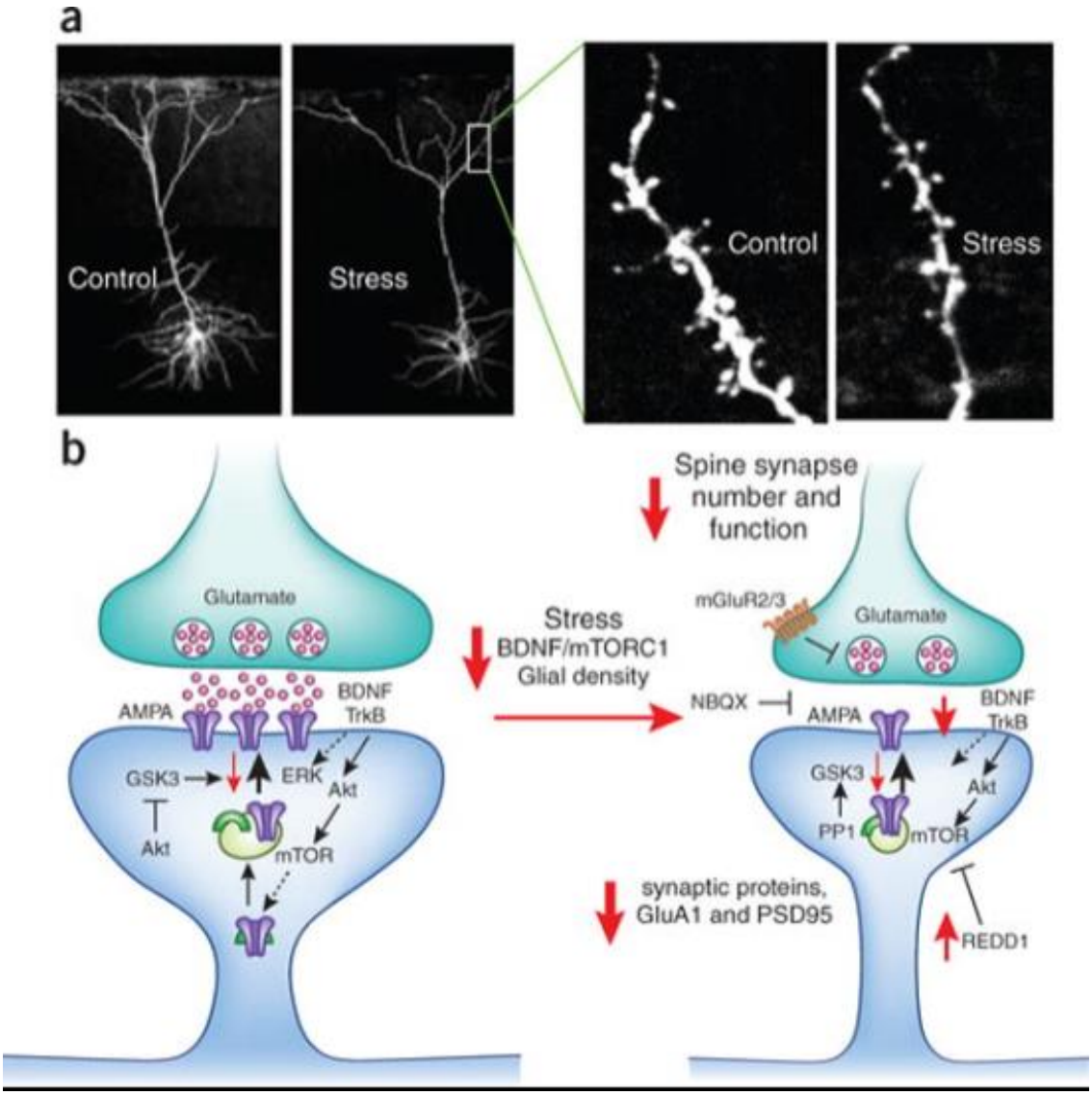


Figure 2.1 Chronic stress dysregulated neuroplasticity in the mPFC.

(a) Repeated stress negatively affects the dendritic architecture of pyramidal neurons in the mPFC, revealing a diminution in both the quantity and length of apical dendrites. The magnified images on the right depict dendritic segments adorned with spines (indicated by arrows), which are the sites of synaptic connections receiving neuronal inputs to the mPFC. (b) Repeated stress decreases the expression of BDNF and mTORC1 signaling to negatively modulate synaptic plasticity, affecting synapse formation and function. The figure was adapted from Duman et al [48].

2.2.2 Downstream targets associated with the rapid antidepressant effect

Recent research has primarily focused on NMDA receptor antagonists like ketamine as an innovative and rapid pharmacological target for treating depression. An increasing number of studies suggest that low-dose ketamine can produce rapid antidepressant effects by promoting adaptive neuroplasticity. The discovery has advanced the understanding of the molecular and cellular changes associated with the antidepressant effect, providing hints for the development of new rapid-acting antidepressant interventions.

2.2.2.1 Glutamate surge and increase in the glutamatergic neuron activity in the PFC

The most extensively discussed rapid action of low-dose ketamine involves the increased excitatory neural activity and glutamate surge in the mPFC [91]. The disinhibition theory postulated that low-dose ketamine selectively blocks the NMDA receptor in the gamma-aminobutyric acid (GABA) interneurons which inhibit the activity of inhibitory neurons, resulting in the disinhibition of excitatory pyramidal neurons, thus enhancing neural activity [91]. In the direct inhibition theory, ketamine directly binds to the NMDA receptor on pyramidal neurons at the resting state, which blocks tonic NMDA receptor activation by spontaneously inducing glutamate release [6]. Preclinical studies show that a sub-anesthetic dose of ketamine causes a continual increase (at least 2 hours) in glutamate release in brain regions including the mPFC [92] and nucleus accumbens [93]. Chowdhury et al further found that a sub-anesthetic dose of ketamine increases the re-cycle of amino acid neurotransmitters in the mPFC, as well as neuronal and glial energy metabolism [94]. Importantly, the transient surge in amino acid neurotransmitter recycling is associated with the induction of rapid antidepressant effects [3].

2.2.2.2 Activate the BDNF-mTOR signaling pathways

BDNF is a neurotrophic factor that regulates synaptic formation, neuronal connection, and synaptic plasticity [95]. Following ketamine administration, evoked released glutamate binds to postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which enhances the release of BDNF. BDNF then activates the tropomyosin receptor kinase B (TrkB) receptors and ultimately promotes protein synthesis via the activation of the mTORC1 pathway [91]. Preclinical studies showed that BDNF knockout abolishes the antidepressant-like effect of low-dose ketamine in mice [6], indicating the

critical role of BDNF in the rapid antidepressant effect of ketamine. As the downstream pathways of BDNF/TrkB, the mTOR pathway plays a crucial role in regulating dendritic spine growth, protein translation initiation, and protein synthesis via phosphorylation of p70S6 kinase and repression of 4E-binding proteins [96]. Additionally, pre-treatment with the mTOR inhibitor rapamycin blocks synaptic changes as well as the antidepressant action induced by ketamine [5].

2.2.2.3 Increases in synaptic protein synthesis, and spinogenesis

The sustained activation of BDNF-mTOR signaling following a single injection of ketamine triggers a cascade of molecular changes. The activation of the mTOR signaling leads to the elongation factor 2-mediated synaptic protein synthesis including postsynaptic density protein 95 (PSD95) and synapsin I and BDNF levels as well as AMPA receptor subunits [6, 97-101]. Furthermore, a single injection of ketamine can change structural plasticity in rodents via increasing dendritic spine density by affecting spine formation/elimination in the prefrontal cortex lasting up to two weeks [102-104]. A recent advanced study utilizing *in vivo* two-photon imaging in conjunction with photoactivatable Rac1 revealed that ketamine can reverse synaptic deficits induced by chronic stress in mice. Furthermore, the targeted elimination of these newly formed synapses abolishes the sustained antidepressant-like effects from day 2 to day 7 following administration [103]. Another study using two-photon imaging and glutamate uncaging demonstrated that ketamine promotes dendritic synaptogenesis following a localized glutamate increase in layer 5 pyramidal neurons. This glutamate surge doubles synapse formation two hours post-injection, with the effect persisting for four hours but returning after twelve hours [104]. These findings support links among ketamine-induced cortical glutamate release, dendritic spine remodeling, and antidepressant responses [103]. Notably, ketamine also induces structural plasticity in non-pyramidal cells, specifically in human-induced pluripotent stem cell-derived dopaminergic neurons [105].

2.2.2.4 Increase in functional plasticity

Functional plasticity involves activity-dependent alterations in synaptic strength. The principal forms of synaptic plasticity at glutamatergic synapses are long-term potentiation (LTP) and long-term depression (LTD), which are widely considered to be the cellular

substrates underpinning learning and memory in the brain [106]. Additionally, an imbalance in LTP/LTD leading to synaptic deficits is also observed in depression [107]. The induction mechanisms of LTP and LTD primarily involve changes in the expression and function of postsynaptic AMPA receptors, resulting in either synaptic strengthening or weakening, respectively [108].

Although there is considerable finding in the molecular, and structural plasticity of ketamine, studies on how ketamine affects the induction, maintenance, and decay of LTP and LTD are relatively rare. An animal study conducted on the Wistar-Kyoto rat model of depression showed that a single injection of ketamine was able to rescue the observed deficit in LTP in the dorsal hippocampus. This effect appeared at 3.5 hours post-injection and resulted in synaptic enhancement at 24 hours [109]. Moreover, compared to control rats, depressed Wistar Kyoto rats exhibited impaired hippocampal-dependent long-term spatial memory, and ketamine effectively restored this capability, consistent with their positive effects on LTP [109]. In addition, a clinical study suggests that low-dose ketamine enhances visual sensory evoked potential LTP in patients with MDD at 3~4-hour post-administration [110]. Future research should further investigate the effects of ketamine on LTP/LTD in depression.

2.3 Physical exercise elicited neuroplasticity changes via exerkines

2.3.1 Definition and classification of the "exerkines"

Although the humoral factors regulating the benefits of exercise were recognized early on, the term "exerkines" was not coined until 2016 [16]. erkines refer to signaling molecules that act through endocrine, paracrine, and/or autocrine pathways and are released after a single-bout or chronic exercise [111]. These molecules are ranging from cytokines, nucleic acids, lipids, and metabolites [111]. Numerous studies have confirmed that BDNF plays a role in mediating exercise-induced adult neurogenesis and synaptic plasticity [112, 113]. In addition to factors produced by the brain during exercise, exerkines secreted by peripheral organs also impact brain health (Fig 2.2).

Depending on the secretory organs, exerkines are known as myokines from the muscles, adipokines from the adipose tissue, hepatokines from the liver, or bone-derived hormones. Given that skeletal muscle accounts for approximately one-third of body weight and has an important function during exercise, myokines have become a research focus [114, 115]. Interleukin 6 (IL-6) is the first muscle-secreted cytokine identified in response to acute muscle contraction [116]. Lactate is the most known myokine and has been studied for over 100 years [117]. In recent years, other myokines such as apelin and irisin have also gained attention. Moreover, other exerkines from other organs including osteocalcin from the bone, fibroblast growth factor 12 from the liver, and adiponectin from adipose tissue [111] have also been reported.

While the roles of exerkines in peripheral metabolism and disease have been extensively studied, their functions in the central nervous system remain largely unexplored. Many exerkines can cross the blood-brain barrier and exert direct effects on the brain. Notably, alterations in their circulatory levels are observed in depressive states, which can be ameliorated through physical exercise, indicating their potential as biomarkers for depression. Given the strong link between depression and neuroplasticity, investigating the influence of exerkines on neuroplasticity may provide valuable insights into the antidepressant effects of exercise.

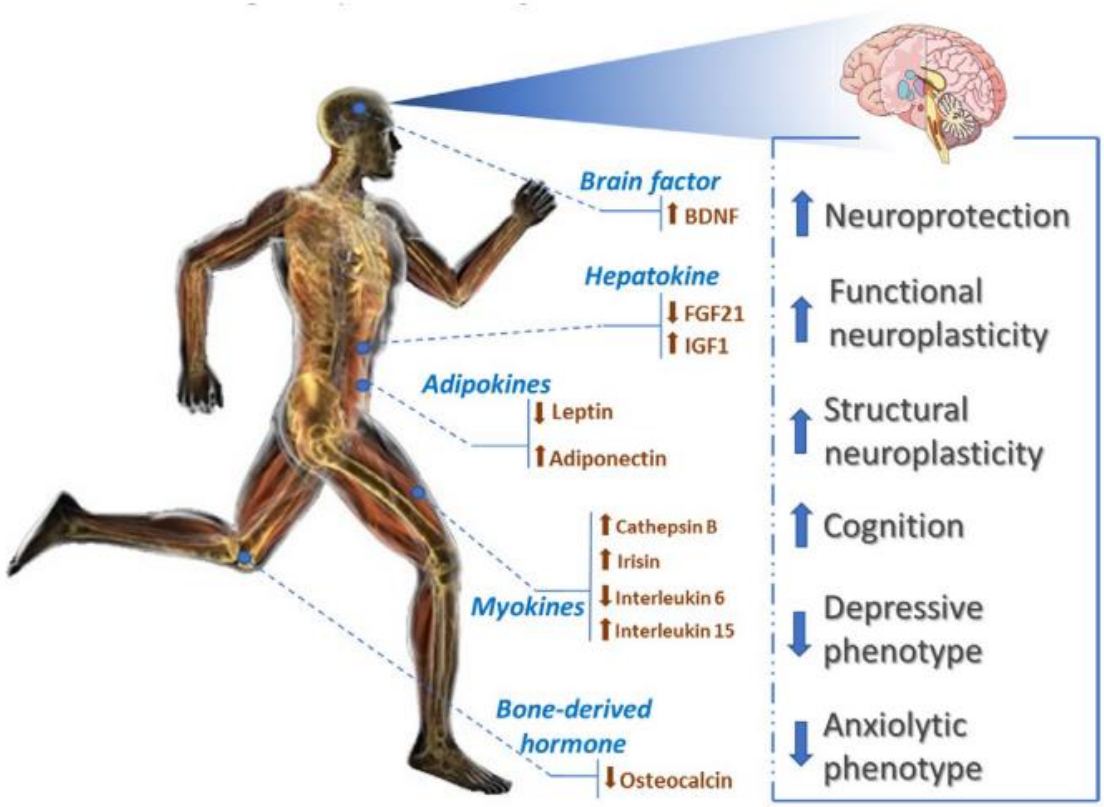


Figure 2.2 Classification and function of the "exerkines" in the brain.

Exercise affects the secretion of exerkines from various tissues, including hepatokines from the liver, myokines from the skeletal muscle, osteokines from the bone, adipokines from the adipose tissue, and neurotrophins from the brain. Increasing evidence suggests that these exerkines mediate the neuroprotective effects, improve neuroplasticity, enhance cognitive function, and relieve depression/anxiety emotion. The figure was adapted from Thomas YH Lee et al [118].

2.3.2 Changes in levels of exerkines in the blood/brain following single-bout physical exercise.Ample research indicates that both acute and chronic exercise confers beneficial effects on brain neuroplasticity through the effect of exerkines induced changes in structural and functional plasticity, which subsequently enhances emotional and cognitive functions. This sub-section will summarize changes in the exerkines including brain factor, hepatokines, adipokines, myokines, and bone-derived hormones in the blood/brain following a single-bout physical exercise in both animal and human studies and introduce its currently known impact on neuroplasticity.

2.3.2.1 BDNF

BDNF is a growth factor that is widely present in the central nervous system and peripheral system. BDNF has neurotrophic and neuroprotective properties [119]. During physical exercise, BDNF in the brain can be produced by neurons [120], astrocytes [121], and endothelial cells [122]. Notably, the expression of BDNF increases during the first few weeks after birth and peaks during the adolescent period in mice [123]. Therefore, BDNF plays a crucial role in adult neurogenesis, including cell proliferation, differentiation, and maturation. Animal studies indicate that chronic exercise with light to moderate intensity can enhance the structural and functional plasticity of the hippocampus, as well as short-term memory, accompanied by an increase in hippocampal BDNF levels [112].

The meta-analysis by Dinoff et al. [124] included 47 studies on cardiovascular exercise and 8 studies on resistance exercise, it was found that both types of acute exercise can temporarily increase the levels of BDNF in circulation. The research also indicates that the increase in BDNF levels in plasma is greater than the increase in serum after exercise. Furthermore, studies on male rats show that acute voluntary wheel running can elevate the levels of BDNF in the hippocampus [125].

2.3.2.2 Hepatokines

The hepatokines play a crucial role in coordinating whole-body energy metabolism and cardiovascular homeostasis [126]. Insulin-like growth factor-1 (IGF-1) is produced not only in the central nervous system but also secreted by the liver and has the potential to cross the blood-brain barrier [127]. IGF-1 is believed to activate signaling pathways that promote neuroplasticity. In vitro studies using hippocampal cultured neurons have shown

that IGF-1 receptors can share downstream signaling cascades with TrkB receptors in both presynaptic and postsynaptic excitatory neurons [128]. Research also indicates that systemic injections of IGF-1 or the elevation of IGF-1 levels induced by physical exercise can increase the expression of BDNF and IGF-1 in the hippocampus [129]. Intraventricular injections of IGF-1 can reverse the age-related reduction in NMDA receptor levels [130]. Blocking the IGF-1 receptor partially inhibits the increase in hippocampal BDNF levels induced by physical exercise and impairs memory recall performance [129].

To the best of our knowledge, there are only two studies that have investigated the impact of acute resistance exercise on IGF-1 levels in the brain [131, 132]. Both studies failed to find a significant effect. This may be due to the timing of sample collection, as brain tissues in these studies were collected 24 hours post-exercise, whereas the circulating levels of IGF-1 typically return to baseline within 15 to 30 minutes after acute exercise [133, 134]. Regarding the effects of acute cardiovascular exercise on the levels of IGF-1, research findings have been divergent [125, 127, 135]. Some evidence suggests that acute cardiovascular exercise can elevate the levels of IGF-1 in the brain [127], but the most recent studies do not support this finding [125].

In summary, single-bout physical exercise may promote the release of IGF-1 from the liver into the bloodstream, increase the uptake of IGF-1 by the brain (and other target organs), and maintain the relative stability of IGF-1 levels in the circulation. Depending on the intensity of uptake, there may be an observable increase, no change, or a decrease in circulating IGF-1 levels after exercise [127].

2.3.2.3 Adipokines:

Adipokines are primarily secreted by white adipose tissue and function to regulate energy metabolism and immune responses. In response to different nutritional states, adipose tissue can secrete factors such as adiponectin and leptin, which coordinate systemic energy metabolism and cardiovascular homeostasis [136]. Recent studies suggest that some adipokines can cross the blood-brain barrier and enter the brain.

Adiponectin circulates in oligomeric forms of low, medium, and high molecular weights in the blood, and the levels of adiponectin are negatively correlated with body fat

content [137]. Notably, the low-molecular-weight form of adiponectin can cross the blood-brain barrier [13]. Adiponectin receptors are widely expressed in multiple brain regions, including the hypothalamus [28], ventral tegmental area [19], frontal cortex, hippocampus, and amygdala [17]. In brain regions responsible for regulating metabolism, such as the hypothalamus, adiponectin not only regulates insulin levels and food intake but also helps balance blood glucose levels [28]. In addition, adiponectin signaling in the brain may be involved in physical exercise-induced neuroplasticity which regulates mood and cognition [13, 138]. Yau et al. demonstrated that adiponectin mediates chronic exercise-induced adult neurogenesis in the hippocampus [13]. In adiponectin knockout mice, the increase in cell proliferation and the anti-depressive response induced by running were diminished. The study suggests that adiponectin may act as a peripheral mediator for exercise-induced adult neurogenesis and anti-depressive effects by activating the AdiopR1-AMPK pathway.

Physical exercise at different exercise intensities and duration can promote changes in peripheral adiponectin levels or its receptor expression [139-141]. Two systematic reviews indicate that acute aerobic exercise can increase adiponectin levels in specific exercise intensity and duration[142, 143]. Simpson and Singh [142] found that high-intensity exercise is required to effectively regulate adiponectin levels in the blood. Bouassida et al. found that an increase in adiponectin levels was only observed with the exercise duration lasted no more than 60 minutes [143]. Additionally, the changes in adiponectin levels induced by acute exercise seem to be limited to aerobic exercise. The beneficial effects of acute resistance exercise may not be reflected in changes in serum adiponectin concentration in healthy young individuals, regardless of whether they are trained or untrained [144, 145].

2.3.2.4 Myokines:

Skeletal muscle communicates with different organs such as the liver, and adipose tissue via the secretion of myokines—peptides synthesized and released by muscle cells upon contraction [146]. The link between muscle and brain function is primarily forged through myokines, including Irisin, IL-6, and cathepsin B, which are thought to mediate exercise-induced neuroplasticity, mood, and cognitive enhancement. Irisin is a potential biomarker for muscle mass [147]. Studies have shown that irisin induced by physical

exercise can cross the blood-brain barrier [148], and regulate the expression of BDNF in the hippocampus after exercise [149]. Additionally, 6 weeks of voluntary wheel running can significantly increase the levels of irisin in the brain in association with its anxiolytic effects [150].

A systematic review has shown that both acute aerobic and resistance exercises can lead to a short-term increase in irisin levels [151]. 30-minute to 1-hour treadmill exercise can raise the concentration of irisin in skeletal muscle and serum [152, 153]. Specifically, the plasma irisin levels can remain elevated for up to 12 hours following 1 hour of exercise [154]. Tsuchiya et al. [155] found that resistance exercise alone significantly increases irisin levels more than aerobic exercise alone or a combination.

2.3.2.5 Bone-derived hormones

Bones are not only the structural support for the body but are also considered an important organ in regulating peripheral energy metabolism. Osteocytes have endocrine functions and can communicate with surrounding organs, such as skeletal muscle [156] and the brain [157]. Osteocalcin, a protein unique to osteoblasts, can regulate the metabolism of skeletal muscles [158], which is crucial for adapting to an optimal state during exercise [159].

Osteocalcin can cross the blood-brain barrier and act in multiple brain regions, including the ventral tegmental area, dorsal and central raphe nuclei, and the hippocampus [157]. Studies suggest that G protein-coupled receptor 158 may be the principal mediator of osteocalcin's effects on the brain [160]. Treatment with osteocalcin in cultured hippocampal neurons increases the mRNA and protein expression levels of BDNF, along with accelerated transport of BDNF-containing vesicles to the synapses. Mice lacking osteocalcin exhibited elevated levels of GABA across multiple brain regions, while monoamine neurotransmitters, such as serotonin, dopamine, and norepinephrine, decreased. These changes in neurotransmitter levels are associated with an increase in anxiety and depression-like behaviors, as well as spatial learning and memory impairments [157].

Single-bout endurance aerobic exercise can double total osteocalcin and undercarboxylated osteocalcin levels in both men [161, 162] and women [162, 163]. However, acute resistance exercise does not affect the levels of osteocalcin [161, 164].

2.4 Increasing adiponectin signaling as a potential treatment for depression

2.4.1 Adiponectin and its receptor in the brain

Adiponectin is one of the most abundant plasma proteins in the circulatory system. It is mainly released by mature white adipocytes, accounting for approximately 0.01% of the total plasma proteins in the human body and 0.05% in rodents [165]. Adiponectin primarily circulates in the blood as three-unit complexes (Trimers), six-unit complexes (hexamers), globular, and high-molecular-weight multimers [166], but only the former two forms can penetrate the blood-brain barrier [28]. The half-life of adiponectin in serum is relatively long, about 2.5 to 6 hours, and its secretion fluctuates with the circadian rhythm [167].

AdipoR1 and AdipoR2 are the main receptors for adiponectin [168]. These receptors are widely expressed in peripheral tissues and the central nervous system. In the brain, AdipoR1 is primarily expressed in the hippocampus, prefrontal cortex, amygdala [17], hypothalamus [28], and ventral tegmental area [19], while AdipoR2 is mainly expressed in regions such as the hippocampal dentate gyrus [18] and hypothalamus [28]. Given the distribution of adiponectin receptors in different brain areas, the role of adiponectin may extend beyond the regulation of peripheral metabolism and could also be a key contributor to the "adipocyte-brain crosstalk".

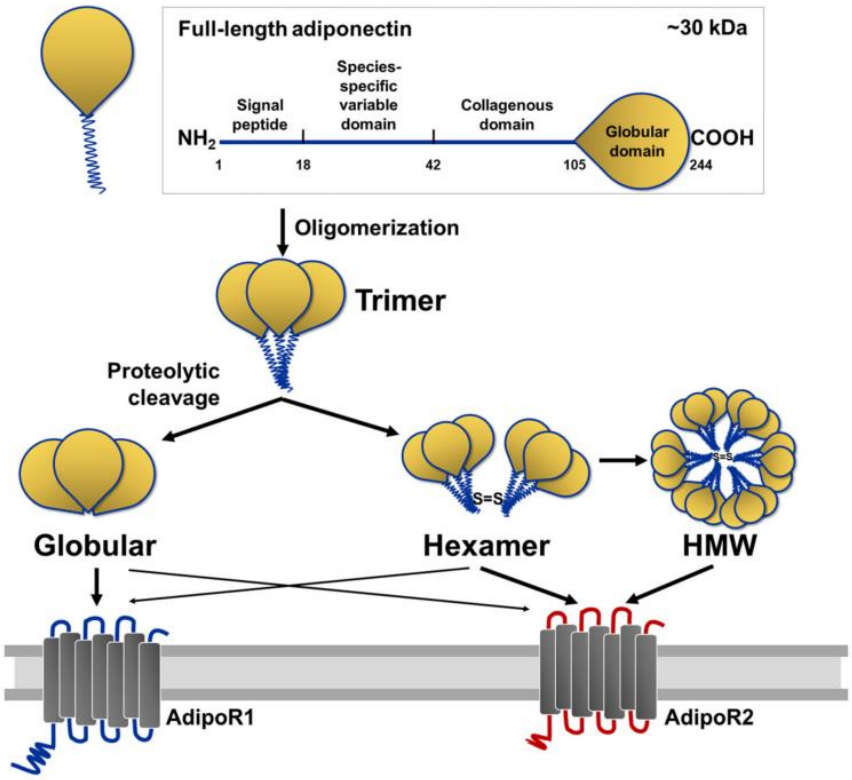


Figure 2.3 Adiponectin structure and its receptors.

Full-length adiponectin oligomerizes to form different formats of adiponectin. AdipoR1 and R2 exhibit different affinity for the adiponectin forms. The figure is adapted from Formolo D et al [22].

2.4.2 Relationship between adiponectin signaling and depression

Both clinical and preclinical research evidence suggests that reduced peripheral adiponectin levels may be associated with major depressive disorder and depression comorbid with certain metabolic and cardiovascular diseases. Clinical reports indicate a causal relationship between peripheral adiponectin levels and depression, with low adiponectin levels strongly correlating with the severity of depression. In depressed women, the average adiponectin levels in the blood over 24 hours are 30% lower than those of the control group and remain at a lower level [21]. Similar associations are found in other disease models comorbid with depression [169-171]. Compared to the general population, the incidence of depression and depressive symptoms in patients with type 2 diabetes is doubled, and there is a significant trend of correlation between reduced adiponectin levels and the severity of depression [169]. In patients with ischemic stroke, serum adiponectin levels can predict the occurrence of post-stroke depression three months later, with low adiponectin levels at admission tripling the risk of post-stroke depression [171]. In patients with hepatitis C, adiponectin may serve as a resilience biomarker for depressive symptoms in hepatitis C virus-infected individuals, with higher adiponectin levels being associated with a lower incidence of depression [170]. Furthermore, preclinical studies also confirm the relevance of adiponectin to depression, for example, in a mouse model of depression induced by chronic social defeat stress, peripheral adiponectin levels are significantly reduced, negatively correlating with an increase in the severity of depressive behaviors [17].

Lower peripheral adiponectin levels are significantly associated with the severity of depression, suggesting that targeted interventions to increase peripheral adiponectin levels may have antidepressant effects. Clinical evidence has shown that, compared to a healthy control group, patients with major depressive disorder who have received monoaminergic antidepressant treatment for at least six months have higher serum adiponectin levels and lower tumor necrosis factor-alpha levels [23]. However, some short-term treatment studies for depression have found that antidepressant treatment may reduce serum adiponectin levels [172] or have no effect [173], which suggests that the impact of peripheral adiponectin levels in indicating antidepressant treatment remains inconclusive. In addition

to pharmacological interventions, physical exercise has been proven to regulate peripheral adiponectin levels and alleviate symptoms of depression. Baduanjin, a form of Chinese martial arts, can significantly increase peripheral adiponectin levels and reduce depressive symptoms in women with chronic fatigue syndrome [174]. Rosiglitazone, a known effective antidiabetic drug, can selectively activate the PPARγ, which is an upstream positive regulator of adiponectin synthesis [175]. Within 24 hours of systemic administration of rosiglitazone, peripheral adiponectin levels significantly increased, triggering an antidepressant response in animal model [176].

Rodent studies also revealed a correlation between adiponectin signaling within the central nervous system and antidepressant responses. For instance, after two weeks of voluntary wheel running, wild-type mice not only exhibited antidepressant effects but also showed increased adiponectin concentrations in the dentate gyrus (DG) [13]. Direct activation of central adiponectin signaling by intracerebroventricular injection of recombinant adiponectin also elicited antidepressant responses [17]. Furthermore, the adiponectin receptor agonist AdipoRon can mimic the multiple effects of adiponectin [175, 177]. As an orally active molecule that can cross the blood-brain barrier, AdipoRon acts on brain regions such as the hippocampus, and its chronic administration promotes adult neurogenesis in the hippocampus and induces antidepressant responses in various animal models of depression [178]. These studies suggest that activating the adiponectin signaling pathway, whether through peripheral or direct central regulation, is a potential strategy for developing antidepressant interventions.

2.4.3 Relationship between adiponectin signaling and neuroplasticity

2.4.3.1 Structural plasticity: Dendritic complexity and spinogenesis

Depression is associated with dendritic spine pathology in brain regions related to emotion like the hippocampus and PFC [48]. Chronic stress dysregulates spinogenesis [103], while low-dose ketamine showing rapid antidepressant effects induces spinogenesis in animal models of depression. Targeting spinogenesis could therefore provide insights into depression and guide antidepressant interventions.

Multiple rodent studies have demonstrated that adiponectin elicits antidepressant events by its action on enhancing structural plasticity. Adiponectin promotes dendritic

growth, arborization, and spine remodeling in the hippocampal DG [179]. Adiponectin null mice exhibit reduced dendritic length, branching, and spine density in granule neurons, particularly in those generated during embryonic development. In contrast, intracerebroventricular infusion of adiponectin for one week promotes spinogenesis and dendritic complexity in adult-born granule neurons [179]. Additionally, upregulating the AdipoR1/Nogo-66 receptor 1 signaling pathway through the adiponectin homolog osmotin can enhance neurite outgrowth and synaptic complexity in a mouse model of Alzheimer's disease [180].

2.4.3.2 Functional plasticity: Neuronal activation and LTD/LTP

MDD patients exhibit reduced dendritic spines and decreased spine complexity [181], which could compromise synaptic integrity and hinder synaptic transmission. Chronic stress, a well-known risk factor for depression, reduces LTP [182] and facilitates LTD [183] in brain regions associated with emotion. Notably, a single low dose of ketamine can induce a rapid antidepressant response and restore LTP and NMDA receptor-dependent excitatory postsynaptic currents in depressed mice [184]. Therefore, alterations in synaptic plasticity are pivotal in the pathophysiology of depression and are promising targets for rapid-acting antidepressants.

Adiponectin exerts a bidirectional effect on neuronal activation by engaging adiponectin receptors, resulting in either neuronal excitatory [185, 186] or inhibitory [18, 19]. This could be because adiponectin's action varies in different types of neurons in different brain regions and the dosage of adiponectin used. AdipoRon, an adiponectin receptor agonist, enhances extinction learning and diminishes the intrinsic excitability of neurons in the DG via an AdipoR2-mediated pathway [18]. Moreover, AdipoRon administration into the ventral tegmental area can mitigate stress-induced anxiety-like behavior and a decrease in dopaminergic neuron activity via activating AdipoR1 [19]. On the other hand, an *in vitro* study demonstrated that adiponectin increased neuron excitability in acute coronal brain slices, specifically in layer V/VI neurons of the ACC by binding to AdipoR2 [185]. Another study revealed that adiponectin stimulates the Cav3.2 channel by interacting with a novel CK2α-dependent PKCβ1 coupling with AdipoR1. This

process induces neuronal hyperexcitability and pain hypersensitivity [186]. This offers a mechanistic explanation for the enhancement of neuronal activity by adiponectin.

Adiponectin has a bidirectional influence on LTP and LTD formation. Intracerebroventricular injection of adiponectin augments LTP and inhibits LTD in the hippocampal DG [187]. Conversely, acute hippocampal slices incubated with AdipoRon exhibit further LTP inhibition in the Cornu Ammonis 1 (CA1) region [188]. Further research into these mechanisms will ultimately reveal how adiponectin signaling regulates synaptic plasticity in the brain.

2.5 Adaptor protein APPL1 in mediating adiponectin signaling

2.5.1 Structure and function of the APPL1

APPL1 is an adaptor protein that contains 3 major functional domains (Fig 2.4): a bin–amphiphysin–rvs (BAR) domain, a pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain [189]. APPL1, through its BAR domain, can form homodimers with APPL1 or heterodimers with APPL2 [190]. In addition, BAR and PH domains together form a functional domain that can bind to the small GTPase Ras-related protein Rab-5 [191] and Ras-related protein 21 [192]. As an adaptor protein with multiple functions, APPL1 interacts with a variety of receptor proteins through its PTB domain, including tropomyosin receptor kinase A [193], AdipoR1 [27], insulin receptor (IR) [194], follicle-stimulating hormone receptor [195], and NMDA receptor [196].

By interacting with the membrane receptors, the adaptor protein APPL1 plays important roles in transducing downstream signalings and regulating different functions, including cellular proliferation, development, cancer cell invasion, and neuroplasticity [197, 198]. It binds to the small GTPase Rab5, facilitating translocation from the cell membrane to the nucleus. The nuclear APPL1 interacts with the nucleosome remodeling and deacetylase (NuRD) / Methyl-CpG-binding protein 1 (MeCP1) to regulate the chromosomal structure, thereby controlling the progression of the cell cycle and cellular development [199]. In addition, the role of APPL protein in the development of different species exhibits species specificity. Studies using the zebrafish model have shown that a

reduction in APPL1 leads to apoptosis in tissue cells; whereas overexpression of APPL1 promotes cell survival [200]. However, APPL1 does not appear to be essential for cellular development in mice. Mice with a global knockout APPL1 can survive and grow normally to adulthood [201]. APPL1 also facilitates the invasion process of cancer cells. In human hepatocellular carcinoma and triple-positive breast cancer tissues, APPL1 directly binds to the leptin receptor and actively mediates leptin signaling to promote leptin-induced proliferation and migration of cancer cells [202]. In aggressive prostate cancer tissues, APPL1 is essential for the nuclear translocation of the transforming growth factor (TGF) β-induced TGFβ type I receptor, a process that contributes to the invasiveness of cancer cells [203].

In addition to regulating common cellular activities, APPL1 expressed in the brain directly interacts with the NMDA receptor complex to regulate neuroplasticity [204-206]. In the cultured hippocampal neurons, a reduction in APPL1 abolishes the spinogenesis in the dendritic. APPL1 can interact with the NMDA receptors via the PI3K/Akt signaling pathway to facilitate NMDA receptors' recruitment and activation [205]. Additionally, APPL1 is located on the dendritic spines, where it translocates to the nuclear triggered by neuron activation. Nuclear APPL1 promotes gene transcription that is required for the maintenance of late-phase LTP [206].

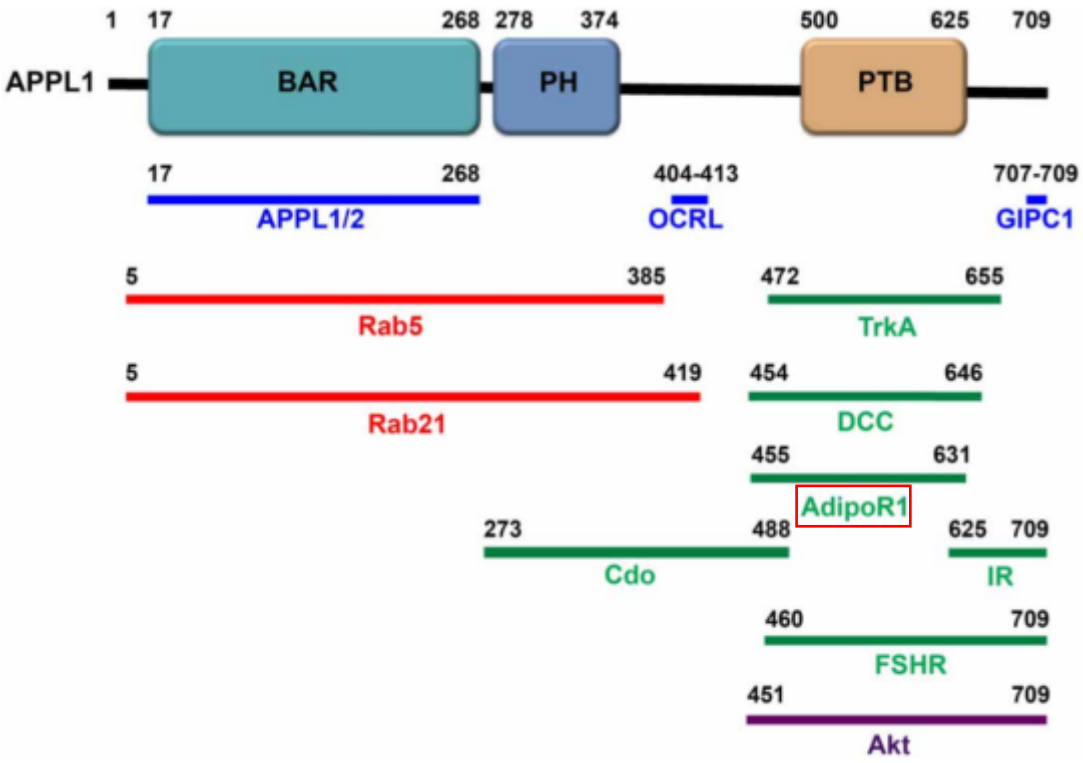


Figure 2.4 Domains and their binding sites of APPL1.

APPL1 is an adaptor protein that contains 3 major functional domains: a BAR domain, a PH domain, and a PTB domain. APPL1, through its BAR domain, can form homodimers with APPL1 or heterodimers with APPL2. In addition, BAR and PH domains together form a functional domain that can bind to the small GTPase Ras-related protein Rab-5 and Ras-related protein 21. APPL1 interacts with a variety of receptor proteins through its PTB domain, including tropomyosin receptor kinase A, AdipoR1, IR, and follicle-stimulating hormone receptors. The figure is adapted from Liu et al [197].

2.5.2 The role of APPL1 in transducing the adiponectin signaling

Adiponectin exerts its physiological functions mainly by activating two specific adiponectin receptors, namely AdipoR1 and AdipoR2 [168]. APPL1 is the first adaptor protein identified to directly interact with these two adiponectin receptors [27] and coupling the upstream adiponectin signaling with the downstream signaling pathway. In detail, the C-terminal extracellular domain of AdipoR1 interacts with adiponectin, while the N-terminal cytoplasmic domain of AdipoR1 interacts with the PTB domain of the APPL1 [27]. Similarly, APPL1 also interacts with AdipoR2 in a yeast two-hybrid library screening, but the interaction domain remains unclear [27].

In the central nervous system, APPL1 is an important mediator for adiponectin signaling transduction (Fig 2.5) by anchoring different effectors [189]. APPL1 promotes the crosstalk between the AdipoR and its downstream signaling targets including AMPK [29], PPARα [207], and PI3K [194]. AdipoR phosphorylates APPL1 to activate the AMPK and PPARα signaling pathways. Concurrently, APPL1 forms a complex with NMDA receptors, linking neuronal activity to the activation of insulin signaling via the PI3K/Akt pathway. In addition, APPL1 also mediated the interaction between adiponectin and insulin signaling, as confirmed by the insulin resistance and impaired signaling observed in APPL1 knockout mice [194]. Specifically, the phosphorylation of APPL1 at the Ser401 site stimulated by adiponectin enhances the binding of insulin receptor substrates 1 and 2 to the insulin receptor, which in turn mediates the insulin-sensitizing effects of adiponectin [194].

APPL1 located in the cellular cytoplasm and nuclear regulates different functions [204, 206]. APPL1 not only coordinates various signal transduction in the cytoplasm [189] but also regulates gene transcription in the nuclear [206]. In non-neuronal cells, adiponectin can directly induce the translocation of APPL1 and promote gene transcription [31, 32]. Chen et al. were the first to demonstrate that adiponectin can increase APPL1 translocation in human adipose stem cells to enhance osteoblast-related gene expression in a time-dependent manner, and these effects are reduced when APPL1 is knockdown with siRNA [31]. A recent study also showed that adiponectin treatment induces APPL1 nuclear translocation in rat aortic endothelial cells, which prevents diabetes-induced pathological vascular remodeling [32]. Nuclear APPL1 interacts with histone deacetylase 2 and

promotes gene expression including angiopoietin 1, occludens, and caveatin 1 [32]. In neurons, APPL1 acts as a messenger transporting signals received at synapses in the nucleus, resulting in changes in transcriptional activity [206]. However, no direct evidence shows that adiponectin-induced APPL1 translocation existed in the neural tissues.

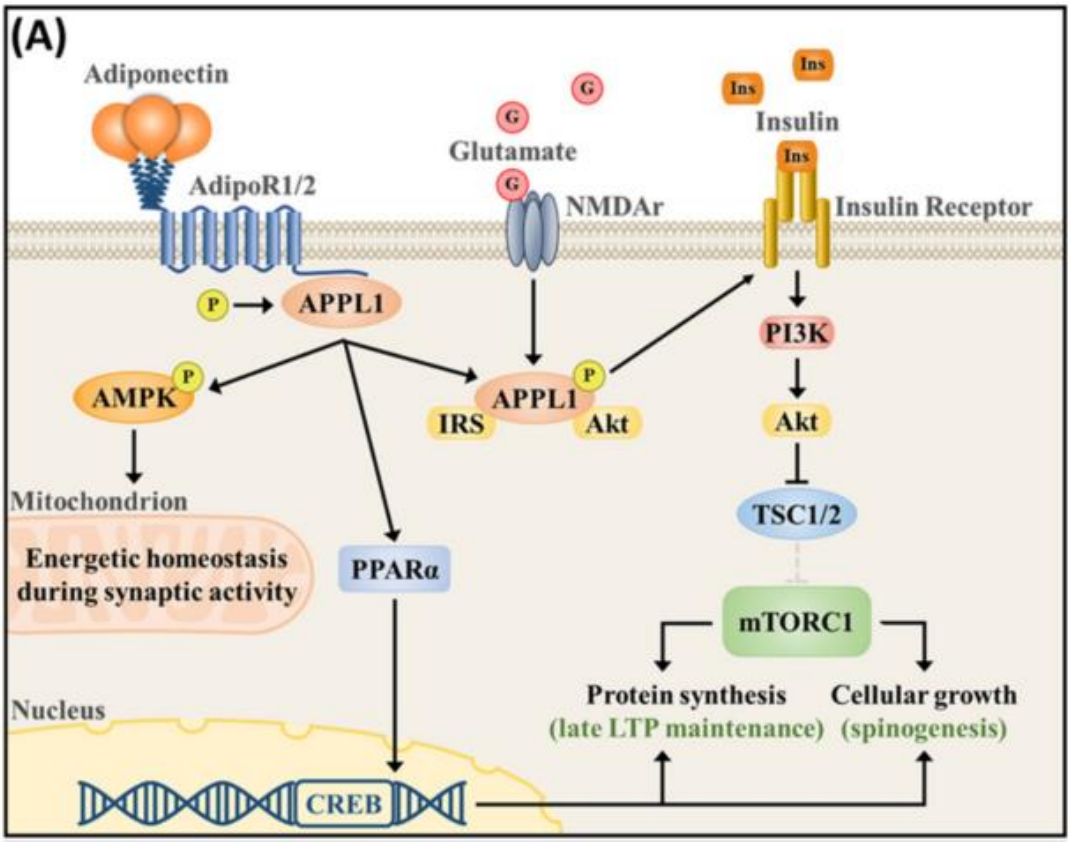


Figure 2.5 APPL1 in the central nervous system is an important adapter protein for downstream transduction of adiponectin signaling.

APPL1 is a key mediator in transducing AMPK, PPAR, and interacting NMDA receptor and insulin receptor signaling pathways. It can regulate energetic homeostasis, cyclic AMP response element-binding (CREB) transcription, and mTORC1 activity-related protein synthesis and cellular growth. The figure is adapted from Formolo D et al [198].

2.6 From synapse to nucleus: the role of APPL1 in histone acetylation

2.6.1 Mechanism and function of APPL1 nuclear translocation.

The transport of macromolecules between the cell nuclear and cytoplasm occurs through the central channel of the nuclear pore complex (NPC) [208]. The NPC is a complex multi-protein structure with a mass of approximately 125 MDa, penetrating the nuclear envelope and extending into the cytoplasm and nucleoplasm [209]. The center of the nuclear pore contains an aqueous channel that allows small molecules to pass through by passive diffusion [210]. However, the larger molecular complexes require a series of complex import/export mechanisms for active transport (Fig 2.6). Early research suggested the nuclear localization signal (NLS) is responsible for the selective nuclear localization of larger molecular proteins. The active nuclear import of proteins containing NLS is mediated by transport molecules of the importin family (importin α and β), which act as adapters and receptors in nuclear import [211]. Importin β can attach to fibrillar proteins projecting from the NPC into the cytoplasm, thereby directing the localization of multi-protein complexes, including importin α/β/NLS nuclear translocation [212]. After the NLS-containing proteins perform their functions in the nucleus, importin α and β are exported back to the cytoplasm, so that they can initiate another cycle of importing proteins containing NLS [213].

APPL1 nuclear translocation initially relies on its interaction with the Ras-related protein Rab-5 (Rab5) protein [199]. Rab5 is a key factor in the membrane transport from the plasma membrane to the early endosome [214]. In response to extracellular stimuli, APPL1 acts as a binding partner and effector of Rab5. It can be released from the membrane and translocated to the nuclear upon activation by Rab5-GTP [199]. Recent bioinformatics analysis and in vitro cell culture studies have further revealed mechanisms underlying APPL1 nuclear translocation: The C-terminus of APPL1 ($APPL1_{633–648}$) contains a predicted NLS, which enables it to bind to importin proteins that can be transported into the nucleus. Blocking the binding of importin proteins to APPL1 prevents the nuclear entry of APPL1 [206]. Notably, abnormalities in the APPL1 nuclear translocation are closely related to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease [215], and neuropsychiatric disorders like post-traumatic stress

disorder [216]. The onset of Alzheimer's disease is tightly linked to the toxic effects of the β-amyloid precursor protein and its cleavage product, the amyloid-β peptide [217]. Endosomes are critical sites for the processing of the β-amyloid precursor protein. In Alzheimer's disease, APPL1 regulates endosomal function and nuclear signaling through its interaction with the small GTPase rab5, while the aberrant mechanisms of APPL1 nuclear entry dysregulates nuclear signaling and endosomal function, thereby exacerbating the progression of the Alzheimer's disease [215]. Furthermore, APPL1 in the ventromedial prefrontal cortex plays a significant role in fear extinction. APPL1 undergoes nuclear translocation during extinction retrieval while blocking the nucleocytoplasmic translocation of APPL1 reduces NMDA receptor currents and impairs extinction retrieval [216].

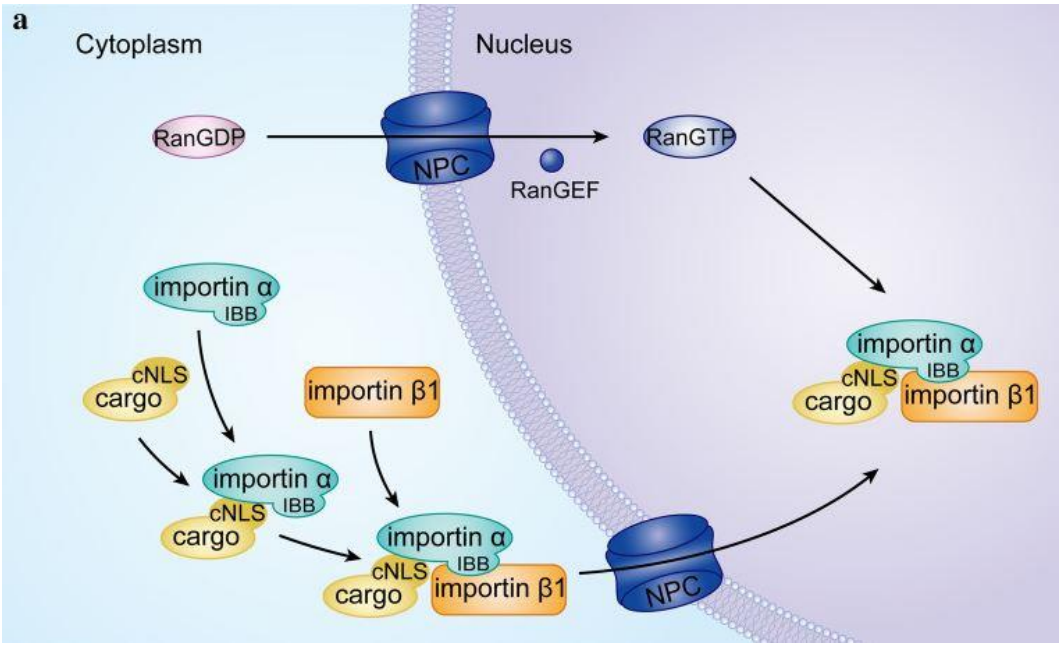


Figure 2.6 Schematic model of nuclear trafficking mediated by NLS.

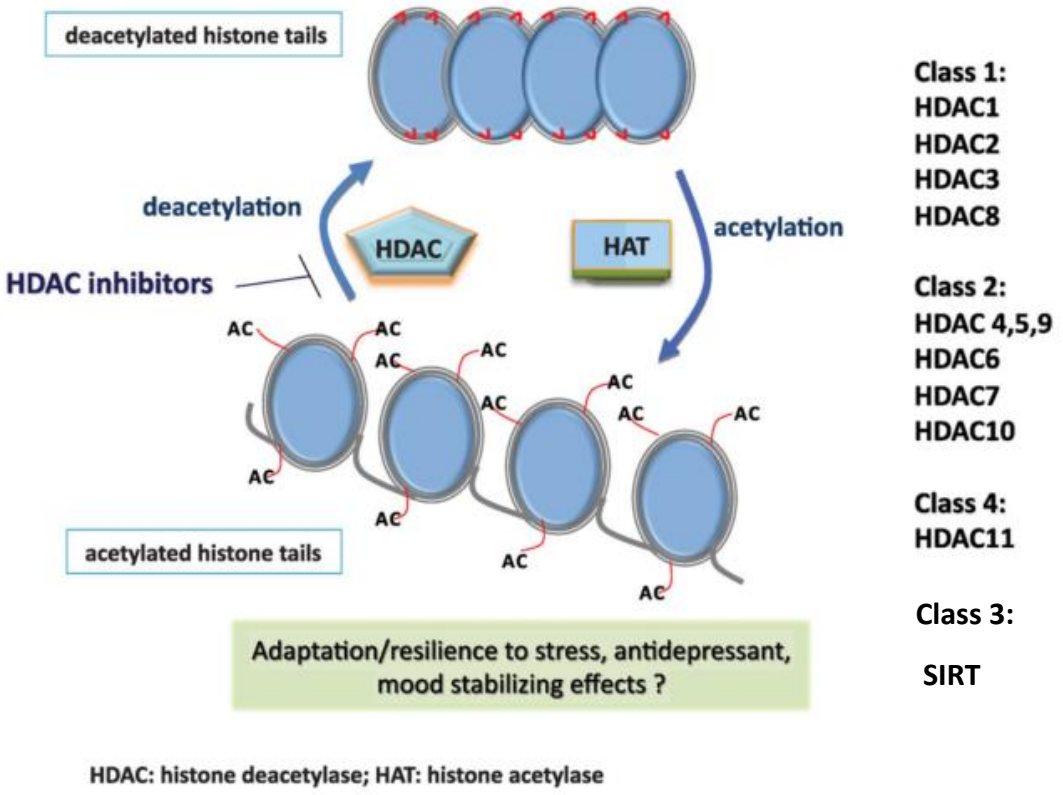
The large molecular proteins with a cNLS （referred to as cargo proteins）are imported by the carrier importin α, further binding importin β1 to form the cNLS-cargo-importin α-importin β1 trimer under the action of various factors. Importin β1 directs importin α to the NPC and transfers the trimer complex to the nucleus. NLS: nuclear localization signal; NPC: nuclear pore complex. The figure is adapted from Lu et al [218].

2.6.2 Histone acetylation in the pathogenesis and treatment of depression.

The term "epigenetics" refers to a process where genetic functions and the regulation of gene expression are mediated by mechanisms other than DNA sequence encoding, influenced by environmental changes [219]. This mechanism can transform environmental signals into precise and stable changes in chromatin structure, which persistently affect gene expressions. Histone acetylation is one of the epigenetic regulatory mechanisms controlled by histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Fig 2.7) [220]. Some transcriptional coactivator complexes possess HAT activity, which adds acetyl groups to the tails of histone to promote gene transcription [221]. In contrast, the transcriptional corepressor complexes contain HDAC, which are enzymes that repress gene expression by removing acetyl groups from histones, leading to a less accessible chromatin configuration [222].

Depression is strongly associated with a reduction in histone acetylation at specific sites as shown in rodent models. The levels of histone 3 (H3K9ac, H3K14ac) and histone 4 (H4K5ac, H4K8ac, H4K12ac, H4K16ac) acetylation have been shown to decrease in the dorsal raphe nucleus and hippocampus of mice exposed to chronic social defeat stress [223]. Interestingly, the acetylation levels of H4K12ac [224] and H3K14ac [225] increased 2 or 3 weeks after the social defeat stress. The results suggest that histone acetylation can be inhibited by stress, but this effect is reversible during recovery. Additionally, HDACs play a key role in regulating histone acetylation in depression, compared to HATs. HDACs are classified into two types: amidohydrolases, which include class 1 HDACs (HDAC 1, 2, 3, 8), class 2 HDACs (HDAC 4, 5, 6, 7, 9, 10), class 4 HDAC (HDAC 11), and the NAD^+-dependent class 3 SIRT enzymes (Fig. 2.7) [226]. Class I HDACs (e.g., HDAC2) and class II HDACs (e.g., HDAC5) have been demonstrated to be the most important HDACs in regulating depression. However, HDAC2 and HDAC5 appear to have opposite functions in the regulation of depression. By measuring the levels of all HDACs in the mouse striatum, the depression model can induce higher HDAC2 expression. Mice injected with an HDAC2 inhibitor exhibited less depressive behavior compared to control mice [227]. However, chronic social defeat stress decreases HDAC5 function in the nucleus accumbens in mice, while antidepressant drug imipramine increases HDAC5 function [228].

The main therapy targeting histone acetylation for depression is the use of histone deacetylase inhibitors (HDACis). Studies have found that MS-275, a selective HDACi that targets class I HDACs, can influence the expression of proteins such as CREB and BDNF in mice subjected to chronic social defeat stress [229, 230]. In addition, studies have found that the combination of the antidepressant drug fluoxetine and HDACis can significantly reduce depression-related behaviors, suggesting the potential of co-treatment with HDACis together with common antidepressant drugs in the future [231]. Vorinostat, the first HDACi approved for clinical use by the U.S. FDA, reverses depression-related behavior and glial cell line-derived neurotrophic factor (GDNF) expression by chronic unpredictable stress exposure in mice [231]. Although HDACis have antidepressive effects in animal models, there are still some limitations to be resolved before their wide application in clinical practice. For example, FDA-approved HDACi Farydak, used to treat multiple myeloma, was found to exhibit serious gastrointestinal toxicity, arrhythmias, and hepatic dysfunction, among other side effects [232]. There is an urgent need to discover new HDACis that can selectively target the promoter regions of depression-related genes and exhibit low toxicity.



HDAC: histone deacetylase; HAT: histone acetylase

Figure 2.7 The process of histone acetylation modification.

Histone deacetylation is to remove acetyl groups from lysine residues on the N-termini of histones by HDAC enzymes. The resulting inactive and silent chromatin blocks gene transcription by interfering with interacting binding to the promoter regions. Deacetylated histone tails bind tightly, resulting in a compact and inaccessible form of chromatin. Conversely, histone tails can be acetylated at different sites by HAT enzymes, generating an accessible form of chromatin. HDACs can be classified into two categories: amidohydrolases, including class 1 HDACs (HDAC 1, 2, 3, 8), class 2 HDACs (HDAC 4, 5, 6, 7, 9, 10), class 4 HDAC (HDAC 11), and the NAD^+-dependent class 3 SIRT enzymes. HDAC inhibitors can target different HDACs to deacetylate histones. HAT: histone acetyltransferases； HDAC: histone deacetylases. The figure is adapted from Rodrigo et al [233].

2.6.3 Nuclear APPL1 interacts with HDAC to regulate histone acetylation

APPL1 in the nuclear is involved in regulating histone acetylation. One early study first indicated that nuclear APPL1 interacts with chromatin, specifically engaging with the nuclear co-repressor complex NuRD [199]. Further study revealed that APPL1 mainly interacts with HDAC1/2 in the NuRD to stimulate chromatin remodeling [234]. The intracellular concentration of HDAC1 modulates the degree of interaction between APPL1 and the NuRD complex, subsequently influencing the distribution of APPL1 between the nuclear and cytoplasm. Silencing of HDAC1 amplifies APPL1's affinity for NuRD, facilitating its accumulation in the nucleus. In contrast, HDAC1 overexpression diminishes this interaction, leading to a reduced presence of APPL1 in the nucleus [234]. Functionally, overexpression of APPL1 affects the composition of the NuRD complex that includes HDAC1, as well as the expression of the HDAC1 target gene p21 Cyclin-Dependent Kinase Inhibitor 1 [234]. In addition, APPL1 can also form a complex with the tumor suppressor factor Reptin, acting in concert with HDAC1 to alleviate translational repression and promote the transcription of target genes in Wnt signaling [235]. A recent animal study showed that APPL1 could also directly interact with HDAC3 to mediate chromatin remodeling and mitochondrial brown fat uncoupling protein 1-related gene expression, which is essential for regulating brown adipocyte thermogenesis and may have potential therapeutic implications for the treatment of obesity [236]. In summary, these findings reveal the complexity of the interaction between APPL1 and HDAC and demonstrate their multifunctional impact on e regulating gene expression.

In the central nervous system, APPL1 mainly interacts with HDAC2 to promote the neuroplasticity-related gene transcription [206], cognition and memory [220], and emotional response [216]. One *in vitro* study showed that neuronal activity in the hippocampus induces retrograde transport of APPL1 to the nucleus, where it interacts with HDAC2 to trigger chromatin remodeling, which in turn regulates synaptic plasticity-related gene transcription and long-term synaptic plasticity [206]. Further animal experiments demonstrated that the nuclear APPL1 is essential for the normal fear extinction response in mice [216]. Notably, HDAC2 in the brain is predominantly localized at the promoters of genes associated with synaptic plasticity, or those responsive to

neuronal activity, such as Bdnf promoter I/II, Fos, candidate plasticity gene 15 (Cpg 15), CREB, and NMDA receptor subunits, etc. HDAC2 represses gene expression to suppress synaptic plasticity [237]. These HDAC2-targeted genes are critical for dendrite spine formation [238] and induction of LTP, which are implicated in the therapeutic effects of antidepressant drugs [239-241]. It is critical to identify the factors that facilitate APPL1's nuclear translocation to significantly influence neuroplasticity-related transcriptional activities linked to antidepressant effects.

2.7 Hypothesis

Clinical and preclinical studies indicate that single-bout physical exercise can produce rapid antidepressant effects (Chapter 2.1). Mechanistic insights from studying the action of rapid-onset antidepressant drugs suggest that targeting the downstream targets associated with enhanced neuroplasticity can result in rapid antidepressant effects (Chapter 2.2). Physical exercise promotes neuroplasticity through the exerkines (Chapter 2.3). Increasing adiponectin (an adipocyte-secreted exerkine) signaling as a potential treatment for enhancing neuroplasticity and treating depression (Chapter 2.4). The adaptor protein APPL1 is a key mediator in transducing adiponectin signaling (Chapter 2.5) and its translocation is crucial for regulating gene expression that promotes neuroplasticity (Chapter 2.6). Based on the above research background, we hypothesized that single-bout physical exercise enhances adiponectin signaling, leading to the nuclear translocation of APPL1. This process subsequently promotes gene transcription related to neuroplasticity enhancement and elicits antidepressant effects. Understanding the detailed mechanisms underlying the rapid antidepressant effects of single-bout physical exercise can provide valuable insights into how acute physical activity benefits brain health and may facilitate the discovery of new rapid antidepressant treatments for rehabilitation.

Chapter 3: Single-bout physical exercise elicits rapid antidepressant effects in humans and mice

3.1 Introduction

Major depressive disorder is a debilitating mental illness with a profound impact on millions globally, leading to significant personal distress and considerable socio-economic costs [242]. Depression is characterized by persistent sadness, feelings of loss, and diminished motivation, which can severely affect an individual's ability to function in daily life [243]. While pharmacological treatments such as selective serotonin reuptake inhibitors and ketamine have proven effective for depression, their drawbacks—such as delayed onset of action, adverse reactions, and increased resistance necessitate the pursuit of alternative therapeutic strategies [244, 245].

Physical exercise is widely recognized as a promising and rapid-acting intervention for mitigating depressive symptoms [7, 8]. Emerging clinical evidence has indicated that single-bout physical exercise can produce rapid antidepressant effects, including remarkable improvement in positive mood and reduction in negative mood in both healthy participants and patients suffering from depression [10, 11]. However, the neural mechanisms underlying this rapid antidepressant effects derived from single-bout physical exercise remain poorly understood. Neurobiological research utilizing animal models may elucidate the detailed cellular and molecular mechanisms underlying the rapid antidepressant effects of single-bout exercise.

This chapter aimed to confirm whether the rapid effect of single-bout physical exercise in reducing depressive phenotypes in humans is also present in a mouse model. The experiments aimed to establish a valid mouse model for investigating the neurological mechanisms underlying the rapid antidepressant effect of single-bout physical exercise. We recruited a cohort of human participants to join in single-bout treadmill running and assessed their mood states pre- and post-exercise using a questionnaire to evaluate mood changes. A mouse model with single-bout treadmill running (similar ~~intensity and~~ duration) was used to test whether the rapid antidepressant effects could be observed in healthy mice and stressed mice with depressive phenotypes.

3.2 Materials and Methods

3.2.1 Recruitment of human participants and experimental design

Human participants referred to by the University Health Clinic or in the university community were recruited. Exclusion criteria: 1) People aged over 40 or below 18 years old, 2) People with injuries that impair the ability to exercise safely, and 3) People with illnesses including metabolic, neurologic, and psychiatric disorders. Initial screening was conducted with potential participants by using an online questionnaire to assess their eligibility for the study based on the exclusion criteria. All eligible participants had signed the consent form and clarified.

Recruited participants were required to complete an online questionnaire of the hospital anxiety and depression scale (HADS) for screening of depression and/or anxiety symptoms. Eligible participants were classified as "symptoms" (HADS > 7) or "no symptoms" (HADS < 7) according to the HADS score for depression and/or anxiety [246]. Shortlisted participants were invited to participate in the exercise experiment. According to the score of the HADS, the number of participants was divided into 2 groups ("symptoms" and "no-symptoms") at around a 1:1 ratio. The required sample size of 40 was estimated using power analysis based on a similar clinical study [79]. The total number of recruited participants was N = 20 for "no symptom" and N = 20 for "symptom". Formal experiments including the mood evaluation questionnaire and treadmill exercise intervention were carried out on the same day at The Hong Kong Polytechnic University.

3.2.2 Evaluation of mood state in participants

To investigate the effects of single-bout physical exercise intervention on the mood state, the Profile of Mood States (POMS) was used to evaluate the mood changes of the participants before and immediately after exercise. POMS is a 30-item questionnaire scored on a 5-point Likert-type scale ranging from "Not at all" to "Extremely", providing seven subscales: 1) anger (e.g., grouchy, furious); 2) confusion (e.g., muddled, forgetful); 3) depression (e.g., sad, unworthy); 4) fatigue (e.g., tired, sluggish); 5) tension (e.g., nervous, anxious); 6) vigor (e.g., lively, active) and 7) esteem [247]. The summary index named "Total Mood Disturbance (TMD)" was calculated by deducting the total score of

the 2 positive emotions from the total score of the 5 negative emotions and then adding 100 points. The lower score describes a better mood state [248].

3.2.3 Single-bout physical exercise training

For the single-bout physical exercise intervention, the running protocol was previously described [79]. It consists of a 5-minute warm-up, and the speed acceleration progressively to the optimal intensity of 70–80% (moderate to high-intensity) of age-predicted maximum heart rate (MHR) [249] and continued for 20 min and ended with a 5-minute cool-down at the speed of 5m/min. A wireless heart rate monitor (Polar 10 series) was worn by the participants during the whole exercise period, and speed adjustments were made by the investigator to maintain the desired exercise intensity. Age-predicted MHR was defined as 220 beats per minute minus age in years.

3.2.4 Timeline for Human Study

Within one week after the HADS test, all assessments and interventions were conducted individually with each participant on the same day, between 9:00 AM and 12:30 PM. Participants were advised to abstain from consuming alcoholic beverages the day before and to maintain their usual sleep and morning routines. Upon arrival for the testing session, participants received instructions on the exercise protocol from a physical educator, donned a heart rate monitor, and completed the Profile of Mood States (POMS) questionnaire immediately before the exercise session. Following the exercise session, participants rested in a seated position for five minutes before completing the POMS questionnaire a second time.

3.2.5 Animal model and experimental design

C57BL/6J male mice (8-9 weeks old) were provided by the Centralized Animal Facility of the Hong Kong Polytechnic University and kept under a half-day dark/light cycle. All the procedures were approved by the Animal Subjects Ethics [(24-194) in DH/HT&A/8/2/4 Pt.19]. All behavioral assessments were performed during the dark phase of the cycle to accommodate the more active activity habits of mice at night.

To mimic the human study grouping (no symptom and symptom), we set the homecage group (no stress) and the chronic unpredictable stress group (with stress) to test the antidepressant-like effect of single-bout physical exercise using animal models. No-stress group mice were held in the cages for 3 weeks without exposure to any stressor while the stressed mice were subjected to 3-week stressors. Chronic unpredictable stress (CUS) (see Table 3.1) was used to induce depression-like behavior in the mouse model.

The single-bout physical exercise protocol was adopted as previously described (Table 3.2) [250-252]. Before exercise intervention, mice underwent a three-day treadmill adaptation to reduce stress (Figure 3.2). On day 1, mice were allowed to adapt to the testing room, handling, and treadmill. On day 2, all mice were acclimated to the treadmill's incline (25°) for 5 min. On day 3, mice were acclimated to the warmup stage with a 5-minute treadmill running at a speed of 5 m/min with the incline (25°). For formal treadmill training on day 4, following a 5-minute warmup at a slow walking speed (5 m/ min), mice were subjected to run for 30s intervals with 60s walking rest periods (5 m/min) interspersed between intervals. The exercise session with speed increase gradually from 15 m/min, 20 m/min, to 25 m/min, followed by a final speed of 30 m/min. The following exercise session included 20 running repeats. In each repeat, mice were running for 30 seconds with the running speed kept at 30 m/min and for 1 minute at 5 m/min. Running speed acceleration of 30 m/ min was performed within 5 s and deceleration to 5 m/min was done within 2 s. The warm-up and the exercise session were performed at an incline of 25° in the treadmill. To maintain running speed without receiving an electrical shock, mice were gently prodded by hand when they closely approached the shock grid. As the control, sedentary mice were put onto the fixed treadmill device to stay for the same time within the same environment.

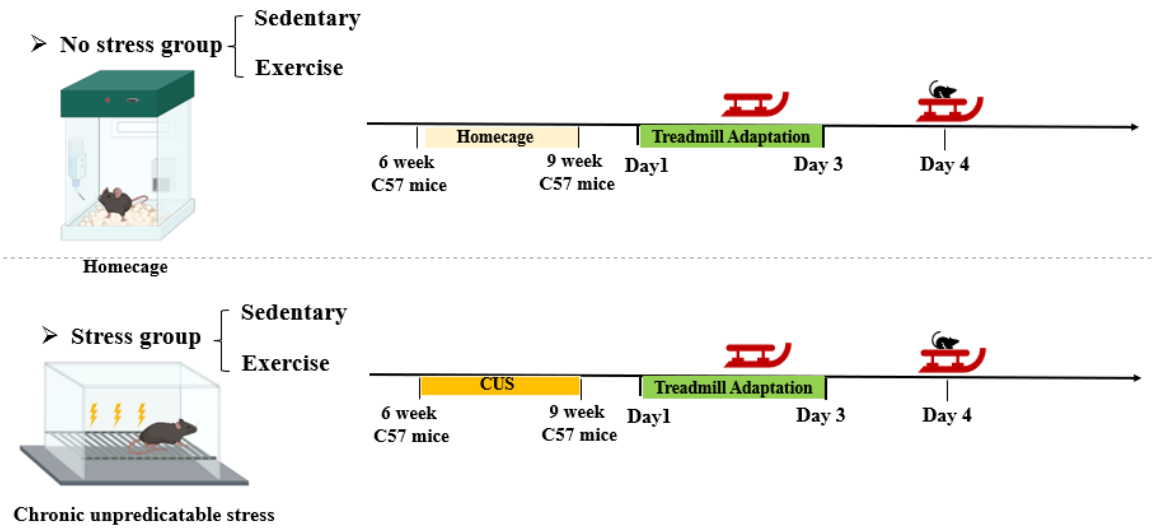


Figure 3.1 Animal models.

The non-stressed control group was housed in their home cages without exposure to any stressors while the stressed control was subjected to 3-week chronic unpredictable stress. The mice were then subdivided into sedentary or exercise groups with a single bout of treadmill running. All mice were briefly handled for one week before the behavioral tests to reduce acute stress response triggered by experimenter handling.

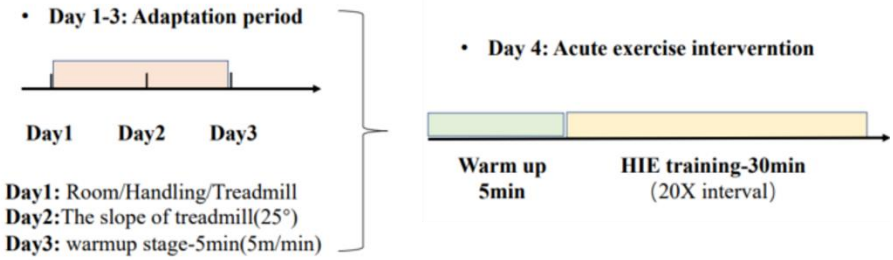


Figure 3.2 The training timeline of single-bout physical exercise for mice.

Before exercise intervention, mice underwent a three-day treadmill adaptation to reduce stressors and one-day formal treadmill training. The mice received a 5-minute warm-up running on the treadmill, followed by high-intensity interval training for 30 min. The training protocol details are described in table 3.2.

Table 3.1 Physical stressors utilized to induce 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 – 5 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 – 5 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 – 5 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)

Table 3.2 Single-bout physical exercise protocol in the mouse model.

Stage		Duration	Speed (m/min)
1	Warm-up	5 min	5
2	Running	30 sec	15
	Rest	1 min	5
3	Running	30 s	20
	Rest	1 min	5
4	Running	30 s	25
	Rest	1 min	5
5	Running	30 s	30
	Rest	1 min	5
6	20 repetitions of stage 5		

3.2.5 Behavioral tests

Open Field Test (OFT): This test examines the basic locomotor activity and anxiety-like behavior of mice. Mice were placed in the center of an open-field apparatus (size = 44×44×30 cm) with dim light in the testing room (20-30 lux). The movements of the animals were tracked by an automatic monitoring system for 10 minutes. Open field was cleaned with 70% ethanol between trials. Anxiety-like behavior and locomotor activity were analyzed using ANYMAZE software (Stoelting Co, Wood Dale, IL, USA). Anxiety-like behavior was evaluated by the percentage of the time spent in the center. The locomotor activity was assessed by the distance travelled in the apparatus.

Sucrose Splash Test (SST): This test is commonly used to evaluate anhedonia-like behavior as one of the core symptoms of depression. The decrease in the grooming behavior of mice is considered a loss of self-care and interest in pleasure. A 10% sucrose solution was sprayed onto the mice's fur. Because of the stickiness and reward of the sucrose, the mice initiated grooming behavior. The time spent grooming the body, face, and paws within 5 minutes was recorded [253]. The testing cages were cleaned with 70% alcohol between trials.

Tail Suspension Test (TST): Briefly, the mouse was suspended at the center of a rectangular compartment using tape, with a climb-stopper (plastic tube) positioned around its tail [13]. The movements of the animals were recorded by an automatic monitoring camera (ispy software) for 6 minutes. The immobility time (all four limbs stopped struggling or only slight body movement occurred) was manually analyzed for the last 4 min of the test by two experienced researchers.

Forced Swim Test (FST): Mice were placed in a plexiglass cylinder (diameter: 15 cm; height: 30 cm) filled with 25-26˚C water (water level: two-thirds of water) for 6 minutes. The immobility of the mice was evaluated for the last four minutes and analyzed in a sample-blinded manner by two observers using stopwatches. An animal will be considered immobile when it remains floating in the water, making only movements necessary to keep its head above water [138].

3.2.6 Statistical analysis.

Figures and data analysis were done using GraphPad Prism 9 (GraphPad Software, USA). The normality of data was analyzed with the Shapiro–Wilk test ($n < 8$) or D' Agostino and Pearson test ($n \geqslant 8$) [101].

For the clinical data, parametric data were analyzed by paired t-test, while non-parametric data were analyzed using the Wilcoxon signed-rank test.

For the pre-clinical data with two independent variables (exercise and stress), two-way ANOVA followed by Tukey's post hoc analysis for multiple comparisons was used. All the data were presented as mean ± SEM. Statistical significance was indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.3 Results

3.3.1 Single-bout physical exercise elicits rapid antidepressant effects in humans.

We initially enlisted 40 volunteers to evaluate the mood elevation effect elicited by single-bout physical exercise (Fig 3.3A). Before the intervention, participants completed the HADS questionnaire to evaluate the presence and severity of depression and anxiety symptoms in outpatient settings [254]. Based on HADS results, participants were classified into two groups: ' no symptom ' (N = 20) and ' symptom ' (N = 20). Their mood states were then measured using the POMS scale [247] before and immediately after exercise. POMS results indicated that single-bout physical exercise reduced TMD scores (Fig 3.3C), enhanced scores in two positive subscales (vigor and esteem) across both groups (Fig 3.3D), and lowered scores in five negative subscales (anxiety, fatigue, depression, confusion, and anger) exclusively in the symptomatic group (Fig 3.3E). These findings corroborate the potential rapid antidepressant effect of single-bout physical exercise.

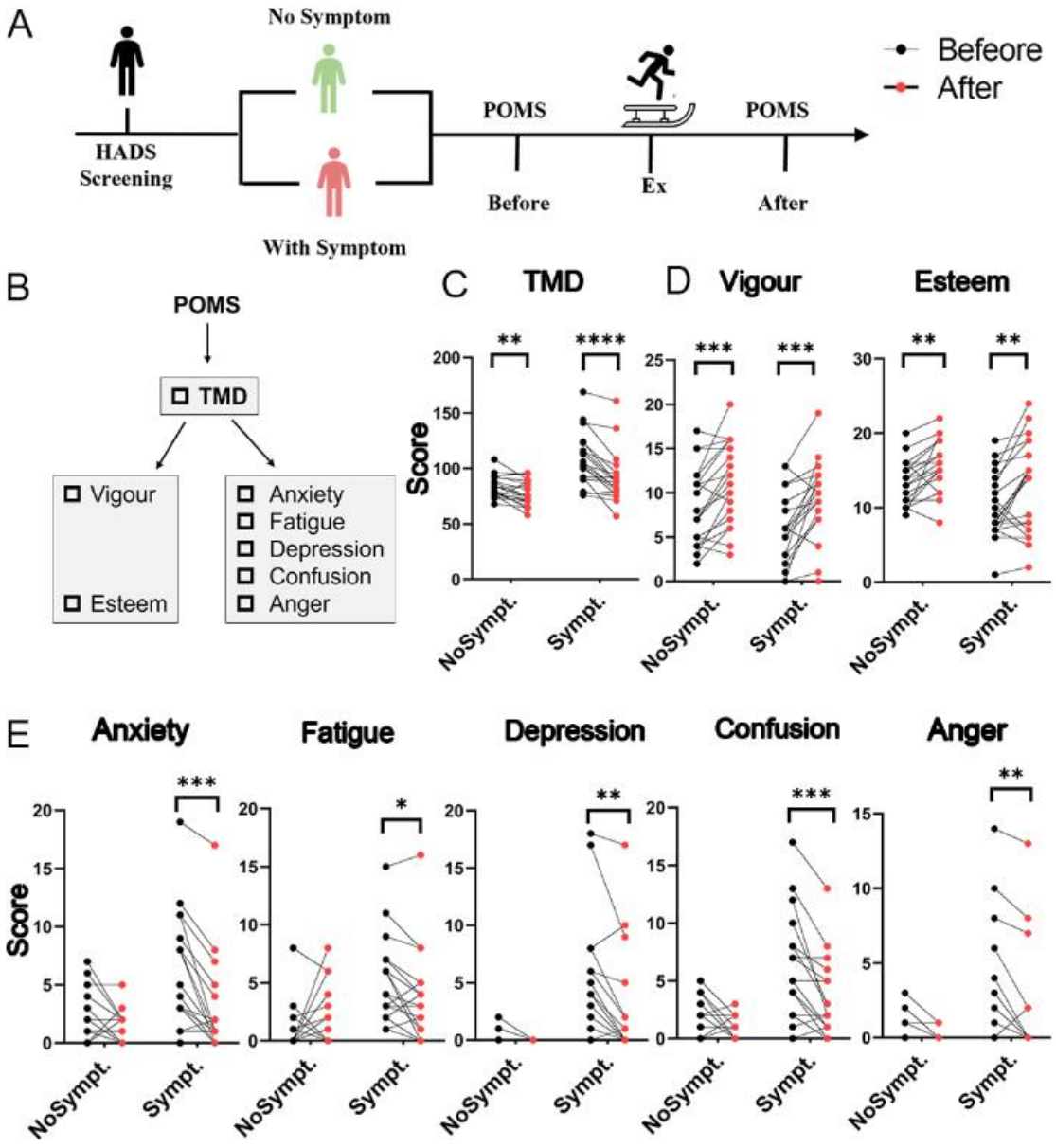


Figure 3.3 Single-bout physical exercise elicits rapid antidepressant effects in humans.

(A) Experimental timeline for clinical study. Based on the result of the hospital anxiety and depression scale, participants were divided into two groups (1) non-symptomatic (NoSympt. N = 20) and (2) symptomatic (Sympt. N = 20), their mood status was then assessed using the Profile of Mood States (POMS) scale pre- and post-exercise.

(B) Schematic diagram of the POMS scale. The scale consists of seven emotion subscales, two positive subscales (vigour and esteem), and five negative subscales (anxiety, fatigue, depression, confusion, and anger). Total Mood Disturbance (TMD) can be calculated by combining the positive and negative subscales.

(C) TMD. Single-bout physical exercise reduced TMD scores in both no symptoms and symptoms. Paired t-test, no symptom: $T(19) = 3.470$, $P = 0.0026$; Wilcoxon matched-pairs signed rank test, symptom: $P < 0.0001$.

(D) Two positive subscales. Single-bout physical exercise increased the positive subscales scores in the groups with no symptoms and symptoms. Vigour: Paired t-test, no symptom: $T(19) = 4.239$, $P = 0.0004$; Paired t-test, symptom: $T(19) = 4.434$, $P = 0.0003$. Esteem: Paired t-test, no symptom: $T(19) = 3.499$, $P = 0.0024$; Paired t-test, symptom: $T(19) = 3.122$, $P = 0.0056$.

(E) Five negative subscales. Single-bout physical exercise decreased the negative subscales scores in the symptoms group. Wilcoxon matched pairs signed rank test was used for the comparison of negative subscales except for the confusion group. Anxiety: no symptom: $P = 0.0801$; symptom: $P = 0.0002$. Fatigue: no symptom: $P = 0.1229$; symptom: $P = 0.0104$. Depression: no symptom: $P = 0.125$; symptom: $P = 0.0024$. Anger: no symptom: $P = 0.0625$; symptom: $P = 0.0032$. Confusion: paired t-test, no symptom: $T(19) = 2.622$, $P = 0.0168$; Wilcoxon matched-pairs signed rank test, symptom: $P = 0.0001$.

3.3.2 Single-bout physical exercise elicits rapid antidepressant effects in mice.

Subsequently, we implemented a comparable experimental design using rodent models (Fig 3.4A). Initially, we assessed the rapid effect of 30 min-post exercise in both non-stressed control and chronically stressed mice (Fig 3.4A). Physical exercise did not alter the basic locomotor activity or anxiety-like behaviors in either model (Fig 3.4B); however, it induced rapid antidepressant-like behaviors, as evidenced by reduced immobility time in the forced swim test among the stressed mice (Fig 3.4C).

A subsequent cohort of mice was evaluated to determine the potential sustained effects at several additional time points using various behavioral tests (Fig 3.4A). Both control and stressed mice demonstrated increased grooming time in the sucrose splash test at 2 hours and decreased immobility time in the tail suspension test at 24 hours post-exercise, but no significant differences were observed in the forced swim test at 48 hours (Fig 3.4D). In sum, these findings imply that single-bout physical exercise can elicit rapid antidepressant effects that persist for at least 24 hours in both non-stressed control and stressed mice.

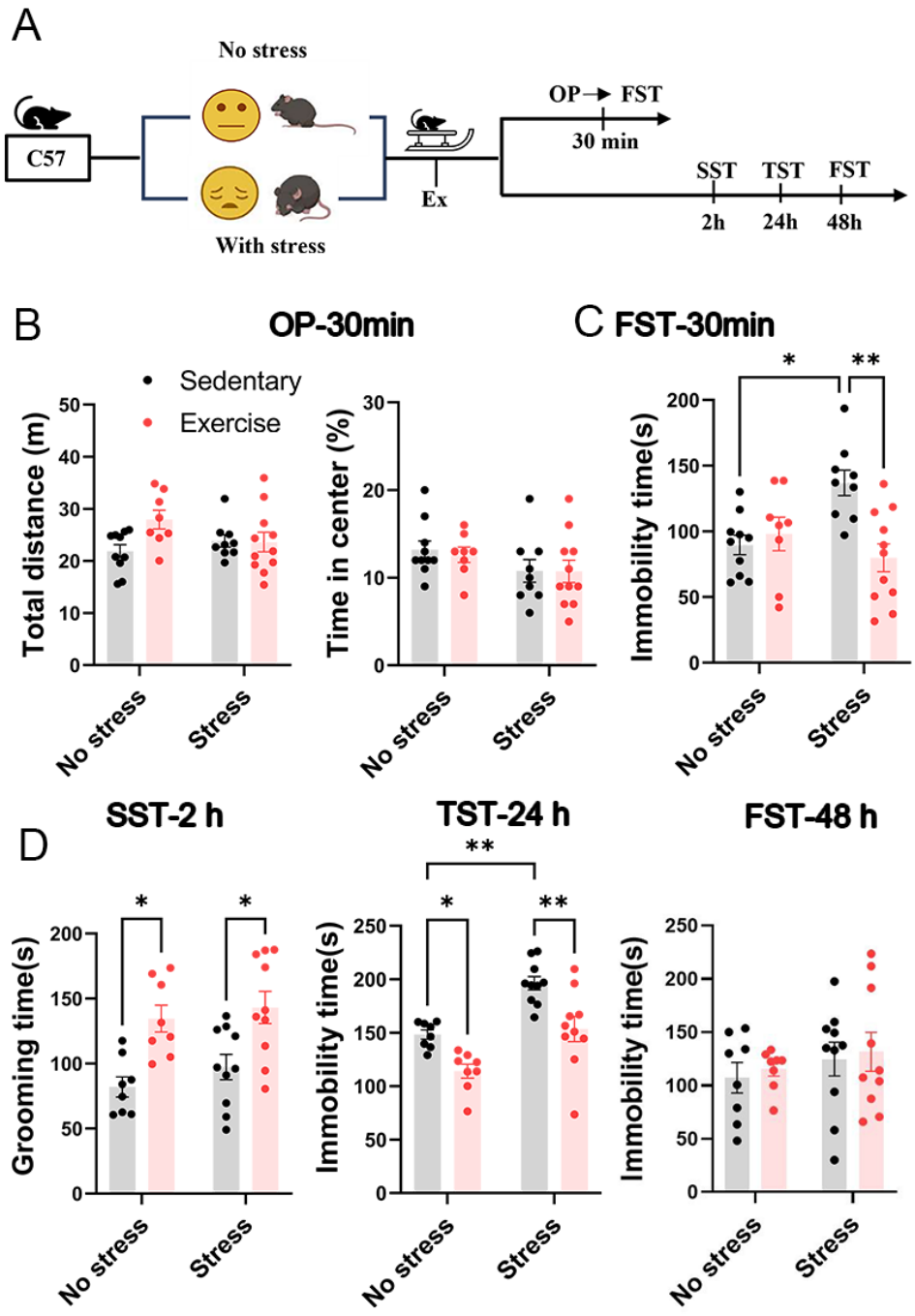


Figure 3.4 Single-bout physical exercise elicits rapid antidepressant effects in mice. (A) Experimental timeline for animal study. The first batch of mice (no stress and stress) was used for the open field test (OFT) and forced swimming tests (FST) 30 minutes post-

exercise. The second batch of mice was used for the sucrose splash test (SST), tail suspension test (TST), and FST for 2-hour, 24-hour, and 48-hour post-exercise, respectively.

(B) OFT. Single-bout physical exercise does not affect locomotor activity (traveled total distance) or anxiety behavior (time spent in the center area) in both the stress and no stress group. Total distance. Interaction: $F(1, 34) = 3.923$, $P = 0.0558$; Stress: $F(1, 34) = 0.5388$, $P = 0.4680$; Exercise: $F(1, 34) = 3.368$, $P = 0.0752$. Time in the center. Interaction: $F(1, 34) = 0.05127$, $P = 0.8222$; Stress: $F(1, 34) = 3.478$, $P = 0.0708$; Exercise: $F(1, 34) = 0.07292$, $P = 0.7888$.

(C) Single-bout physical exercise decreased the immobility time of the stress group in the FST test at a 30-minute time point. FST. Interaction: $F(1, 34) = 10.30$, $P = 0.0029$; Exercise: $F(1, 34) = 2.041$, $P = 0.1622$; Stress: $F(1, 34) = 5.710$, $P = 0.0226$.

(D) Single-bout physical exercise elicited antidepressant effect at 2-hour and 24-hour time points but no effect at 48-hour time points. SST. Interaction: $F(1, 32) = 0.1046$, $P = 0.7484$; Stress: $F(1, 32) = 1.281$, $P = 0.2662$; Exercise: $F(1, 32) = 21.76$, $P < 0.0001$. TST. Interaction: $F(1, 32) = 0.2699$, $P = 0.6070$; Stress: $F(1, 32) = 27.85$, $P < 0.0001$; Exercise: $F(1, 32) = 21.57$, $P < 0.0001$. FST. Interaction: $F(1, 32) = 0.001289$, $P = 0.9716$; Stress: $F(1, 32) = 1.208$, $P = 0.2799$; Exercise: $F(1, 32) = 0.2366$, $P = 0.63$. All the data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.4 Discussion

In this chapter, we demonstrated that single-bout physical exercise (in terms of 30-minute treadmill running) induced rapid antidepressant effects in participants with or without depressive symptoms. Single-bout physical exercise is sufficient to elevate positive mood states (vigor and esteem) in both symptomatic and asymptomatic participants and to reduce negative mood states (anxiety, fatigue, depression, confusion, and anger) in the symptomatic participants (Fig 3.3 A-E). A similar rapid antidepressant effect was observed in the control mice without chronic stress and stressed mice displaying an increase in depression-like behavior. Notably, the antidepressant response in the mice persisted up to 24 hours post-exercise (Fig 3.4 A-D). These findings have confirmed that the rapid action of physical exercise in reducing depressive symptoms is consistent in humans and rodents. The promising behavioral benefits brought by single-bout physical exercise strongly support the clinical significance of using single-bout physical exercise as an effective intervention for reducing depressive mood, not only in depression patients but also in the general population who have encountered stressful events in life to prevent further development of clinical depression. Also, the mouse model with rapid antidepressant response induced by single-bout physical exercise was successfully established for the follow-up studies for the detailed underlying neural mechanisms.

Acute effects of single-bout physical exercise in mood changes have been investigated in a few clinical studies [79, 255]. Our data suggested that single-bout physical exercise benefits exercisers with or without depression symptoms in terms of the improvement of positive mood states (vigor and esteem) (Fig. 3.3 D). This aligns with a previous study demonstrating that exercise improves vigor scores in patients with MDD [256]. Regarding the negative mood state, existing literature suggests that individuals with clinical mental health disorders, including MDD, generally experience greater emotional improvements from exercise than their healthy counterparts [257]. Our data showed that exercise benefited those with symptoms in terms of the improvement of the negative mood state but not in the no-symptom group (Fig. 3.3E), potentially due to the “floor effect” since some participants without symptoms had a very low baseline in negative mood-related scores before benefiting from treadmill exercise. Our findings underscore the potential of single-

bout physical exercise as a rapid and non-pharmacological mood enhancer. Future research should explore the use of physical exercise intervention in people at the initial signs of depressive disorders to achieve immediate mood improvement and prevent the progression of depressive symptoms. Physical exercise should be emphasized as an important complementary intervention to conventional antidepressant pharmacological treatments, which may take weeks to elicit the onset of its therapeutic effects [244].

The pre-clinical study primarily focuses on the impact of chronic exercise intervention on mood improvement [13, 258], while studies exploring the antidepressant effects of acute exercise are still in their infancy. In our study, we established a mouse model with single-bout treadmill running which mimicked the protocol of the single-bout exercise treadmill running in humans. Behavioral results suggest that single-bout physical exercise did not affect locomotor activity and anxiety-like behavior as examined in the OPT (Fig 3.4 B). This finding appears to be contradictory to previous study reporting the anxiolytic effects of acute treadmill running in the open field test [82]. The inconsistency could be attributed to post-exercise fatigue during the behavioral test immediately after finishing high-intensity interval training, while our testing was done 30 minutes after exercise. However, the forced swim test shows that single-bout physical exercise rapidly decreased depressive symptoms in stressed mice but not in non-stressed mice (Fig 3.4 C), echoing findings in human studies showing that single-bout physical exercise benefits more in individuals with depressive symptoms (Fig 3.3 C-E). Moreover, the sucrose splash test [259] and tail suspension test [260] were used to evaluate anhedonia and despair-like symptoms which are the core depressive characteristics in patients with depression [38]. Our findings reveal a sustained antidepressant effect up to 24 hours following single-bout exercise. This finding is supported by another rodent study showing a significant antidepressant effect in the forced swimming test on the second day following a single bout of treadmill training in rats [80].

Taken together, our findings confirmed that single-bout physical exercise elicits a rapid antidepressant effect in both human participants and animals and highlighted the rapid onset of antidepressant response even with a single-bout session in people with or without depressive mood. Single-bout physical exercise is a promising non-

pharmacological intervention for managing depressive mood and should be implemented early to arrest symptomatic development into clinical depression. Future research will aim to elucidate the neurological mechanisms underlying the rapid antidepressant effects of single-bout physical exercise.

Chapter 4: Activation of glutamatergic neurons in the ACC is required for single-bout physical exercise to elicit rapid antidepressant effect

4.1 Introduction

Depression is a widespread and devastating mental health condition with significant negative individual and societal impacts [261]. The discovery of ketamine, a fast and long-lasting antidepressant, has given hope for solving the delayed response and treatment resistance issues. A single injection of low-dose ketamine can rapidly alleviate depressive symptoms within a few hours and last for at least 7 days [5]. Nonetheless, the potential for abuse and the dissociative effect of ketamine constrain its long-term use in patients [63]. Considering the adverse effects of the currently available antidepressant drugs, it is imperative to explore safer and more efficacious non-pharmacological interventions for depression. The findings from clinical and animal studies presented in the preceding chapters provide scientific evidence showing the potential of single-bout physical exercise as an effective, rapid-onset antidepressant intervention.

Depressed patients are reported to have reduced activity of the glutamatergic system in the mPFC [262]. Neuroimaging studies have shown that antidepressants like ketamine can enhance the activity of the mPFC [263]. Preclinical research indicates that activation of the glutamatergic neurons in the mPFC is crucial for the therapeutic effects of ketamine [264]. Low dosages of ketamine can induce a burst release of glutamate and rapid activation of the glutamatergic neurons in the mPFC. In contrast, silencing the glutamatergic neurons in the mPFC can inhibit the rapid antidepressant effects [264]. Notably, single-bout physical exercise elicited significant glutamate surge in cortex regions observed in both animals [265, 266] and clinical studies [267, 268]. While running on a freely rotating spherical treadmill, the balance of cortical excitation and inhibition in the mouse cortex shifts toward excitation, making glutamatergic neurons more active compared to the sedentary state [268, 269]. In addition, several clinical studies using magnetic resonance spectroscopy showed an increase in glutamate levels in several cortex

regions, including the ACC, after single-bout physical exercise in healthy participants [265, 266]. However, whether the enhanced function of the glutamatergic system by single-bout physical exercise is involved in rapid antidepressant responses has not been examined.

In this chapter, we aim to clarify the neural mechanism by which single-bout physical exercise elicits its rapid antidepressant effects. By employing multiple approaches including whole-brain c-Fos mapping, *in vivo* two-photon calcium imaging, and chemogenetic manipulation of specific neuronal populations, we want to investigate whether the glutamatergic neurons in the mPFC participate in the rapid antidepressant effects of single-bout physical exercise.

4.2 Materials and methods

4.2.1 Animals and tissue preparation

C57BL/6J male mice and CAMKII-cre mice (6-8 weeks old) were housed in the centralized animal facility (CAF) of the Hong Kong Polytechnic University. Unless otherwise specified, the housing conditions follow the same described in Chapter 3.

Experimental mice for the whole brain c-Fos mapping were separated into the sedentary group (N = 4 males) and the exercised group (N = 4 males). After treadmill running, we anesthetized mice with isoflurane and subsequently perfused them with 1× PBS and paraformaldehyde (PFA). The brain tissues were fixed overnight in PFA and subsequently immersed in 30% sucrose until they sank. For the experiment with brain-wide mapping for neuronal activation, consecutive 40-um-thick coronal sections were sliced between 2.34 and -3.40 mm from bregma along the rostral-caudal axis. Each brain slice of every 0.7-0.8 mm was used for immunostaining using antibodies against c-Fos, giving a total of nine brain slices per mouse. Eight key regions of the entire brain were included in the analysis: the brainstem, cortical Subplate, hypothalamus, olfactory, striatum pallidum, thalamus, hippocampus, and cortex.

4.2.2 Immunofluorescence staining

Brain slices were washed with PBS and incubated with citric acid buffer (10 mM) for 10 min at 95 ◦C for antigen retrieval and then incubated with 2N HCl for 10 min at 37 ◦C

for denaturing double-strand DNA. After PBS washing, slices were incubated with primary antibodies (Table 1) at 4 °C overnight, followed by a 2-hour incubation with the second antibody (Table 1) at room temperature. The following antibodies were used: Rabbit anti-c-Fos (1:200, Abcam, ab214672), Mouse anti-c-Fos (1:200, Abcam, ab208942), Rabbit anti-CAMKII (1:200, Abcam ab214672), Rabbit anti-GABA (1:200, Sigma, A2052), Goat anti-rabbit 568 (1:200, Thermofisher, A-11011), and Goat anti-mouse 488 (1:200, Abcam, ab150113). In the final step, sections were coverslipped with fluorescent mounting medium with DAPI (Fluoroshield™ with DAPI, Sigma).

4.2.3 Brain-wide mapping for neuronal activation following single-bout physical exercise

The Z-stacks of brain slices stained with c-Fos were captured using a 10× objective on a Zeiss confocal microscope. Acquisition settings (laser power, gain/offset, averaging, dwell time, etc.) were kept constants for all samples. Before quantification, maximum intensity projections of each slice were made. The intensity of c-Fos expression calculated for all nine slices was used in a custom-made analysis software named the “brein” shared by Prof. Thomas Sudhof's group at Stanford University. The accuracy of the software was previously validated with results from manual counting. Briefly, the Brein software is used to locate the positions of nine brain sections within its built-in brain atlas which accurately match confocal images with the most suitable images, aligning anatomical features of the corresponding brain regions. The "Calculate Intensities" function was used to analyze the data and export fluorescence intensity measurement to an Excel spreadsheet for further analysis. The statistical results include the relative fluorescence intensity values for each region in the brain section. This experiment specifically focuses on the fluorescence expression of the c-Fos protein. Therefore, the level of c-Fos expression is directly proportional to its fluorescence intensity and presented as relative fluorescence intensity in the target brain subregions.

4.2.4 Stereotaxic injection of adeno-associated viruses (AAV)

For AAV injection, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus (RWD, Shenzhen, China).

After local sterilization and making an incision in the head skin, a high-speed microdrill was used to open the skull with an injection hole bilaterally. A glass micropipette connected to an ultra-micro injection pump (Nanoliter2010, WPI, USA) with an injection controller (Micro4, WPI, USA) was used for AAV injections targeting the ACC (anterior-posterior - AP 0.8 mm, medial-lateral - ML 0.3 mm, and subpial -SP 0.6 mm) [270]. The micropipette was retained for 5 min before retraction. After surgery, animals were treated with Flunixin (2.5 mg/kg, s.c.) for three consecutive days and allowed for a 3-week recovery. The viruses used are following: AAV2/9-CaMKIIα -GCaMP6 (Brain VTA), AAV-Syn-DIO-hM3Dq-mCherry (Addgene plasmid #44361), AAV-Syn-DIO-hM4Di-mCherry (Addgene plasmid #44362). Detailed information on the viruses can be found in Table 2.

4.2.5 *In vivo* transcranial two-photon calcium imaging

To image neuronal activity located in the ACC brain region, surgical procedures were conducted as previously described [270]. After 3 weeks of recovery from the AAV injection with sufficient target gene expression in infected neurons, the head skin was opened, and the skull over the ACC was carefully removed by a microdrill. The imaging window was covered with a round glass coverslip. A customized metal scaffold was fixed onto the mouse skull using dental cement. The awake mouse was fixed on the imaging stage using the metal bar, followed by 30 min of acclimation. For the imaging session, mice were recorded to examine the time course of ACC-glutamatergic neuron activity at different time points, including pre-exercise, 30 min, 2 h, and 4 h post-exercise. The calcium activities of ACC-glutamatergic neurons under 200 to 300 μm in depth were recorded at 2 Hz with a water-immersed objective during a 2.5 min period under a multiphoton microscope A1 MP (Nikon, Japan). During imaging, the laser was tuned to 920 nm.

Imaging data were analyzed with ImageJ as previously reported [271]. All acquired time series images were corrected by the TurboReg module of ImageJ. The same neurons were circled as regions of interest (ROI) at each time point and analyzed in a sample-blinded manner. The fluorescent value of each neuron was normalized as $(F - F_0)/F_0$, where the F_0 (basal value) was the minimum of all values during each recording series. The

averaged integrated calcium was calculated as the average of $\Delta F/F_0$ of all recording neurons.

4.2.6 Chemogenetic experiments and clozapine-N-oxide (CNO) treatment

CNO (Brand: APExBIO) was dissolved in saline and delivered intraperitoneally at a dose of 1 mg/kg. The applied doses were used for chemogenetic activation (hM3Dq) and inhibition (hM4Di) experiments as previously reported [272]. For the chemogenetic inhibition experiment, the CNO was injected half an hour before the treadmill running. For the chemogenetic activation experiment, the CNO was injected 2 hours before the behavioral tests.

4.2.7 Behavioral tests

The behavioral assessment was carried out following the same procedures described in Chapter 3, including the SST, TST, and FST.

4.2.8 Statistical analysis

Graph figures and data analysis were performed using GraphPad Prism 9 (GraphPad Software, USA). The normality of the data distribution was assessed using the Shapiro–Wilk test for sample sizes less than 8 ($n < 8$) or the D'Agostino and Pearson test for sample sizes of 8 or more ($n \geqslant 8$) [101].

For data with a normal distribution, comparisons between the two groups were conducted using an unpaired t-test. For non-parametric data, the Mann-Whitney analysis was used. For single-variable animal group comparisons, one-way ANOVA followed by Dunn's multiple comparison test was employed. For comparisons involving two independent variables (e.g., exercise and drug treatment), two-way ANOVA followed by Tukey's post hoc analysis for multiple comparisons was used.

All behavioral tests were analyzed by well-trained researchers who were blinded to the treatment groups. Data are presented as mean ± SEM. Statistical significance was indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.3 Results

4.3.1 Whole-brain analysis of c-Fos expression following single-bout physical exercise.

To elucidate the neural mechanisms underlying the antidepressant effect of single-bout physical exercise, we employed whole-brain imaging of the immediate early gene c-Fos to identify the key brain areas in response to exercise intervention in the non-stressed mice. We examined the relative c-Fos immunofluorescence intensity in 146 brain regions across the whole brain in the “non-stressed mice”(Fig.4.1).

We identified significant changes in neuronal activation or suppression across approximately 17 brain regions in response to a single bout of exercise. In detail, changes in c-Fos expression were observed in the fasciola cinerea (Fc) of the hippocampus; the nucleus of Darkschewitsch (Dk), the superior colliculus (SuG), and the Edinger-Westphal nucleus (EW) in the brainstem; the organum vasculosum laminae terminalis (VOLT), the lateral hypothalamic area (LH), and the hypothalamus (HYP) in the hypothalamus; the secondary motor area (M2), the prelimbic area (Prl), the ACC, and the gustatory areas (Gus) in the cortex; the striatum-like amygdala nuclei (Astr), the central amygdala nucleus (CeC), the caudoputamen (CP), and the anterior amygdala area (AA) in the striatum/pallidum systems; and the anterodorsal nucleus (AD) and the intermediodorsal nucleus (IMD) in the thalamus. In contrast, no significant changes were detected in the olfactory bulb and cortical subplate.

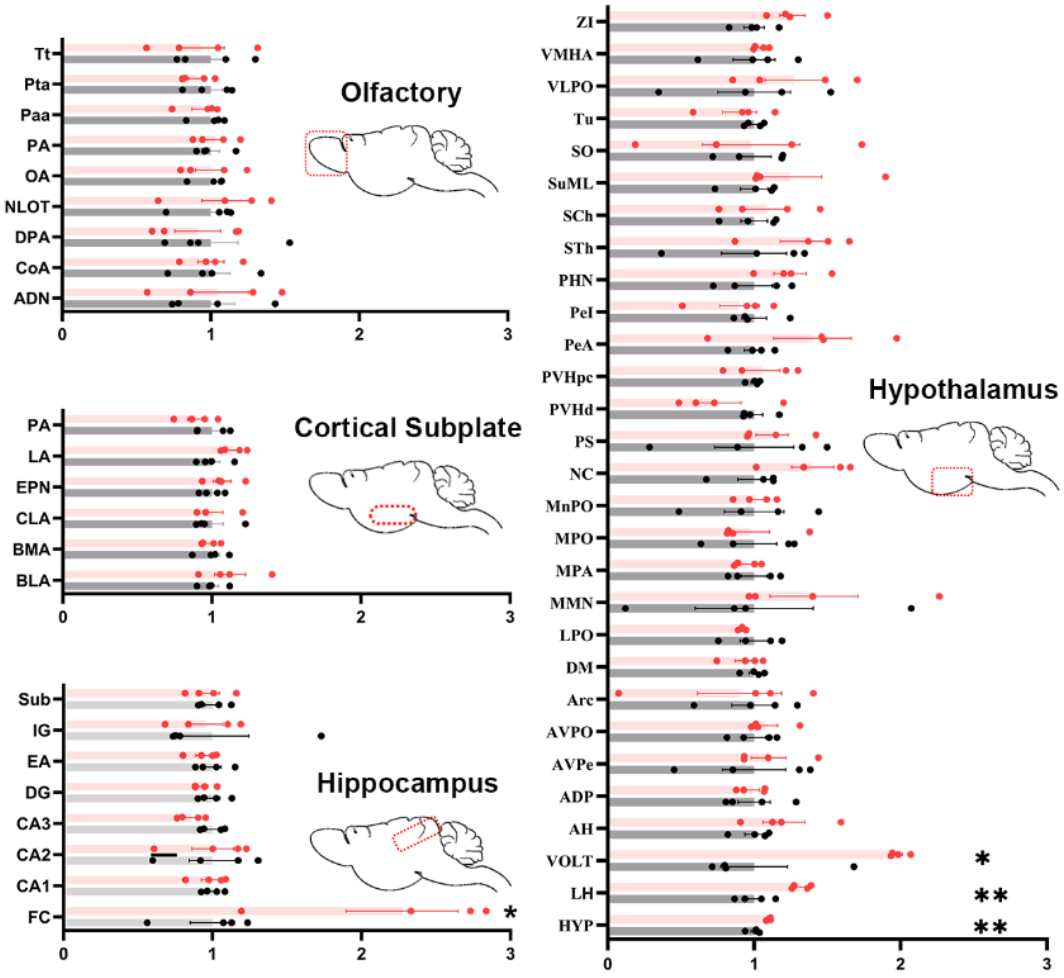


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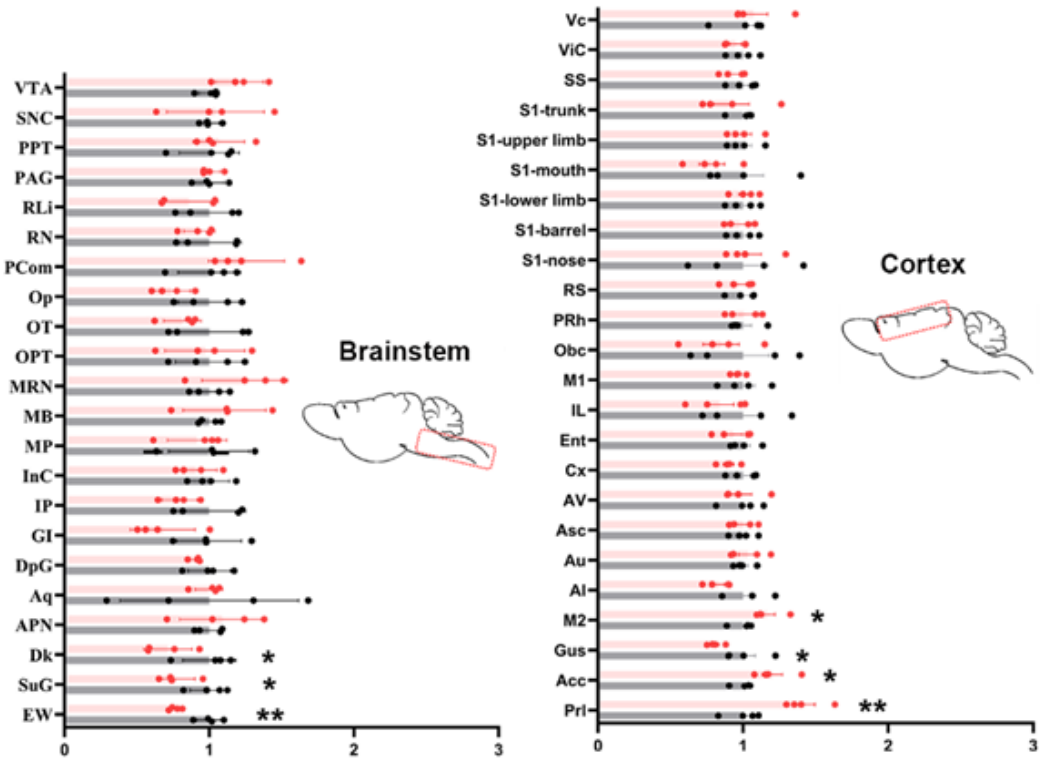
Figure 4.1 Whole-brain analysis of c-Fos expression following single-bout physical exercise.

Relative fluorescence intensity of c-Fos^{+} cells in different brain regions of sedentary and exercised mice (30 mins-post single-bout of treadmill running) (N = 4 mice/group). The black dot represents the sedentary group while the red dot represents the exercise one. *, $P < 0.05$; **, $P < 0.01$. Data are presented as mean ± SEM.



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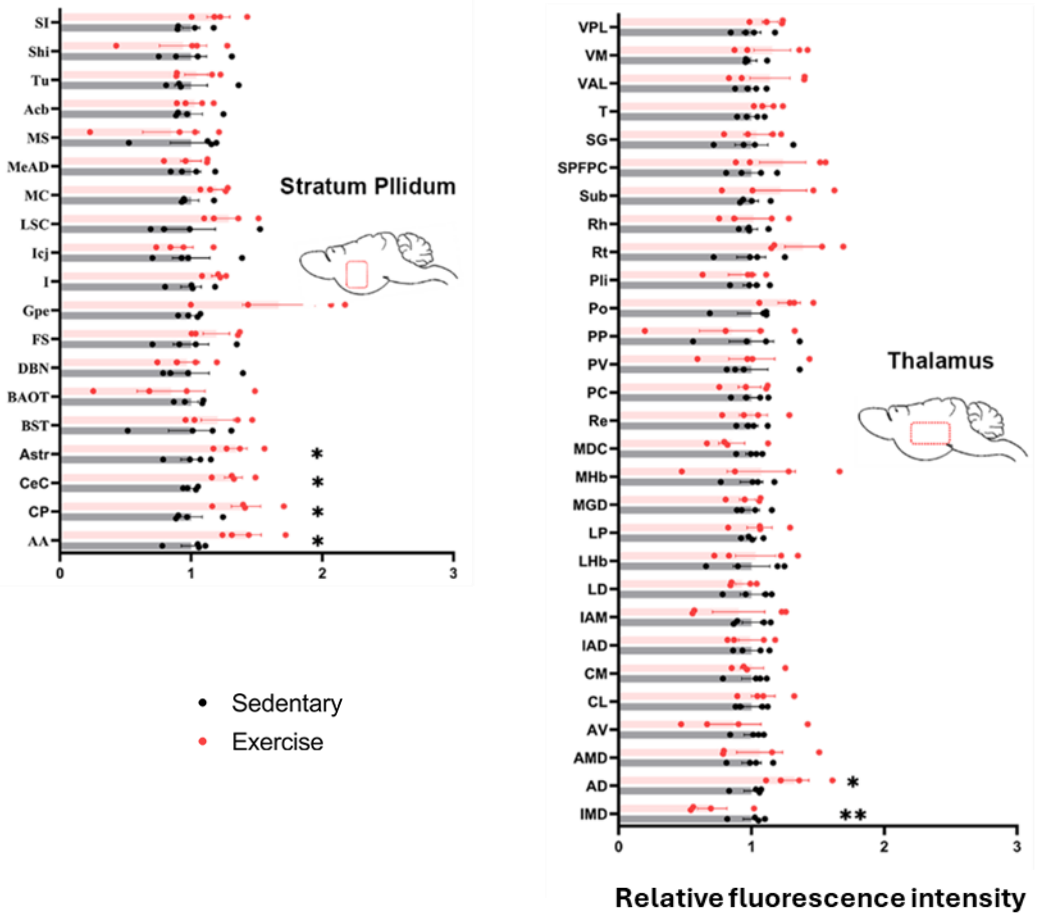
Relative fluorescence intensity of c-Fos^{+} cells in different brain regions of sedentary and exercised mice (30 mins-post single-bout of treadmill running) (N = 4 mice/group). The black dot represents the sedentary group while the red dot represents the exercise one. *, $P < 0.05$; **, $P < 0.01$. Data are presented as mean ± SEM.



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4.3.2 Single-bout physical exercise activates glutamatergic neurons in the ACC.

Results from the c-Fos brain mapping identified 17 brain regions with increased and decreased neural activity by single-bout physical exercise in mice (Fig 4.2 A-B). Notably, the ACC (a subregion of the mPFC) is implicated as a key brain region in the pathogenesis and antidepressant action involved in depression in both human [273, 274] and animal studies [275]. In addition, the activation effect of ACC was further confirmed by using manual counting of c-Fos^{+} cell numbers (Fig 4.2 C). Given the pivotal role of ACC in depression research across species and its marked and rapid alterations in c-fos expression after single-bout physical exercise, we hypothesized that the sensitization of the ACC neuronal response may serve a key role in mediating the antidepressant effect inflicted by single-bout physical exercise.

To determine which cell types contributed to the increased neuronal activity in the ACC of exercised mice, we performed double immunohistochemistry in brain slices of C57BL/6J mice in both sedentary and exercised groups. The percentage of activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)$^{+}$ neurons was significantly higher in the ACC of exercised mice (Fig 4.2 D). In addition, the percentage of activated GABA-interneurons decreased in the ACC in the exercise group compared to sedentary mice (Fig 4.3). These results indicated that the increased neural activation was mediated by activated glutamatergic neurons in the ACC of mice following single-bout physical exercise.

We further performed calcium imaging using the CAMKII-GCaMP6s virus to detect the activation state at several different time points in the ACC-glutamatergic neurons in response to single-bout physical exercise (Fig 4.2 E). Significant changes in calcium activity were observed in the ACC-glutamatergic neurons at 30 minutes post-exercise. Intriguingly, the neuronal activation by single-bout exercise persisted for up to 2 hours and returned to baseline 4 hours post-exercise (Fig 4.2 F). Notably, no significant changes in calcium activity were observed across these timepoints in sedentary mice (Fig 4.5). These findings indicated that the ACC-glutamatergic neurons underwent rapid and sustained activation in response to single-bout physical exercise intervention.

4.3.3. Chemogenetic inhibition of ACC-glutamatergic neurons diminishes the rapid antidepressant effects of single-bout physical exercise.

To investigate whether the activation of ACC-glutamatergic neurons is necessary for the rapid antidepressant response following exercise, a chemogenetic approach was adopted to modulate the activity of ACC-glutamatergic neurons in the exercised mice. Silencing ACC-glutamatergic neurons was achieved by CNO injection using the DIO-hM4Di-DREADD virus combined with CAMKII-Cre mice (Fig 4.2 G). Immunostaining of c-Fos confirmed the efficacy of the chemogenetic inhibition on neuronal activation in the ACC (Fig 4.4 A). Single-bout physical exercise significantly increased c-Fos expression at a 2-hour time point in the ACC-glutamatergic neurons. In contrast, this activation was blocked by pre-treatment with CNO (30 min before exercise intervention) (Fig 4.4 B-C), suggesting successful inhibition of ACC-glutamatergic neuronal activation in the exercised mice.

To further investigate behavioral outcomes in exercised mice with inhibited neural activation in the ACC glutamatergic neurons, mice were subjected to behavioral tests including the SST, TST, and FST (Fig 4.2 G). As expected, exercised mice with chemogenetic inhibition of ACC-glutamatergic neurons exhibited decreased grooming behavior in the SST and increased immobility time in TST and FST compared to their saline-treated counterparts (Fig 4.2 H). The above result showed that chemogenetic inhibition of the activation of ACC-glutamatergic neurons abolished the single-bout exercise-elicited rapid antidepressant effect, indicating the indispensable role of ACC-glutamatergic neurons in the rapid antidepressant response.

4.3.4 Chemogenetic activation of ACC-glutamatergic neurons produces rapid antidepressant effects.

To further confirm the critical role of ACC-glutamatergic neurons in rapid antidepressant action, a chemogenetic activation experiment was performed to test whether specifically activating ACC-glutamatergic neurons mimicked the rapid antidepressant effect elicited by single-bout physical exercise (Fig 4.2 I). The c-Fos immunofluorescence staining was performed to validate the efficiency of chemogenetic activation (Fig.4.4 D). CNO treatment yielded a higher number of c-Fos^{+} cells in mCherry-positive glutamatergic neurons (Fig 4.4 E-F), suggesting successful chemogenetic activation of ACC-glutamatergic neurons.

Then, the mice were subjected to behavioral tests including SST, TST, and FST to examine changes in depression-like behavior (Fig.4.2 I). Chemogenetic activation of ACC-glutamatergic neurons with CNO injection increased grooming behaviors in SST and reduced immobility time in TST and FST when compared to mice with saline injection (Fig.4.2 J). These results indicate that chemogenetic activation of the ACC-glutamatergic neurons mimicked the antidepressant effect of single-bout physical exercise, and these antidepressant effects were sustained for up to 48 hrs. Collectively, these findings indicated the critical role of activation of ACC-glutamatergic neurons in rapid antidepressant response in the exercised mice.

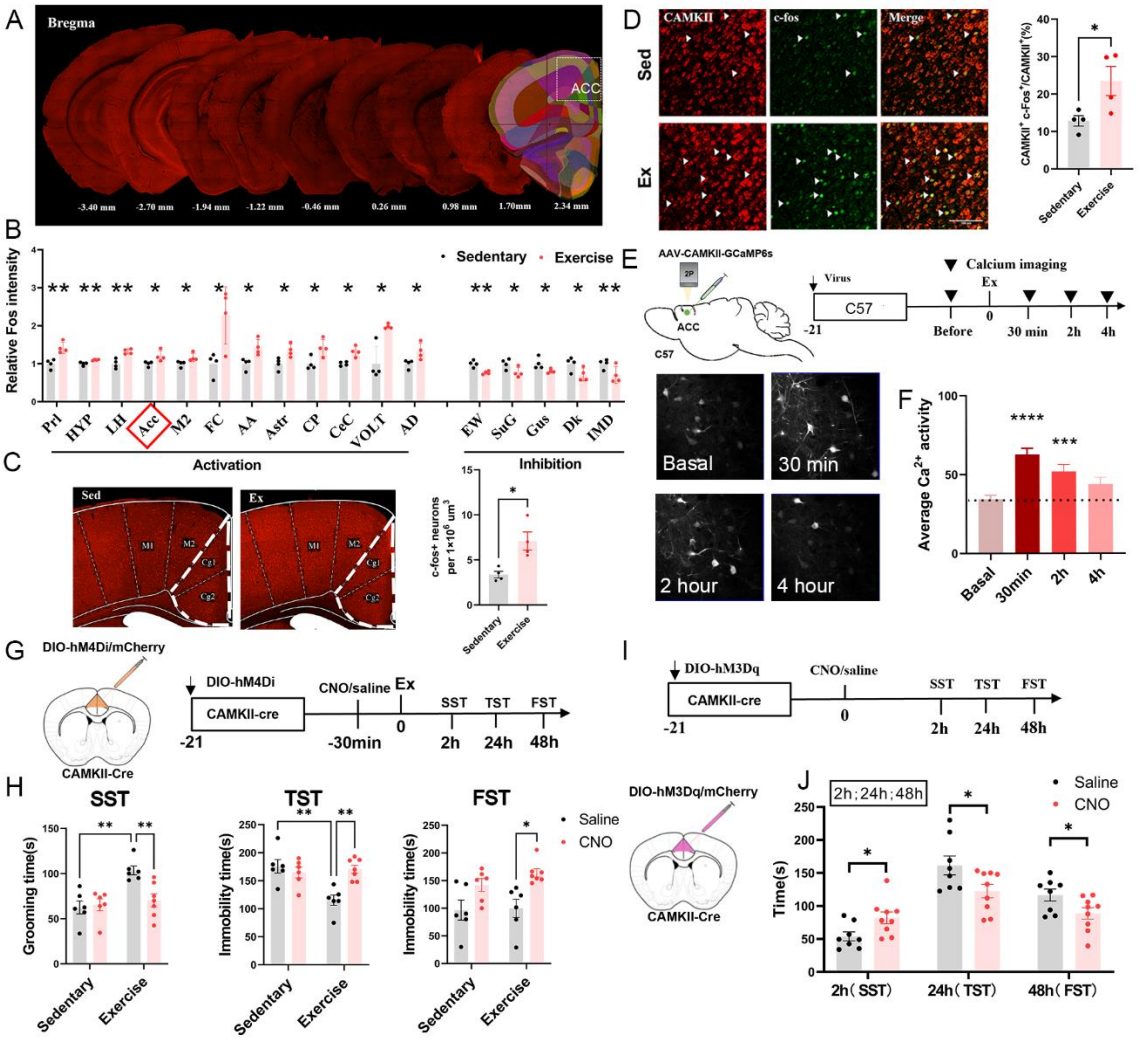

Figure 4.2 Single-bout physical exercise activates the ACC-glutamatergic neurons to elicit a rapid antidepressant effect.

(A) Representative images of c-Fos immunostaining result in nine coronal sections.

(B) Brain regions with significant differences in relative c-Fos fluorescence intensity between sedentary and exercise groups. N = 4 per group.

(C) Representative images of c-Fos immunostaining in ACC between sedentary and exercise groups. Quantification of c-Fos$^+$ cells in the ACC. Unpaired t-test. T (6) = 3.481, P = 0.0131.

(D) Representative images of the $CaMKII^+$ (red) and $c\text{-}Fos^+$ (green) cells between the sedentary and exercise groups in ACC. Scale bar, 50 μm. Quantification of the percentage of the co-labeling of the $CaMKII^+$ (red) & $c\text{-}Fos^+$ (green) cells in the $CaMKII^+$ population. Unpaired t-test. T (6) = 2.589, P = 0.0413.

(E) Experimental design for in vivo calcium imaging of ACC glutamatergic neurons.

(F) Average calcium activity of the pre-exercise and post-exercise at 30 min, 2h and 4 h. Kruskal-Wallis test statistic = 37.87, P < 0.0001.

(G) The experimental design for virus injection and behavioral tests for chemogenetic inhibition.

(H) Acute inhibition of the ACC-glutamatergic neurons before exercise abolished the antidepressant effect at 2-hour, 24-hour, and still elicited the inhibition effect at 48-hour time points. SST. Decreased grooming time in exercised mice injected with CNO. Main effect of exercise: F (1, 21) = 11.94, P = 0.0024; Main effect of Drug: F (1, 21) = 5.249, P = 0.0324; Interaction: F (1, 21) = 7.391, P = 0.0129. TST. Increased immobility time in exercised mice injected with CNO. Main effect of exercise: F (1, 21) = 8.275, P=0.009; Main effect of Drug: F (1, 21) = 5.474, P = 0.0293; Interaction: F (1, 21) = 12.18, P = 0.0022. FST. Increased immobility time in exercised mice injected with CNO. Main effect of exercise: F (1, 21) = 0.8456, P = 0.3682; Main effect of Drug: F (1, 21) = 16.05, P = 0.0006; interaction: F (1, 21) = 0.4563, P = 0.5067.

(I) The experimental design for virus injection and behavioral tests for chemogenetic activation.

(J) Acute activation of ACC-glutamatergic neurons elicited antidepressant effects at 2-hour, 24-hour, and 48-hour time points. SST. Increased grooming time after CNO injection. Unpaired t-test, T (15) = 2.443, P = 0.0274; TST. Decreased immobility time after CNO injection. Unpaired t-test, T (15) = 2.245, P = 0.0403. FST. Decreased immobility time after CNO injection. Unpaired t-test, T (15) = 2.230, P = 0.0414. All the data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001.

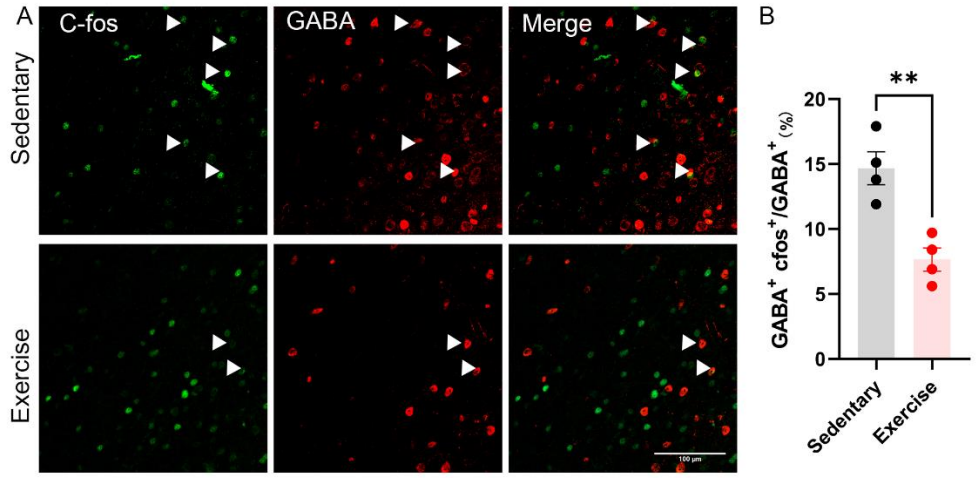

Figure 4.3 Single-bout physical exercise reduces the activity of ACC-GABAergic neurons.

(A) Representative images of the $GABA^+$ (red) and c-Fos^+ (green) cells of sedentary and exercised mice. Scale bar, 100 μm. (B) Quantification of $GABA^+$ (red)/c-Fos^+ (green) co-labeled neurons in the $GABA^+$ neurons (N = 4 mice/group). Unpaired t-test, T (6) = 4.552, P = 0.0039. **, P < 0.01. Data are presented as mean ± SEM.

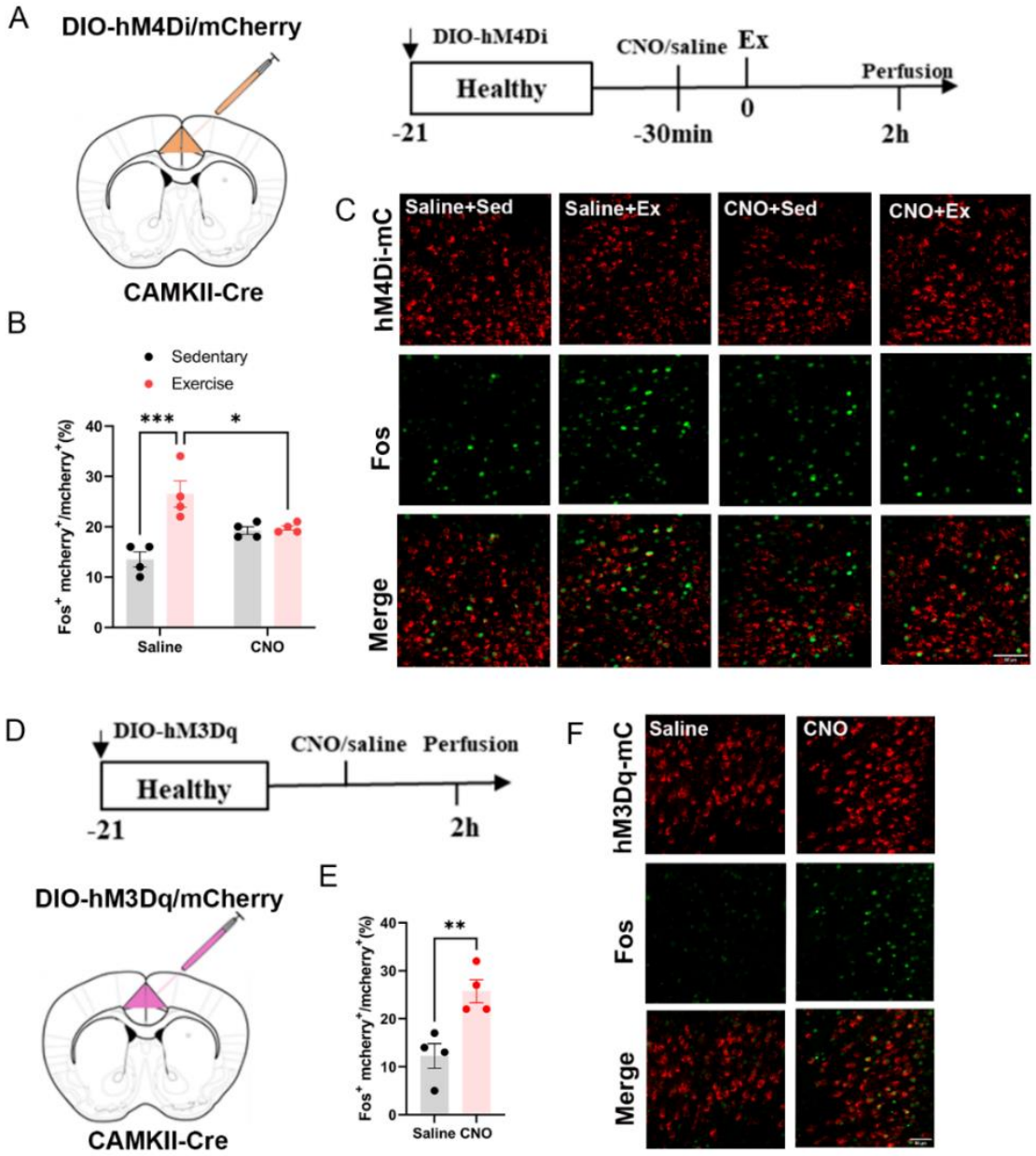

Figure 4.4 The validation experiments for chemogenetic regulation.

(A) The experimental design for hM4Di-mCherry validation in CAMKII-Cre mice.

(B) Quantification of the mCherry$^+$ (red)/c-Fos$^+$ (green) co-labeled neurons in the mCherry$^+$ neurons. Two-way ANOVA, Drug x Exercise interaction, F (1, 12) = 15.69, P = 0.0019; Exercise: F (1, 12) = 18.3, P = 0.0011; Drug: F (1, 12) = 0.1004, P = 0.7568.

(C) Immunostaining showing c-Fos and hM4Di-mCherry$^+$ cells. Scale bar, 50 μm.

(D) The experimental design for hM3Dq-mCherry validation in CAMKII-Cre mice.

(E) Quantification of the mCherry^{+} (red)/c-Fos^{+} (green) co-labeled neurons in the mCherry^{+} neurons. Unpaired t-test, CNO vs. Saline, P = 0.0085.

(F)Immunostaining showing c-Fos and hM3Dq-mCherry^{+} cells. Scale bar, 50 μm.

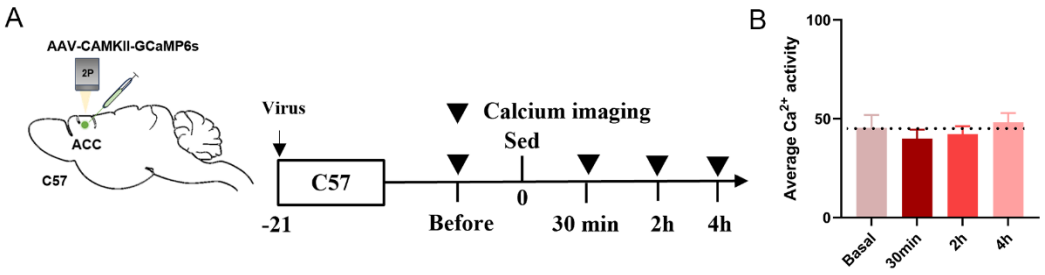

Figure 4.5. Calcium activity in sedentary mice.

(A) Experimental design for in vivo calcium imaging of ACC-glutamatergic neurons in sedentary mice.

(B) Average calcium activity of ACC-glutamatergic neurons in the sedentary mice detected pre-exercise and 30 min, 2 hours, and 4 hours post-exercise (n = 3 mice for recording). Friedman test followed by Dunn's test for multiple comparison.

4.4 Discussion

In this chapter, we elucidated the neural mechanism underlying single-bout physical exercise-induced rapid antidepressant effect and found the critical role of the activation of the ACC-glutamatergic neurons in the rapid antidepressant effect elicited by single-bout physical exercise. The comprehensive brain-wide c-Fos mapping revealed neural activation and inhibition in various brain regions in mice with single-bout physical exercise. The result suggested that the ACC subregion of the mPFC could be a pivotal subregion involved in single-bout exercise-induced rapid antidepressant effect (Fig 4.2A-C). ACC glutamatergic neurons were rapidly activated by single-bout exercise, with this effect

lasting at least 2 hours (Fig 4.2D-F). Together with behavioral data using chemogenetic manipulation of activation and inhibition of the ACC glutamatergic neurons, it is found that activation of ACC-glutamatergic neurons is essential for rapid antidepressant response following exercise（Fig 4.2G-J).

Immediate-early genes, as the “first wave” of genes that respond to changes in neural activity, play key roles in inducing protein synthesis, enhancing synaptic transmission, and remodeling neural architecture [276], are proposed to be key molecular mechanisms underlying the action of rapid antidepressant response. Understanding neuroplastic alterations indicated by the immediate early genes following single-bout physical exercise is crucial to identifying therapeutic targets for fast-acting antidepressant treatment and to informing the research field regarding neural mechanisms underlying rapid antidepressant response of acute physical exercise. The quantification of c-Fos^{+} cells in different brain regions revealed 17 brain regions with significant changes in neuronal activation or suppression in response to single-bout exercise. An increase in c-Fos expression was observed in the brainstem (Dk, SuG, EW), striatum/pallidum systems (Astr, CeC, CP, AA), and cortex (M2, Prl, Acc, Gus) (Fig 4.1). Such findings are consistent with previous research indicating that central motor control systems—including the brainstem, basal ganglia, and motor cortex—are integral to initiating and regulating motor activities [277].

Notably, single-bout physical exercise also markedly activated regions associated with emotional regulation within the limbic system, such as the prelimbic cortex and the anterior cingulate cortex in the medial prefrontal cortex, the fasciola cinerea in the hippocampus, the anterior amygdala area and the central amygdala nucleus in the amygdala, and the hypothalamus. These brain regions may serve as the neural substrate of the antidepressant response to exercise. The ACC was selected for further investigation mainly because it is a common brain region implicated in the neuropathology of depression, and antidepressant treatment response as shown in both human [273, 278] and animal studies [279]. This selection also aligns with our findings in Chapter 3, which demonstrate that single-bout physical exercise can produce rapid antidepressant effects in both humans and mice. In addition, the c-Fos expression of the ACC can also be significantly activated by antidepressant drugs such as ketamine and psilocybin, according to the published data of

whole-brain c-Fos mapping from antidepressant drugs [241]. These findings collectively provide strong evidence that the ACC is a key region in mediating the antidepressant effects of single-bout physical exercise.

At the cellular level, we observed the rapid activation of glutamatergic neurons in ACC after single-bout exercise and found that its activation lasted at least 2 hours (Fig 4.2D-F). This enhanced glutamatergic activity may likely reflect an adaptive shift in intracellular metabolic processes that support increased neurotransmission during physical exercise [265]. Given the association between reduced glutamatergic activity in the frontal cortex and depressive symptoms in both humans and animal models [280-282], the enhanced activity of the glutamatergic system could be linked to the rapid antidepressant effects of acute exercise. Our data demonstrated that inhibiting the ACC glutamatergic neurons abolished acute exercise-induced antidepressant effect (Fig. 4G-H), suggesting the necessary role of activating glutamatergic signaling in the ACC for physical exercise to elicit rapid antidepressant action. Notably, the cellular mechanisms underlying the rapid onset of antidepressant action of ketamine involve the activation of glutamatergic neurons in the mPFC too [264], suggesting that increasing the activity of the glutamatergic neurons could be the common cellular target for initiating rapid antidepressant effects. In addition, the chemogenetic activation of the ACC-glutamatergic could partially mimic exercise to elicit the antidepressant effect and persist up to 48 hours post-CNO injection while the antidepressant effect of single-bout physical exercise only lasted up to 24 hours (Fig. 4I-J). This discrepancy may be attributed to the extended half-life of CNO that can activate or inhibit target neurons within 9-12 hours [283], suggesting the potential limitations of chemogenetic manipulation in replicating actual exercise effects. However, this data emphasized that activating the ACC-glutamatergic neurons could be the target to sustain the antidepressant effects. Future research will focus on investigating the brain circuit involved with the ACC glutamatergic neurons following physical exercise.

In summary, we elucidated the neural mechanism underlying single-bout physical exercise-induced rapid antidepressant effect from the brain region and cellular levels. Our study identifies the pivotal role of the activation of ACC-glutamatergic neurons involved in the rapid antidepressant effects of physical exercise. The next chapter examined the

“exerkine” that mediates the action of a single bout physical exercise on inducing activity of the ACC-glutamatergic neurons.

Chapter 5: Activation of the Adiponectin-AdipoR1 signaling in the ACC-glutamatergic neurons mediate single-bout physical exercise-induced rapid antidepressant effects.

5.1 Introduction

The therapeutic benefits of physical exercise in treating major depressive disorders are increasingly recognized [7]. The antidepressant effects of physical exercise are attributed to its role in enhancing neuroplasticity [12], as evidenced by the promotion of neurogenesis [13], synaptogenesis [14], and functional synaptic plasticity [15]. The findings from Chapter 4 indicated that single-bout physical exercise significantly increased the activation of the ACC-glutamatergic neurons, and its activation was essential for the rapid antidepressant effect of acute exercise. However, it is unclear how physical exercise elicits its effect on activating ACC-glutamatergic neurons.

A plethora of factors, known as “exerkines”, which include brain-derived neurotrophic factors and those secreted from peripheral organs during exercise, contribute to exercise-induced neuroplasticity [16]. Yau et al. have provided an overview of the latest clinical and preclinical research on various exerkines released from different organs in response to exercise training [118]. Adiponectin, an adipocyte-secreted protein hormone, can mimic many metabolic effects of physical exercise. Adiponectin exists in the circulation in different isoforms, its trimeric and low-molecular-weight forms can cross the blood-brain barrier and access the central nervous system [13]. Adiponectin exerts its biological effects via two receptors, AdipoR1 and AdipoR2, which are mainly located in various brain regions involved in emotional regulation, including the medial prefrontal cortex [17], the hippocampus [18], and the ventral tegmental area [19] and the dorsal raphe nucleus [20].1/1

Clinical and preclinical studies have established a close link between adiponectin signaling and mood regulation [22]. Adiponectin signaling is not only a key indicator in the onset and progression of depression, but its regulation also holds potential antidepressant value [22]. Central overexpressing adiponectin by intracerebroventricular infusion of recombinant adiponectin elicited rapid antidepressant responses within hours

[17, 24]. In addition, Yau et al. have shown that adiponectin is a crucial mediator of the antidepressant effects of chronic physical exercise via promoting hippocampal neurogenesis [13, 25]. Recent clinical research has demonstrated that single-bout physical exercise can significantly elevate adiponectin levels in the blood of healthy participants [26]. Based on all these findings, we hypothesized that adiponectin may serve as a potential mediator for single-bout physical exercise elicited antidepressant effect.

In this chapter, we aimed to investigate: 1) whether single-bout physical exercise elicited rapid antidepressant effects via adiponectin signaling; and 2) whether the adiponectin signaling participates in activating the ACC-glutamatergic neurons following single-bout physical exercise. Focusing on the peripheral-central crosstalk, we want to know whether single-bout physical exercise can increase adiponectin levels to modulate the activity of the ACC-glutamatergic neurons and consequently elicit rapid antidepressant effects.

5.2 Materials and methods

5.2.1 Animals

C57BL/6J male mice (6-8 weeks old) were provided and kept in the Centralized Animal Facility of the Hong Kong Polytechnic University. Adiponectin knockout (KO) breeder mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and kept in the Hong Kong Polytechnic University Shenzhen Research Institute. Unless otherwise specified, the housing conditions follow the same described in Chapter 3. The Animal Subjects Ethics approved all the procedures, and the student was licensed to conduct experiments.

5.2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for measuring adiponectin protein expression

The blood was obtained from the hearts of the mice and centrifuged to get serum. The adiponectin levels in serum were determined using commercially available ELISA kits (ImmunoDiagnostics Limited, Cat. No.32010). The serum samples were diluted 600 folds when performing ELISA. The proteins were extracted from the isolated mPFC and hippocampal tissues. The levels of adiponectin in the brain were measured using

commercially available adiponectin ELISA kits for brain tissues (AdipoGen Life Sciences, AG-45A-0004Y).

5.2.3 Behavioral tests

The behavioral assessments including SST, TST, and FST followed the same procedures as described in Chapter 3.

5.2.4 Immunofluorescence staining

The immunofluorescence staining experiment followed the same procedures as described in Chapter 4.

5.2.5 Generation of AAV transgenes/Stereotaxic injection of viral vectors

The AAV injection was carried out following the same procedures as described in Chapter 4. The used viruses are the following: AAV2/9-CMV-DIO-RFP- mir30shRNA (AdipoR1) (WZ Biosciences Ince), AAV2- CaMKIIa-Cre (Addgene plasmid #105558). Detailed information on the used viruses can be found in Table 2.

5.2.6 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from brain cortical tissues of different time points after exercise using ReliaPrep™ RNA Tissue Miniprep System (Promega) and reversely transcribed into cDNA with GoScript™ Reverse Transcriptase Kit (Promega). Using the CFX96 Real-Time PCR Detection System (Bio-Rad) and the GoTaq® qPCR Master Mix (Promega), real-time PCR was carried out. The primer sequences used were listed (Table 3). The analysis of the relative mRNA expression was calculated by using a delta-CT ($\Delta\Delta$Ct) relative quantification model and normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

5.2.7 Statistical analysis.

Graph figures and data analysis were done using GraphPad Prism 9 (GraphPad Software, USA). The normality of the data distribution was assessed using the Shapiro–Wilk test for sample sizes less than 8 ($n < 8$) or the D'Agostino and Pearson test for sample sizes of 8 or more ($n \geqslant 8$) [101].

For data involving four groups with two independent variables, a two-way ANOVA followed by Tukey's multiple comparisons post hoc test was used. For data involving three groups with one independent variable, one-way ANOVA followed by Tukey's multiple comparisons post hoc test was used. Data are presented as mean ± SEM. Statistical significance was indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

5.3 Results

5.3.1 Increased adiponectin levels in the medial prefrontal cortex correlate with the rapid antidepressant effect of single-bout physical exercise.

After identifying the specific brain and cellular target (ACC-glutamatergic neurons) mediating the antidepressant effect elicited by single-bout physical exercise, we further investigate how physical exercise elicits its antidepressant effect by activating ACC-glutamatergic neuronal activity. Most of our current knowledge specifies that central overexpressing adiponectin by intracerebroventricular infusion of recombinant adiponectin elicited rapid antidepressant responses within hours [17], and the adiponectin signaling in the hippocampus mediated the antidepressant effect elicited by chronic physical exercise [13]. We thus detect whether the adiponectin signaling mediated the rapid antidepressant effect elicited by single-bout physical exercise. Firstly, we measured mice adiponectin levels at a rapid time (30 minutes) following exercise in both serum and brain tissues (Fig 5.1 A). Our results indicated that exercise did not alter serum adiponectin levels in control mice, but elevated adiponectin levels in stressed mice (Fig 5.1 B). Additionally, single-bout exercise increased mPFC adiponectin levels in both no-stress and stressed mice (Fig 5.1 C). Interestingly, a significant negative correlation was found between adiponectin concentrations in the mPFC and immobility times in FST (Fig 5.1 D), suggesting the potential role of adiponectin in rapid changes in antidepressant behaviors following single-bout exercise.

Given the established link between adiponectin levels in the hippocampus and antidepressant effects [13], we also examined the hippocampal adiponectin levels in these mice. Single-bout physical exercise increased adiponectin levels in the hippocampus of the no-stress mice but not in stressed mice (Fig 5.1 E). Furthermore, there was no significant correlation between hippocampal adiponectin levels and immobility times in the FST (Fig 5.1 F). These findings suggest that the rapid antidepressant effect of single-bout physical exercise could be mediated by increased adiponectin levels in the mPFC rather than the hippocampus.

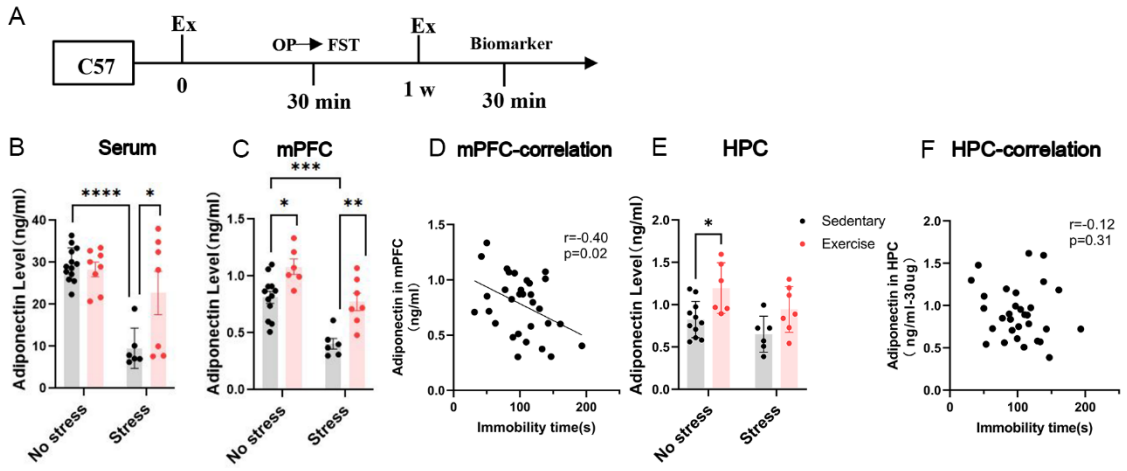

Figure 5.1 Increased adiponectin levels in the medial prefrontal cortex are negatively correlated with a decrease in depressive behavior following single-bout physical exercise.

(A) Experimental timeline. Mice were subjected to a rapid time-point (30 minutes) behavioral test. Brain tissue and blood samples were collected 30 minutes post-exercise.

(B) Exercise increased serum adiponectin levels in stressed mice. Main effect of exercise: $F(1, 30) = 5.335$, $P = 0.028$; Main effect of stress: $F(1, 30) = 24.12$, $P < 0.0001$; Interaction: $F(1, 30) = 7.566$, $P = 0.01$.

(C) An increase in adiponectin level in the mPFC 30 min post-exercise in both the non-stressed and stressed groups. Main effect of exercise: $F(1, 27) = 23.80$, $P < 0.0001$; Main effect of stress: $F(1, 27) = 30.31$, $P < 0.0001$; Interaction: $F(1, 27) = 0.5787$, $P = 0.4534$.

(D) Levels of adiponectin in the mPFC were negatively correlated with immobility time in FST. $r = -0.4091$; $P = 0.0223$.

(E) Single-bout exercise increased adiponectin levels in the hippocampus in the non-stressed mice. Main effect of exercise: $F(1, 27) = 13.90$, $P = 0.0009$; Main effect of stress: $F(1, 27) = 4.948$, $P = 0.0347$; Interaction: $F(1, 27) = 0.2746$, $P = 0.6046$.

(F) There was no correlation between hippocampal adiponectin levels and immobility time in the FST. $r = -0.1999$; $P = 0.2808$. All data represented as mean ± SEM; *$P < 0.05$.

5.3.2 Adiponectin-AdipoR1 signaling is involved in ACC-glutamatergic neuronal activation and the antidepressant effects elicited by single-bout physical exercise.

Prior studies have indicated that mPFC has higher protein expression of AdipoR1 over AdipoR2 [17]. To determine whether AdipoR1 is expressed in the ACC-glutamatergic neurons, we conducted immunostaining to co-localize AdipoR1^{+} and the glutamatergic neuron marker CAMKII^{+} cells. The result revealed significant co-localization (Fig 5.2A), establishing a neuroanatomical foundation for adiponectin's direct influence on glutamatergic neurons. Since the activation of the ACC-glutamatergic neurons is essential for the rapid antidepressant effect of single-bout physical exercise, we further test whether interfering adiponectin-AdipoR1 signaling could affect the neural activation in the ACC following exercise. We first examined using adiponectin KO mice to see whether the absence of adiponectin reduces activation of the ACC-glutamatergic neurons 2 hours following exercise (Fig 5.2 B). The c-Fos analysis of ACC-glutamatergic neuron activation showed that adiponectin deficiency significantly diminished neuronal activity 2-hour after exercise (Fig 5.2 C-D). Additionally, we specifically knock down AdipoR1 in ACC-glutamatergic neurons (**Fig 5.2 E**) and confirmed the efficacy of the viral knockdown using qPCR (**Fig 5.3**). The specifical AdipoR1 knockdown in ACC-glutamatergic neurons also significantly diminished neuronal activation 2-hour post-exercise (Fig 5.2 F-G). These data suggest that the presence of both adiponectin and its receptor AdipoR1 are essential for the activation of the ACC-glutamatergic neurons by single-bout exercise.

Lastly, we investigated whether knockdown of the AdipoR1 in ACC-glutamatergic neurons affects the antidepressant response elicited by single-bout physical exercise (Fig 5.2 E). We found that the knockdown effectively abolished the exercise-induced increase in grooming time in the SST and decreased immobility time in the TST (Fig 5.2 H), indicating that the disruption of the adiponectin-AdipoR1 signaling effectively abolished the antidepressant response triggered by a single bout of exercise. These findings highlight adiponectin-AdipoR1 signaling as a key modulator of ACC-glutamatergic neuron activation, thereby influencing the antidepressant effects elicited by physical exercise.

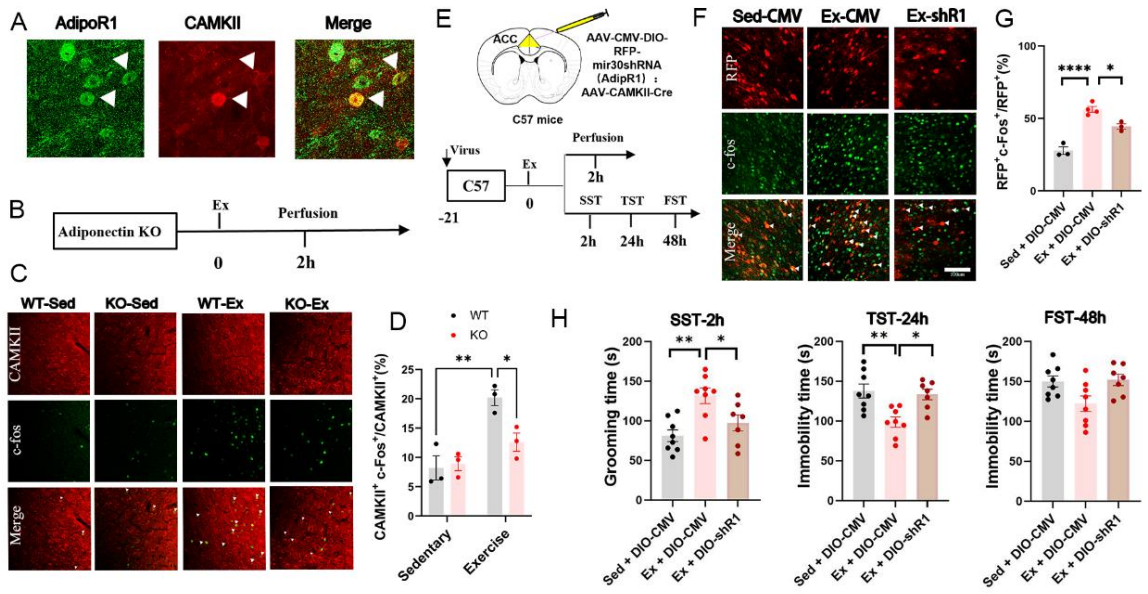

Figure 5.2 Adiponectin-AdipoR1 signaling is involved in ACC-glutamatergic neuron activation and the antidepressant effects elicited by single-bout physical exercise.

(A) Representative co-labelling images of the AdipoR1$^+$ (green) and CAMKII$^+$ (red) cells.

(B) Experimental timeline.

(C&D) Representative images of the CaMKII$^+$ (red) and c-Fos$^+$ (green) cells in Sed + WT, Sed + KO, Ex + WT, Ex + KO groups (N = 3 mice/group). Quantification of the percentage of CaMKII$^+$ (red)/c-Fos$^+$ (green) cells in the CaMKII$^+$ population. Main effect of exercise: F (1, 8) = 24.64, P = 0.0011; main effect of genotype: F (1, 8) = 4.734, P = 0.0613; Interaction: F (1, 8) = 6.972, P = 0.0297.

(E) The experimental design for virus injection and timeline.

(F&G) Representative images of the RFP$^+$ (red) and c-Fos$^+$ (green) cells in Sed + DIO-CMV, Ex + DIO-CMV, and Ex + DIO-shRNA-AdipoR1 groups (N = 3-4 mice/group). Quantification of the percentage of RFP$^+$ (red) /c-Fos$^+$ (green) cells in the RFP$^+$population. One-way ANOVA with Tukey's multiple comparisons test, F (2, 7) = 57, P <0.0001.

(H) Knockdown of the AdipoR1 in ACC-glutamatergic neurons abolished exercise-elicited

antidepressant effects. SST. One-way ANOVA with Tukey's post hoc analysis, $F(2, 20) = 8.2$, $P = 0.0025$; TST. One-way ANOVA with Tukey's multiple comparisons test, $F(2, 20) = 8.6$, $P = 0.002$; FST. One-way ANOVA with Tukey's multiple comparisons test, $F(2, 20) = 4.1$, $P = 0.0321$. All data represented as mean ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

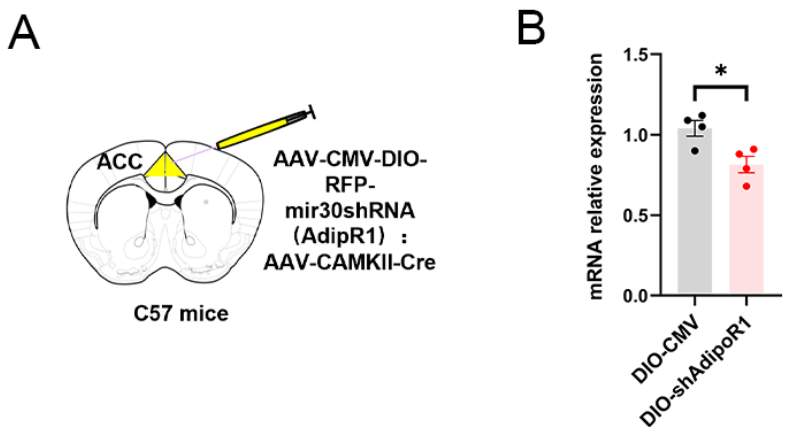

Figure. 5.3 Verify the virus efficacy using qPCR.

(A) Experimental design for the knockdown AdipoR1 virus in ACC-glutamatergic neurons.

(B) RT-qPCR showed a significant reduction of mRNA AdipoR1 expression in the knockdown group (n=4 mice per group). Unpaired t-test.

Data were presented as mean ± SEM. *P < 0.05.

5.4 Discussion

In this chapter, we found that single-bout physical exercise can increase adiponectin levels to modulate the activity of the ACC-glutamatergic neurons and consequently elicit rapid antidepressant effects. An increase in adiponectin levels in mPFC was associated with the rapid antidepressant response in the exercised mice. In addition, we discovered that knockout adiponectin or knockdown AdipoR1 signaling impaired the activation of the ACC-glutamatergic neurons following single-bout physical exercise. These findings offer insights into the antidepressant effects of physical exercise, highlighting the role of the peripheral-central crosstalk mediated by adiponectin. This involves binding to AdipoR1 receptors on ACC-glutamatergic neurons, affecting neural activation and behavior changes.

Chronic voluntary exercise elevated serum adiponectin levels in mice subjected to chronic stress [33], but did not alter its expression levels in healthy mice [13]. Notably, we observed a similar change trend in serum adiponectin following single-bout physical exercise: increased in stressed groups but no changes in no-stress mice (Fig. 5.1 B). These data suggest that acute/chronic physical exercise-induced significant increase in serum

adiponectin may specifically exist in the depression disease model. Both clinical and preclinical studies have established a positive correlation between the upregulation of adiponectin levels and antidepressant effects. Low serum adiponectin levels have been observed in depressed patients [284], which can be elevated after antidepressant treatment [23]. Adiponectin haploinsufficient mice have a greater susceptibility to stress-induced depressive phenotype, but increasing central adiponectin levels by infusing low molecular weight recombinant adiponectin directly into the ventricle [17] or by overexpressing adiponectin by tail vein injection with adeno-associated virus [13] can induce an acute antidepressant response. Our correlation results consistent with previous findings, showed that acute elevation of the adiponectin level in the mPFC after exercise negatively correlated with immobility time in the forced swimming tests (Fig. 5.1 D). Notably, the significant correlation only existed in the mPFC but not in the hippocampus (Fig. 5.1 F), which suggested that the neural basis for the rapid antidepressant effects of single-bout physical exercise mediated by adiponectin is initiated in the prefrontal cortex region.

The effects of adiponectin on synaptic plasticity have been widely reported [22]. Of note, adiponectin and its receptor activation have a bidirectional role in regulating neuronal activities, either excitatory [185, 186] or inhibitory effect [18, 19, 285]. Results vary according to the recording neuron type across different brain regions and the dosage of adiponectin applied. Our results reported that mice lacking adiponectin or with specific knockdown of AdipoR1 in the glutamatergic neurons in the ACC showed diminished glutamatergic neuronal activation after exercise (Fig 5.2 D &G), which suggested that the adiponectin-AdipoR1 signaling elicited by exercise is essential for activating ACC-glutamatergic neurons. Our finding is consistent with one in vitro study that adiponectin increased the excitability of neurons in acute coronal brain slices in layer V/VI neurons of ACC [185]. Another study revealed that adiponectin induces neuronal hyperexcitability by stimulating the Cav3.2 channel through its interaction with adipoR1 [186]. This offers a mechanistic explanation for the enhancement of neuronal activity by adiponectin. In addition, our behavioral results revealed that mice with AdipoR1 knockdown in the ACC glutamatergic neurons did not show rapid antidepressant-like behaviors following exercise (Fig 5.2 H), indicating that the adiponectin-AdipoR1 signaling in ACC is critical for the

antidepressant response. Consistently, other studies also reported that adiponectin-AdipoR signaling in different brain regions can regulate the intrinsic excitability of neurons to regulate emotional behaviors such as fear extinction or anxiety-like behavior [18, 19]. Together, these studies highlight the broad role of adiponectin/AdipoR signaling in modulating emotional-related responses by affecting the neuronal activation state. Further research should elucidate the detailed mechanisms by which exercise-induced elevation of adiponectin signaling regulates the activation of glutamatergic neurons, which involves understanding the functional plasticity mechanisms of adiponectin signaling during neuronal activation following exercise.

Our research results demonstrated a significant role of adiponectin/AdipoR1 signaling in activating ACC-glutamatergic neurons and antidepressant effect by single-bout exercise. Adiponectin levels can be increased by single-bout physical exercise in the mPFC, which then interacts with AdipoR1 in the ACC, leading to the activation of the glutamatergic neurons. This process is required for rapid antidepressant response elicited by single-bout exercise. In the next chapter, we will explore the detailed mechanism involved with the downstream signaling of adiponectin-AdipoR1 mediates the antidepressant effects induced by single-bout physical exercise.

Chapter 6: Adaptor protein APPL1 in ACC-glutamatergic neurons is essential for the rapid antidepressant effects of single-bout physical exercise

6.1 Introduction

Adiponectin is an adipocyte-secreted hormone with anti-depressant effects [13, 286] and an exercise mimetic hormone in improving hippocampal functions [25]. Adiponectin binds to its receptors AdipoR1 and AdipoR2 to exert its physiological functions [198]. APPL1 is the first identified adaptor protein to interact with AdipoR directly [27], facilitating the transduction of the adiponectin signaling by interacting with multiple downstream targets, including AMPK [28], PPARα [29], and the PI3K [30]. Physical exercise induces antidepressant effects by activating the adiponectin/AdipoR1 signaling pathway in the hippocampus of both mice without stress [13] and with chronic unpredictable stress [33]. However, the adaptor protein APPL1's contribution to the adiponectin-AdipoR signaling-mediated antidepressant effects of physical exercise remains fully elucidated.

APPL1 not only orchestrates adiponectin downstream signaling transduction events in the cytoplasm [198] but also can translocate to the nuclear in response to adiponectin stimulus to participate in gene transcription [31, 32]. Our findings in Chapter 5 showed that adiponectin-AdipoR1 signaling in activating the ACC-glutamatergic neurons mediates single-bout physical exercise-induced rapid antidepressant effects. Notably, APPL1 in the neurons serves as a mediator linking neuronal activity to gene transcription through translocation from the synapse to the nucleus [206]. Given the dual role of APPL1 nuclear translocation in regulating adiponectin signaling and connecting neural activity, we can reasonably speculate that adiponectin binds to AdipoR1 to enhance neuronal activation, further triggering APPL1 nuclear translocation and mediating the antidepressant effect of single-bout physical exercise.

This chapter aims to investigate 1) whether APPL1 is involved in the rapid antidepressant effect elicited by single-bout physical exercise, and 2) whether APPL1 in ACC-glutamatergic neurons translocates into nuclear in response to adiponectin-AdipoR1

signaling enhanced by single-bout physical exercise. Focusing on these two research aims, we hope to elucidate the cellular mechanisms underlying the antidepressant impact of single-bout physical exercise, particularly through the role of adaptor protein APPL1 within the ACC glutamatergic neurons in triggering downstream adiponectin-AdipoR1 signaling.

6.2 Materials and methods.

6.2.1 Animals

C57BL/6J male mice (6-8 weeks old) were provided and kept in the Centralized Animal Facility of the Hong Kong Polytechnic University. APPL1 KO breeding pairs were given as gifts from Dr. Kenneth Cheung's group at PolyU and were kept in the CAF. Adiponectin KO mice (6-8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and kept in the Hong Kong Polytechnic University Shenzhen Research Institute. Unless otherwise specified, the housing conditions follow the same described in Chapter 3. The Animal Subjects Ethics Committee approved all the procedures, and the student was licensed to conduct experiments.

6.2.2 Behavioral tests

The behavioral assessment was carried out following the same procedures as described in Chapter 3, including the SST, TST, and FST protocols.

6.2.3 Generation of AAV transgenes/Stereotaxic injection of viral vectors

The AAV injection followed the same procedures described in Chapter 4. The used viruses are the following: AAV2/9-CMV-DIO-RFP- mir30shRNA (AdipoR1) (WZ Biosciences Ince), AAV2- CaMKIIa -Cre (Addgene plasmid #105558). AAV9-CMV-DIO-RFP-mir30shRNA (APPL1) (WZ Biosciences Ince), AAV9-CMV-DIO-RFP-mir30shRNA (Control) (WZ Biosciences Ince), AAV9-CaMKIIα-APPL1/mcherry (WZ Biosciences Ince). Detailed information on the APPL1 knockdown/overexpression virus and AdipoR1 knockdown virus can be found in Table 2.

6.2.4 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR followed the same procedures described in Chapter 5.

6.2.5 Analysis of APPL1 nuclear translocalization by immunofluorescence staining

Immunostaining procedures were the same as described in Chapter 4, brain slices were incubated overnight with antibodies against APPL1, and CAMKII at 4 °C, followed by a 2-hour incubation with secondary antibodies (see Table 1 for details) at room temperature. In the final step, sections were coverslipped with fluorescent mounting medium with DAPI to label the nuclear (Fluoroshield™ with DAPI, Sigma). Images were captured using the Zeiss confocal microscope (magnification: ×60).

To characterize the nuclear translocalization of APPL1 in the neuron, the neurons with double-labeled CAMKII and DAPI staining were selected for analysis. The ROI function of ImageJ was utilized to outline the cell contour (Alexa 568 for CAMKII) and the nuclear using DAPI staining. The cell and nuclear contour were applied to the APPL1 images (Alexa 488 for APPL1) simultaneously to get the distribution area of APPL1 in the glutamatergic neurons. All analyses were performed by two well-trained researchers in a sample-blinded manner.

6.2.6 Statistical analysis.

Graph figures and data analysis were performed using GraphPad Prism 9 (GraphPad Software, USA). The normal distribution of data was analyzed using the Shapiro–Wilk test for sample sizes less than 8 ($n < 8$) or the D'Agostino and Pearson test for sample sizes of 8 or more ($n \geqslant 8$) [101].

For comparisons between the two groups, an unpaired t-test was conducted once normality was confirmed. For comparisons involving more than two groups with a single variable, one-way ANOVA was employed, followed by Dunn's multiple comparison test when comparing with the baseline, or Tukey's multiple comparison test when comparing between groups. For comparisons involving four groups with two independent variables, two-way ANOVA followed by Tukey's multiple comparisons post hoc test was used.

All data analyses were performed by two lab members who were blinded to the group assignments. Data are presented as mean ± SEM. Statistical significance was indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.001$.

6.3 Results

6.3.1 Adaptor protein APPL1 in ACC-glutamatergic neurons is essential for the rapid antidepressant effects of single-bout physical exercise.

The adaptor protein APPL1 interacts with adiponectin receptors to initiate its downstream signaling cascades [27]. The previous chapter has confirmed the critical role of adiponectin-AdipoR1 signaling in the rapid antidepressant effects of single-bout physical exercise, we next examined the involvement of the downstream APPL1. We first used the global APPL1 knockout (KO) mice for a series of behavioral tests (Fig 6.1 A-B). APPL1 KO did not affect grooming time and immobility time in the sedentary state when compared to the wild-type mice, indicating the absence of APPL1 did not affect the basal depression-like behaviors (Fig 6.1 C). However, APPL1 KO mice with exercise showed lower grooming time in SST and higher immobility time in TST compared with the exercised WT mice (Fig 6.1 C), which showed that APPL1 loss abolished the exercise-elicited antidepressant behaviors. These findings provided preliminary evidence that APPL1 is involved in single-bout exercise-mediated behavioral changes.

Considering the activation of the ACC-glutamatergic neurons by the adiponectin-AdipoR1 signaling is required for the rapid antidepressant effects of exercise, we further investigate the critical role of downstream adaptor protein APPL1 presence in the ACC-glutamatergic neurons. On the one hand, we generated brain region-specific APPL1 knockdown conditions in ACC-glutamatergic neurons by using virus intervention in WT mice (Fig 6.1 D) and verified the virus knockdown efficiency (Fig 6.1 E). The behavioral tests showed that specific knockout APPL1 in the ACC-glutamatergic neurons does not affect basal depressive-like behaviors in the sedentary state but abolished the antidepressant effect of exercise (Fig 6.1 F), which is consistent with the global knockdown result. On the other hand, we also generated brain region-specific APPL1 overexpression conditions in ACC- glutamatergic neurons by using virus intervention in KO mice (Fig 6.1 G) and verified the virus overexpression efficiency (Fig 6.1 H). Although APPL1 loss abolished exercise elicits antidepressant effects, re-expressing APPL1 in ACC-glutamatergic neurons of APPL1 KO mice increased the grooming in SST and decreased the immobility time in TST (Fig 6.1 I). The above results suggested the indispensable role

of APPL1 in ACC-glutamatergic neurons in mediating single-bout physical exercise-elicited antidepressant effects.

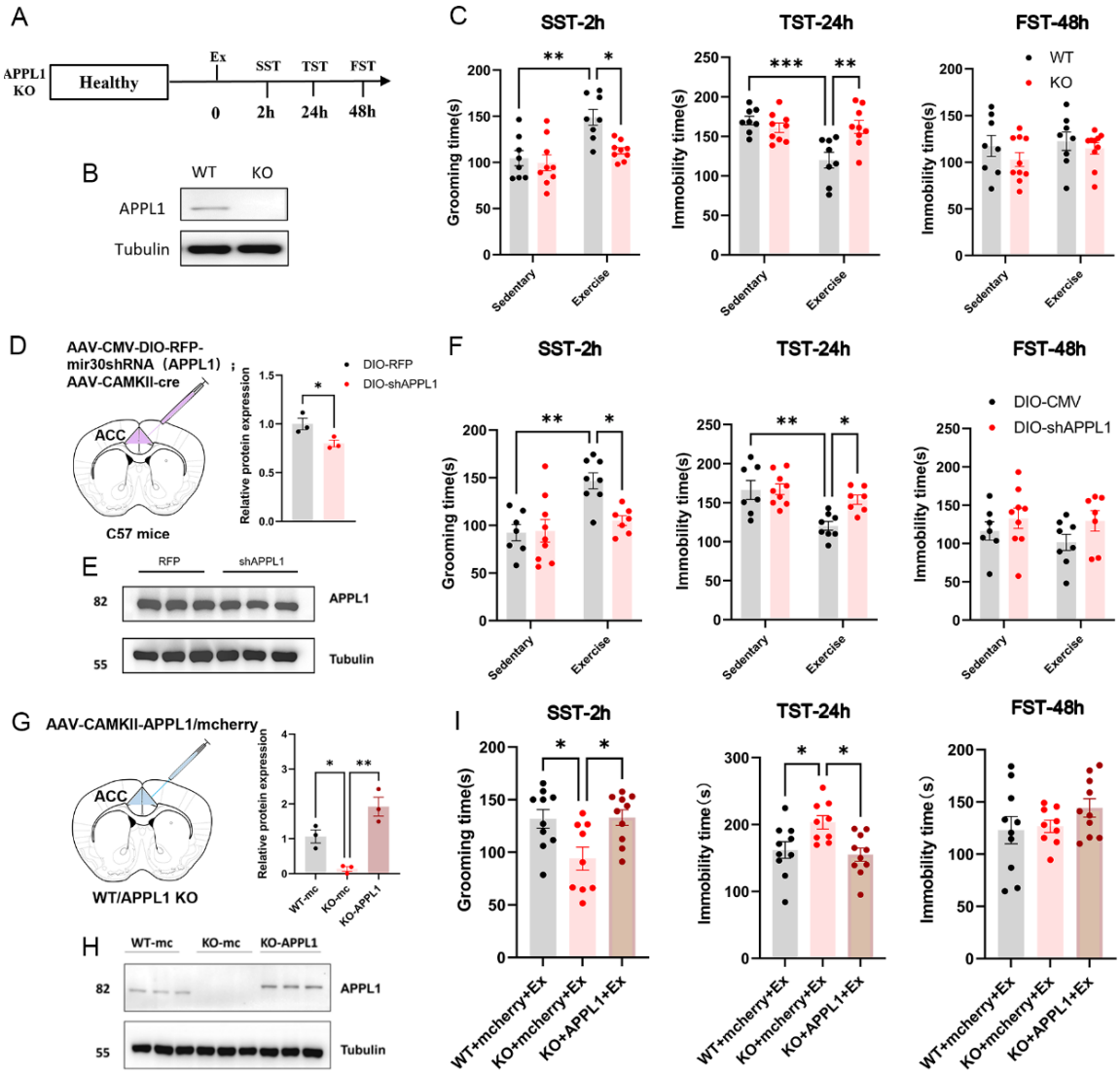

Figure 6.1 Adaptor protein APPL1 in ACC-glutamatergic neurons is essential for rapid antidepressant effects of a single-bout physical exercise.

(A) Experimental timeline.

(B) Western blot verified that no APPL1 expression in APPL1 KO mice.

(C) Global knockout APPL1 abolished the rapid antidepressant effect elicited by exercise. SST. Main effect of exercise: F (1, 30) = 14.77, P = 0.0006; Main effect of genotype: F (1, 30) = 7.64, P = 0.0097; Interaction: F (1, 30) = 4.378, P = 0.045. TST. Main effect of

exercise: F (1, 30) = 10.33, P = 0.0031; Main effect of genotype: F (1, 30) = 4.633, P = 0.0395; Interaction: F (1, 30) = 11.19, P = 0.0022. FST. Main effect of exercise: F (1, 30) = 0.6603, P = 0.4229; Main effect of genotype: F (1, 30) = 1.455, P = 0.2372; Interaction: F (1, 30) = 0.05909, P = 0.8096.

(D) Experimental timeline. Specifically knockdown APPL1 in ACC-glutamatergic neurons in C57/BL6J mice

(E) Representative western blot band showing APPL1 expression in DIO-CMV and DIO-shAPPL1 group. Quantification of APPL1 expression levels between DIO-RFP (N = 3) and DIO-shAPPL1 (N = 3) groups. Unpaired t-test, T (4) =2.898, P=0.0442.

(F) Specific knockdown APPL1 in ACC-glutamatergic neurons abolished the antidepressant effect elicited by exercise. SST. Main effect of exercise: F (1, 27) = 12.11, P = 0.0017; Main effect of virus: F (1, 27) = 4.518, P = 0.0428; Interaction: F (1, 27) = 5.430, P = 0.0275. TST. Main effect of exercise: F (1, 27) = 14.25, P = 0.0008; Main effect of virus: F (1, 27) = 4.826, P = 0.0368; Interaction: F (1, 27) = 4.318, P = 0.0474. FST. Main effect of exercise: F (1, 27) = 0.5318, P = 0.4721; Main effect of virus: F (1, 27) = 3.142, P = 0.0876; Interaction: F (1, 27) = 0.2175, P = 0.6447.

(G) Using overexpression virus to re-express APPL1 in ACC-glutamatergic neurons in KO mice and C57 mice.

(H) Representative western blotting bands of APPL1. Quantification of APPL1 expression levels in WT + mcherry (N = 3), KO + mcherry (N = 3), and KO + APPL1 (N = 3) groups. One-way ANOVA with Tukey's multiple comparisons test, F (2,6) = 20.46, P = 0.0021.

(I) Re-introduction of APPL1 in the ACC-glutamatergic neurons of APPL1 KO mice rescued the antidepressant effect of single-bout physical exercise. One-way ANOVA with Tukey's post hoc analysis. SST. F (2,27) = 5.267, P = 0.0117; TST. F (2,27) = 5.015, P = 0.0141. FST. F (2,27) = 1.396, P = 0.2650.

6.3.2 APPL1 nuclear translocation induced by a single-bout exercise depends on the adiponectin-AdipoR1 signaling in ACC-glutamatergic neurons.

After verifying the key role of APPL1 expressed in ACC-glutamatergic neurons in mediating antidepressant changes, we further investigate how adaptor protein APPL1 is involved in transducing the adiponectin signaling to elicit antidepressant effects after single-bout physical exercise. Initially, we examined the temporal changes in APPL1 levels post-exercise (Fig 6.2 A). Our analysis revealed that exercise did not influence APPL1 mRNA levels at various time points (Fig 6.2 B), indicating that single-bout physical exercise does not alter APPL1 expression. Notably, APPL1 in the neurons translocates from the synapse to the nuclear in response to the activated neurons [206]. We further detect APPL1's spatial distribution changes in neurons after exercise. At the cellular level, the APPL1 underwent rapid and sustained translocation to nuclear in the ACC- glutamatergic neurons and back to baseline 24 hours post-exercise (Fig 6.2 C). Together, these results suggest that the adaptor protein APPL1 undergoes a time-dependent nuclear translocation in ACC glutamatergic neurons in response to single-bout physical exercise without changes in total expression levels.

To investigate whether adiponectin-AdipoR1 signaling is the key to mediating the single-bout physical exercise elicited APPL1 translocation, we further detected the spatial distribution of APPL1 in the ACC-glutamatergic neurons in the adiponectin KO mice following exercise (Fig 6.2 D). We found no changes in the distribution of APPL1 in the cytoplasm and nuclear at a 2-hour timepoint (peak time point) following the exercise in adiponectin KO mice (Fig 6.2 E), indicating that adiponectin is crucial for mediating APPL1 translocation following single-bout physical exercise. Additionally, upon AdipoR1 knockdown in ACC glutamatergic neurons, the APPL1 translocation observed at the 2-hour timepoint post-exercise was nullified (Fig 6.2 F-G), suggesting that AdipoR1 is essential for this process. Collectively, these results demonstrate that APPL1 nuclear translocation in ACC-glutamatergic neurons following single-bout physical exercise is dependent on adiponectin-AdipoR1 signalling.

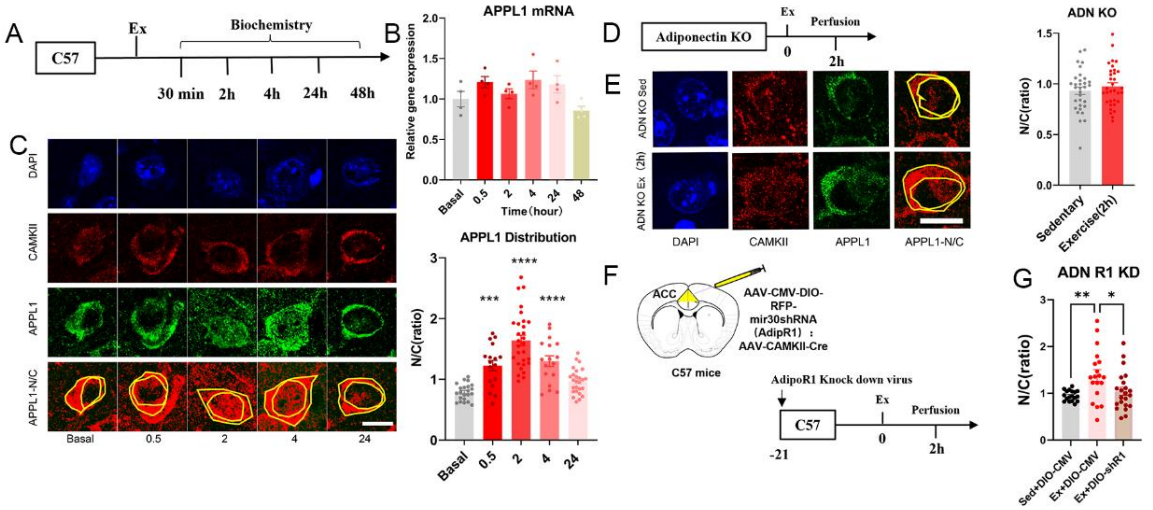

Figure 6.2 Single-bout physical exercise triggers adaptor protein APPL1 nuclear translocation in ACC-glutamatergic neurons depending on adiponectin-AdipoR1 signaling.

(A) Experimental timeline for tissue collection.

(B) Expression of APPL1 mRNA in the medial prefrontal cortex at various time points after single-bout physical exercise.

(C) Representative images to show the subcellular localization of APPL1 in ACC-glutamatergic neurons at various time points after single-bout physical exercise. Images were taken using a fluorescence microscope with a 63x oil lens. Scale bar, 50 μm. Statistical analysis of the nuclear/cytoplasmic ratio of APPL1.

(D) Experimental timeline using adiponectin global knockout mice.

(E) Representative images and analysis of the nuclear-to-cytoplasmic ratio of APPL1 immunofluorescence intensity in ACC-glutamatergic neurons of adiponectin KO mice 2 hours after single-bout physical exercise. Unpaired t-test, T (63) = 0.8405, P = 0.4038. Scale bar, 50 μm.

(F) Experimental timeline for experiment with AdipoR1 knockdown using AAV-shRNA targeting ACC-glutamatergic neurons.

(G) Analysis of the nuclear-to-cytoplasmic ratio of APPL1 immunofluorescence intensity in ACC-glutamatergic neurons with AdipoR1 knockdown 2 hours after single-bout physical exercise. One-way ANOVA with Tukey's post hoc analysis. N/C ratio. F (2, 56) = 5.944, P = 0.0046

6.4 Discussion

This study revealed the key role of APPL1 in ACC glutamatergic neurons in mediating the rapid antidepressant effect of single-bout physical exercise. We found that APPL1 underwent rapid nuclear translocation from the cytoplasm in ACC-glutamatergic neurons following exercise and its nuclear translocation relies on the adiponectin-AdipoR1 signaling. These findings provide mechanistic insights into the rapid antidepressant action of single-bout physical exercise, which involves the transduction of the adiponectin-AdipoR1 signaling to the nuclear translocation of APPL1.

APPL1 is a crucial adaptor protein that interacts with AdipoR receptors to facilitate the transduction of adiponectin signaling [27]. A previous study in Yau's lab showed that chronic physical exercise increases hippocampal adiponectin levels, contributing to the antidepressant effect via an AdipoR1-AMPK-dependent mechanism, without altering APPL1 protein expression in the hippocampal tissues [13]. The precise function of APPL1 as a central component of the adiponectin signaling pathway in the context of antidepressant effects triggered by physical exercise warrants further investigation. We first found that APPL1 is necessary to mediate the antidepressant effect of physical exercise because APPL1 global knockout abolished the antidepressant effect induced by a single-bout physical exercise (Fig. 6.1 C). In addition, our findings demonstrate that APPL1 knockdown and overexpression specifically in the ACC glutamatergic neurons interfered with the rapid antidepressant effect of single-bout physical exercise (Fig. 6.1 F&I). Our results echoed one previous finding reporting that APPL1 knockout in the ventromedial prefrontal cortex impaired fear extinction retrieval [216], highlighting the critical role of APPL1 in mPFC neurons in modulating emotional responses.

APPL1 undergoes nuclear translocation in response to enhanced neuronal activity [206, 216]. Our data (Fig 6.2 C) showed that APPL1 translocated into the nuclear in activated ACC glutamatergic neurons, and this translocation persisted longer (at least 4 hours) than the duration of neuronal activation as shown by calcium imaging data (Fig 4.2 F). This phenomenon aligns with one *in vitro* study that activity-dependent APPL1 translocation in the cultured hippocampus neurons happens after neuronal activation [206],

suggesting an inductive relationship between changes in neuronal activity and nuclear translocation of APPL1. Our data in Chapter 5 reported that blocking the adiponectin-AdipoR1 pathway in the ACC diminished neuronal activation of glutamatergic neurons induced by single-bout physical exercise. Consistently, APPL1 nuclear translocation is absent not only in adiponectin knockout mice but also in mice with AdipoR1 knockdown in ACC glutamatergic neurons (Fig.6.2 H-I). Collectively, the APPL1 translocation depends on the adiponectin-AdipoR1 signaling. This phenomenon is also shown in the non-neuronal cells [31, 32]. Chen et al. [31] demonstrated for the first time that adiponectin increased the nuclear translocation of the adaptor protein APPL1 in human adipose-derived stem cells in a time-dependent manner. Similarly, a recent animal study showed that adiponectin induces the nuclear translocation of APPL1 in rat aortic endothelial cells [32].

In summary, the peripheral-central crosstalk of single-bout physical exercise involves adiponectin's action on the activation of the ACC glutamatergic neurons through binding to its AdipoR1, which in turn induces APPL1 nuclear translocation. The function of nuclear APPL1 as the potential therapeutic target for rapid antidepressant treatment in depression warrants further investigation.

Chapter 7: Critical role of nuclear translocation of APPL1 in spinogenesis underlying the rapid antidepressant-like effect of single-bout physical exercise

7.1 Introduction.

Synapse-to-nuclear signaling is pivotal in converting signals received at synapses into synaptic gene transcription essential for regulating neuroplasticity [287]. APPL1 can function as a messenger from synapse to the nuclear by its nuclear translocation from the cytoplasm upon neuronal activation in the central nervous system [204, 206]. Chapters 5 and 6 have reported that adiponectin-AdipoR1 signaling modulates ACC-glutamatergic neuronal activity, which further induces APPL1's nuclear translocation in neurons following single-bout physical exercise. Nevertheless, the precise role of nuclear APPL1 in mediating the downstream transduction of adiponectin signaling and elucidating the antidepressant effects induced by single-bout physical exercise remains to be established.

Histone acetylation plays a critical role in the pathophysiology of depression and the therapeutic action of typical antidepressants [225, 288]. Histone deacetylases (HDACs), enzymes that repress gene expression by removing acetyl groups from histones, lead to a less accessible chromatin configuration [222]. HDAC2, a member of the HDAC family, epigenetically modulates the pathogenesis of major depressive disorder [220]. Animals that overexpress a chromatin-bound variant of HDAC2 display increased depression-like behaviors [289]. Of note, nuclear APPL1, by interacting with the NuRD/MeCP1/HDAC2 complex [199, 234], indirectly contributes to histone acetylation and gene transcription in non-neuronal cells [234, 235]. In the central nervous system, nuclear APPL1 in neurons mainly interacts with HDAC2, disrupting HDAC2’s binding to chromatin and indirectly promoting gene transcription [216, 290]. Therefore, elucidating how nuclear APPL1's interaction with HDAC2 affects subsequent transcriptional events may be intimately connected to explain the antidepressant effects induced by physical exercise.

The function of the HDAC2 in the brain is primarily related to gene transcription regulating synaptic plasticity [220]. HDAC2 is predominantly localized at the promoters of genes associated with synaptic plasticity, or those responsive to neuronal activity, such

as BDNF promoter I/II, Fos, Cpg15, CamkII, CREB1, and NMDA receptor subunits, etc. Notably, these HDAC2-targeted genes are critical for dendritic spine formation [238] and are implicated in the behavioral effects of antidepressant drugs [239-241]. HDAC2 acts to repress the expression of these genes, thereby inhibiting synaptic plasticity [237]. Based on the above research, we hypothesized that the nuclear APPL1 in the ACC glutamatergic neurons may interact with HDAC2 to promote histone acetylation, which in turn enhances synaptic-related gene transcription, and consequently results in an increase in spine formation, which may underlie the neural basis of the antidepressant effect of single-bout physical exercise.

In this chapter, we aimed to investigate: 1) whether single-bout physical exercise-induced nuclear translocation of APPL1 promotes histone acetylation, which in turn enhances synaptic gene transcription and expression, thus increases spinogenesis in the ACC; and 2) whether nuclear translocation of APPL1 in ACC glutamatergic neurons is necessary for the rapid antidepressant effects elicited by single-bout physical exercise.

7.2 Materials and methods

7.2.1 Animals

C57BL/6J male mice (6-8 weeks old) were provided and kept in the Centralized Animal Facility (CAF) of the Hong Kong Polytechnic University. The breeding pair of the Thy1-yellow fluorescent protein (Thy1-YFP) transgenic mice was obtained from the Animal Center of The University of Hong Kong (provided by Dr. Cora Lai) and kept in the CAF of the Hong Kong Polytechnic University and was used for in vivo dendritic spine imaging. Unless otherwise specified, the housing conditions follow the same described in Chapter 3. The Animal Subjects Ethics approved all the procedures.

7.2.2 Separation and preparation of cytoplasmic and nuclear protein extracts from the mPFC

The cytosolic and nuclear protein extraction kit (Minute TM Cytosolic and Nuclear Extraction Kit for Frozen/Fresh Tissues; Cat. No. NT-032) was used to collect cytosolic and nuclear fractions of proteins. After the exercise, mice were sacrificed, and the brain

tissues were collected. Tissues were ground with the pestle for about 1 min (40-60 times). The homogenate was used to separate the cytosolic and nuclear proteins following the protocol provided by the kit. The nuclear protein was used to detect the acetylation of the histone protein.

7.2.3 Synaptoneurosome and total protein preparations

To extract synaptoneurosome fractions, the mPFC was isolated, homogenized, and purified to collect synaptoneurosome by using the ice-cold Syn-PER synaptic protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA). Then, the homogenates were centrifuged for 10 min at 1200× g at 4 ◦C, and cytosolic fraction was collected and centrifuged for 30 min at 15,000× g at 4 ◦C. The synaptosomal fraction was resuspended in the Syn-PER reagent and was used for the synaptic protein quantification in the western blotting experiment.

To get the total proteins from homogenate, the mPFC tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer (Abcam, Cambridge, UK) containing Halt© phosphatase/protease cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Samples were centrifuged at 14,000× g at 4 ◦C for 30 min. The protein concentrations of synaptoneurosome and homogenates were quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

7.2.4 Western blotting

Protein (30 μg) of each sample was loaded and separated using SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes with targeted protein were blocked in 5% bovine serum albumin (Sigma-Aldrich, St. Loius, MO, USA) for 1 hour, and then incubated overnight with primary antibodies. After washing the primary antibody, the membranes with targeted protein were incubated with the secondary HRP-linked antibodies for 1 h at room temperature. The following antibodies were used (see Table S1): GluR1 (1:1000, CST, 13185S), PSD95 (1:1000, CST, 2507S), Synapsin I (1:1000, CST, 5297T), Synaptophysin (1:1000, CST, 5461S), Histone 4 (1:1000, CST, 2935), Acetyl-Histone 4 (Lys12) (1:1000, CST, 13944S), Actin (1:1000, CST, 4967S)， Tubulin(1:1000,

CST, 2144S)，GAPDH (1:1000, CST, 51744)，Goat anti-rabbit IgG（1:2000, CST, 7074）and Goat anti-mouse IgG (1:2000, CST, 7076). Finally, the protein membranes were visualized using one imaging system (Bio-Rad, Hercules, USA) and were analyzed using ImageJ (National Institutes of Health, Bethesda, USA).

Total protein and nuclear protein were used to quantify the total histone H4 and acetylated H4 respectively in the western blotting. Tubulin and Histone H3 were used separately as the internal proteins for the total and nuclear extracts. Synaptoneurosome was used to quantify the synaptic protein expression. Tubulin was used as the internal protein.

7.2.5 Cannula implantation and drug infusion

For the cannula implantation, 6~7 weeks male mice were deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then fixed on a stereotaxic frame (RWD Life Science). The guide cannulas (RWD Life Science) were bilaterally implanted into the ACC (anteroposterior, +0.98 mm; mediolateral, ±0.4 mm; dorsoventral −1.2 mm from the bregma) and fixed to the skull by cement and three skull screws. Metal cannula caps (RWD Life Science) were tightened to protect the cannula from gnawing by mice. After the implantation, mice were allowed to recover for 2 weeks and were pre-handled by the researcher 1-week before the exercise intervention to reduce stress on the experimental mice.

As previously described, importazole was dissolved in Dimethyl sulfoxide (DMSO) to yield a final concentration of 40 μM and administered 0.3 μl per side for the cannula infusion and local drug infusion experiment [216]. For the cannula drug infusion, mice were lightly anesthetized with isoflurane in a glass bottle for 10-15 seconds. The metal cap was removed from the guide cannula, and then the injection cannulas were inserted (RWD Life Science) into the guide cannulas. Importazole was bilaterally infused into the ACC using microinjection at a flow rate of 0.1 μl per minute. After infusion with importazole was completed, the injection cannula was left in the ACC for 5 minutes to facilitate diffusion of the solution. Mice were lightly anesthetized again by isoflurane to remove the injection cannula and tighten the cap. After 10 minutes of rest, the mice recovered to a normal awake state, and they were then undergoing treadmill intervention.

7.2.6 Dendritic spine imaging

To image the ACC brain region, we took a similar surgery as previously described [291]. Before the start of the imaging experiment, Thy1-YFP male mice were used to create a glass window the same as a calcium imaging window. The multiphoton microscope A1 MP (Nikon, Japan) with the laser excitation wavelength (920 nm) was used to image the dendritic spine. During the process of the imaging session, the mouse was head fixed and kept awake. To target the ACC region, we performed imaging within 0 – 400 μm of the midline demarcated by the sagittal sinus, and we kept the same imaging parameters for the different imaging time points. Each thy1-YFP mouse will be imaged in the same field of view by locating it from the same blood vessel. For the importazole infusion experiment, the Thy1-YFP mice were imaged at a 2-hour time point.

To solve the problem of the local application of importazole, we adopted a similar protocol as previously used [292]. A small skull bone flap close to the imaging window was removed by a high-speed microdrill. The same dose and concentration of the importazole (40 μM,0.3 μl per side in ACC) were applied onto the superficial layer with an angle of 60° using the virus injection pump. Besides, the injection site was away from the imaging region (>100 μm) to avoid tissue injury. The importazole immersion lasted for 15 minutes to facilitate diffusion of the solution, followed by the single-bout physical exercise intervention.

7.2.7 Behavioral tests

The behavioral tests were carried out following the same procedures as described in Chapter 3, including the SST, TST, and FST protocols.

7.2.8 Statistical analysis

Graph figures and data analysis were done using GraphPad Prism 9 (GraphPad Software, USA). The normal distribution of data was analyzed with the Shapiro–Wilk test ($n < 8$) or D'Agostino and Pearson test ($n \geqslant 8$) [101].

If the data passed the normality test, Student's t-tests were used to compare biochemical results with the basal time point. For data involving more than two groups

with two independent variables, two-way ANOVA followed by Tukey's multiple comparisons post hoc test was employed. All analyses were conducted by two lab members who were blinded by the group assignments. Data are presented as mean ± SEM. Statistical significance was set at *P < 0.05, **P < 0.01, and ***P < 0.001.

7.3 Results

7.3.1 Nuclear accumulation of APPL1 increases histone acetylation, synaptic gene transcription, and synaptic protein expression.

An *in vitro* study suggested that the nuclear APPL1 in neurons can regulate histone acetylation by interrupting HDAC2 binding to the CREB promoter (Fig 7.1A) [206]. After demonstrating that single-bout physical exercise can induce nuclear translocation of APPL1 in the ACC-glutamatergic neurons (Fig 6.2 C-D), we further investigated the subsequent event initiated by nuclear APPL1. Consistent with previous findings, we observed a peak level in the histone H4 acetylation at the H4K12 site at the 2-hour following physical exercise (Fig 7.1B), coinciding with the peak induction time point (2-hour) of APPL1 nuclear translocation (Fig 6.2 C-D). These results suggest that nuclear accumulation of APPL1 may lead to increased histone acetylation with a temporal change.

The transcription factor CREB facilitates the transcription of several synaptic genes, including cpg15, c-Fos, and BDNF (Fig 7.1A) [293]. To investigate whether nuclear APPL1 accumulation leading to increased histone acetylation promotes CREB-dependent gene transcription, we examined the mRNA levels of CREB genes at different time points after exercise. As expected, APPL1 nuclear accumulation led to a rapid increase in gene transcription as early as 30 minutes post-exercise, and this increase remained significant up to 24 hours post-exercise, then before returning to the baseline levels 48 hours post-exercise (Fig 7.1C). CREB-targeted genes are key factors in promoting presynaptic axonal elaboration, dendritic outgrowth, and AMPA receptor insertion for synapse formation in neurons [293]. We also detected the synaptic protein expression in the synaptoneurosomes at different time points and found that nuclear APPL1 rapidly increased protein expression of the PSD95 and AMPA receptor, as well as the presynaptic protein synapsin I and

synaptophysin as early as 2 hours following exercise, and the elevation lasted up to 24-hours following exercise (Fig 7.1 D). These results illustrated that the rapid and sustained nuclear translocation of APPL1 in the ACC-glutamatergic neurons inflicted by exercise induced several molecular events, including increased histone acetylation, and enhanced CREB-targeted gene transcriptions and synaptic protein expression.

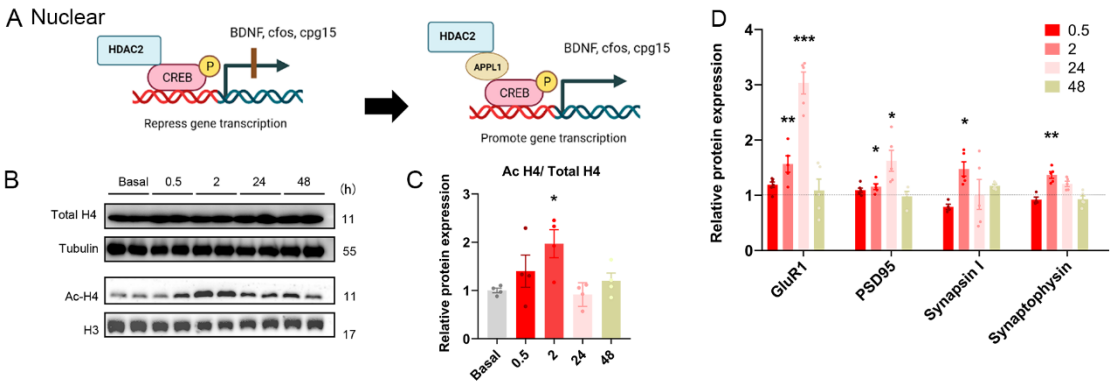

Figure 7.1 Nuclear translocation of APPL1 increases histone acetylation leading to increased synaptic genes and protein expression in exercised mice.

(A) Regulation of synaptic gene transcription by nuclear APPL1 in neurons. HDAC2 suppresses CREB-targeted gene transcription while nuclear APPL1 interrupts the interaction between HDAC2 and chromatin, indirectly promoting CREB-targeted gene (BDNF, c-Fos, and cpg15) transcription.

(B) Temporal changes of acetylated histone H4 and total histone H4 proteins after exercise. Results of Acy-H4 levels were normalized with the level of total Histone and compared with time point 0 hr (no exercise group) as the baseline level. Representative western blotting images of total histone H4 and acetylated histone H4 protein.

(C) Temporal changes of BDNF, c-Fos, and cpg15 mRNAs after exercise. The results of mRNA levels were normalized with the level of GAPDH and are shown as fold changes relative to the value at the baseline level.

(D) Temporal changes of AMPA, PSD95, synapsin I, and synaptophysin proteins after exercise. The results of each time point were normalized and compared with the baseline level.

(B-D) The animal number is around 4-5 mice per time point from independent experiments. Unpaired Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001 compared with the baseline time point.

7.3.2 Pharmacological inhibition of APPL1 translocation abolishes exercise-induced nuclear events and rapid antidepressant effects.

After examining downstream molecular events triggered by APPL1 translocation, we further detected whether these molecular changes are necessary for the rapid antidepressant effect of single-bout physical exercise. We administered the nuclear transport inhibitor importazole utilizing intracranial administration via cannulation targeting the ACC. The infusion was performed 10 minutes before exercise to block APPL1 nuclear translocation (Fig 7.2 A). Importazole effectively prevented APPL1 nuclear translocation induced by exercise for at least two hours (Fig 7.2 B).

We further examined the effect of the inhibition of APPL1 nuclear translocation on the molecular events 2 hours after exercise (Fig 7.3 A). Firstly, we found that inhibiting APPL1 nuclear translocation reduced levels of the histone H4 acetylation induced by exercise (Fig 7.3 B). Secondly, we found that the inhibition of APPL1 nuclear translocation prevents exercise-increased synaptic protein expression (Fig 7.3 C). An increase/decrease in synapse-associated proteins indicates that intervention rapidly increases spine formation/elimination [5]. We, therefore, examined the influence of importazole on spine changes by two-photon imaging of the apical tuft of the ACC-glutamatergic neurons using the Thy1-YFP mice (Fig 7.3 D). The result showed that importazole administration significantly abolished exercise-induced spine formation and had no effect on spine elimination (Fig 7.3 E-F). Altogether, these results demonstrate that the pharmacological inhibition of APPL1 nuclear translocation abolished exercise-elicited molecular changes and new spine formation.

Lastly, we investigated whether the pharmacological inhibition of APPL1 nuclear translocation affects the rapid antidepressant response of single-bout physical exercise (Fig 7.3 G). We found that blocking APPL1 nuclear translocation effectively diminished the rapid antidepressant effects of single-bout physical exercise, as indicated by no significant increase in grooming time in SST and no decreased immobility time in TST (Fig 7.3 H). The above results suggest that the APPL1 translocation and its downstream molecular events in ACC play an essential role in mediating single-bout physical exercise-elicited antidepressant effects.

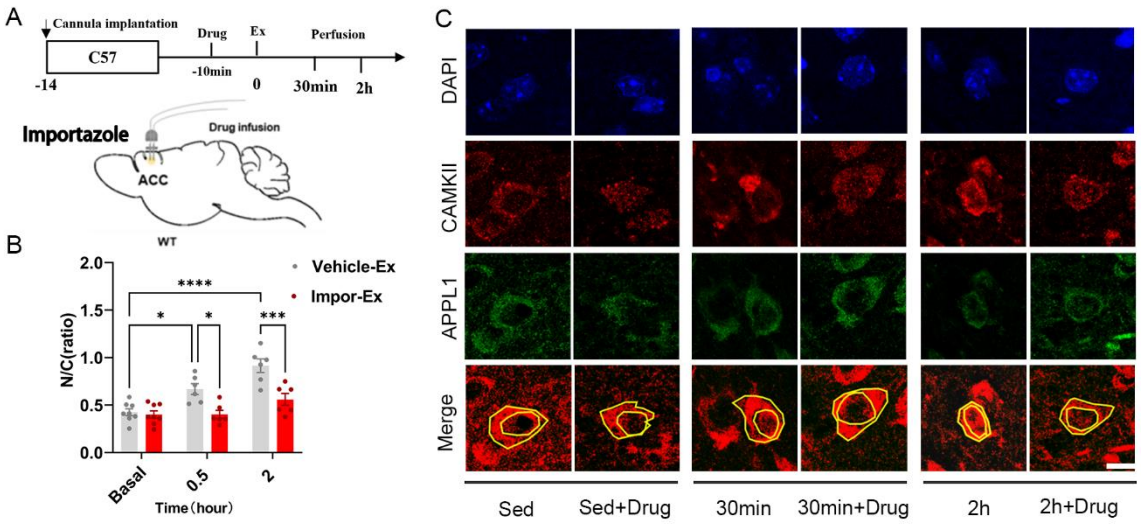

Figure 7.2 Administration with importazole in the ACC effectively inhibits exercise-induced nuclear translocation of APPL1.

(A) The experimental timeline.

(B) Effects of importazole on the APPL1 nuclear translocation at different time points following single-bout physical exercise. Main effect of time: $F(2, 34) = 21.31$, $P < 0.0001$; Main effect of drug: $F(2, 34) = 26.81$, $P < 0.0001$; Interaction: $F(2, 34) = 6.125$, $P = 0.0053$. Each point means one ROI and each ROI has 3-4 neurons. The animal number is 3 for each group. *P < 0.05, ***P < 0.001 and ****P < 0.0001. Bar graphs: the mean ± SEM.

(C) Immunofluorescence staining showing temporal changes of APPL1 distribution for two groups after a single-bout physical exercise. Scale bar, 50 μm.

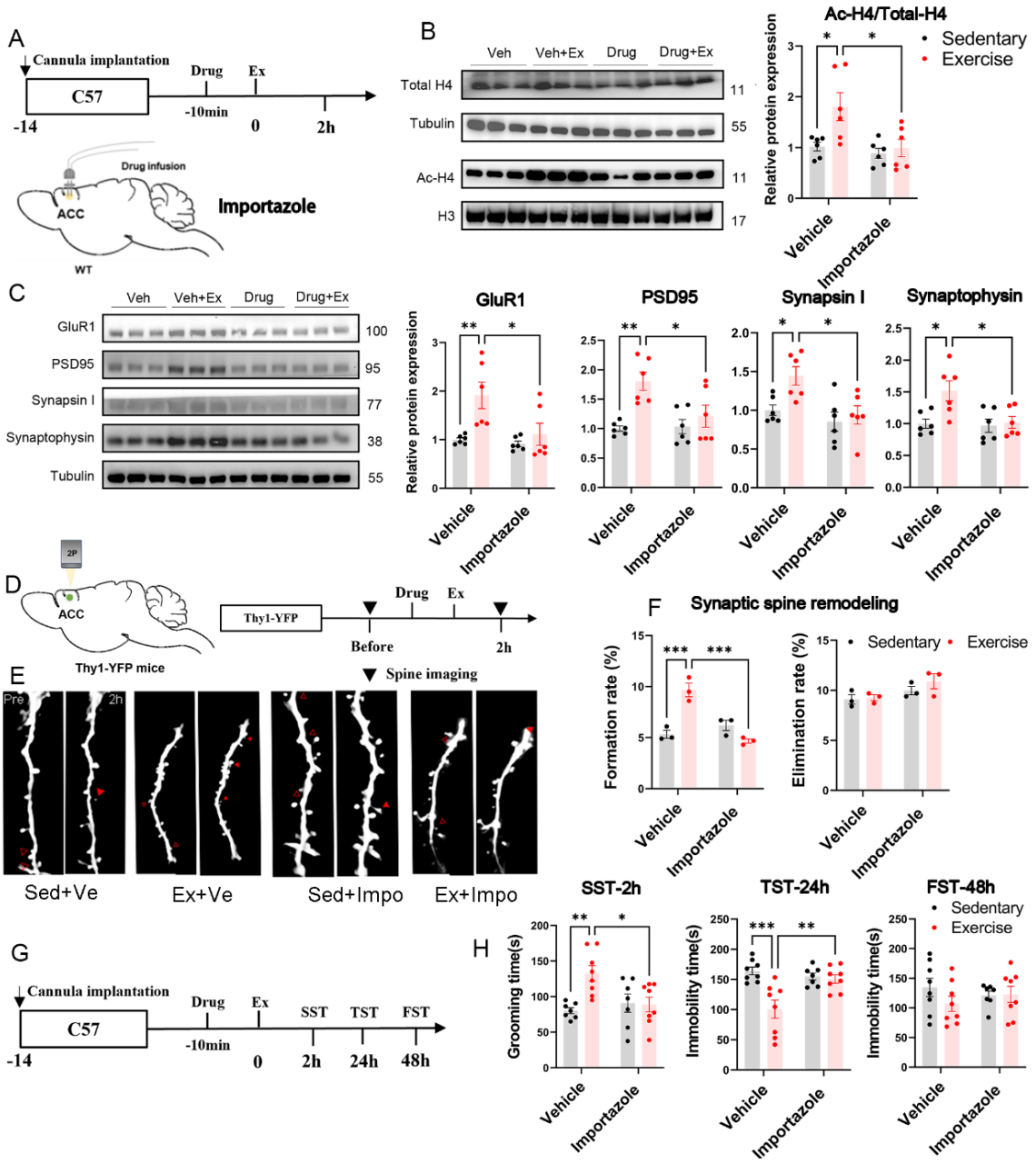

Figure 7.3 Blocking APPL1 nuclear translocation with importazole administration significantly abolishes molecular changes and the rapid antidepressant effects induced by single-bout exercise.

(A) Experimental timeline for brain infusion with importazole.

(B) Representative western blotting images of total histone H4 and histone H4 acetylation proteins. Results of Acy-H4 levels were normalized with the level of total Histone. N = 6

mice/timepoint. The main effect of exercise: F (1, 20) = 6.466, P = 0.0194; main effect of importazole: F (1, 20) = 7.307, P = 0.0137; interaction: F (1, 20) = 3.819, P = 0.0648.

(C) Representative western blotting images of synaptic proteins. Quantification results of synaptic protein expression. The results of each group were normalized with the vehicle/sedentary group. N = 6 mice per group. GluR1: main effect of exercise: F (1, 20) = 28.88, P = 0.0031; main effect of the importazole: F (1, 20) = 12.73, P = 0.0371; interaction: F (1, 20) = 0.1061, P = 0.1061; PSD 95: main effect of exercise: F (1, 20) = 12.76, P = 0.0019; main effect of the importazole: F (1, 20) = 4.125, P = 0.0558; interaction: F (1, 20) = 5.213, P = 0.0335; synapsin I: main effect of exercise: F (1, 20) = 12.76, P = 0.0254; main effect of the importazole: F (1, 20) = 23.72, P = 0.0075; interaction: F (1, 20) = 7.104, P = 0.119; synaptophysin: main effect of exercise: F (1, 20) = 17.80, P = 0.0191; main effect of the importazole: F (1, 20) = 15.43, P = 0.0278; interaction: F (1, 20) = 11.94, P = 0.0499.

(D) Experimental timeline for two-photon spine imaging using Thy1-YFP mice.

(E) Z-axis stacked images showing the same dendritic branches of ACC-glutamatergic neurons at baseline 0 hr (first imaging), 2 hr (second imaging). Hollow arrowheads in the first imagin: eliminated spines. Solid arrowheads in the second imaging: newly formed spines.

(F) The spine formation rate was increased in exercise mice compared to sedentary controls, while the drug treatment abolished the exercise-elicited new spine formation. Main effect of exercise: F (1, 8) = 5.880, P = 0.0178; main effect of importazole: F (1, 8) = 19.27, P = 0.0023; Interaction: F (1, 8) = 38.82, P = 0.0003. The spine elimination rate was not affected.

(G) Experimental timeline for the behavioral tests.

(H) Pharmacological inhibition of APPL1 nuclear translocation abolished exercise-induced rapid antidepressant effects. SST: Main effect of exercise: F (1, 26) = 2.602, P = 0.1188; main effect of importazole: F (1, 26) = 6.070, P = 0.0207; interaction: F (1, 26) = 7.033, P

= 0.0135; TST: main effect of exercise: F (1, 26) = 4.021, P = 0.0554; main effect of importazole: F (1, 26) = 12.03, P = 0.0018; interaction: F (1, 26) = 9.393, P = 0.005.

(B-H) Two-way ANOVA followed with Tukey's multiple comparisons test; *P < 0.05, **P < 0.01, ***P < 0.001. Bar graphs: the mean ± SEM.

7.4 Discussion

In this chapter, we examined the role of nuclear APPL1 in the ACC-glutamatergic neurons. The findings indicate that nuclear APPL1 promotes histone acetylation, thereby enhancing the transcription of synaptic genes and synaptic protein expression. Concurrently, the inhibition of APPL1 nuclear translocation abolished increases in histone acetylation and synaptic protein expression, and the rapid antidepressant effect elicited by exercise. Of note, our finding provides in vivo and cell-specific evidence that single-bout exercise induces new spine formation through the APPL1 nuclear translocation in ACC glutamatergic neurons. These data provide molecular mechanisms underlying the rapid antidepressant effects of single-bout physical exercise, revealing that exercise-induced neural remodeling includes epigenetic regulation on histone acetylation, synaptic protein expressions, and dendritic spine formation.

Our data revealed that exercise-induced nuclear translocation of APPL1 in the ACC glutamatergic neurons leads to time-course changes in histone acetylation and synaptic gene expression (Fig 7.1 A-C). These findings align with one *in vitro* study that APPL1 located in dendritic spines in the cultured hippocampus neurons responds to neuronal activation by translocating to the nuclear to facilitate transcription for late-phase LTP maintenance [206]. Our current study extends the scope from gene transcription to synaptic protein expression, highlighting increased synthesis of postsynaptic proteins such as PSD95 and AMPA receptors, as well as presynaptic proteins synapsin I and synaptophysin (Fig 7.1 D). Notably, our findings are consistent with the current theory established by the Duman lab that rapid antidepressant action involves a rapid increase in synaptic protein synthesis. This occurs through the activation of the mTOR-S6 pathway following a single sub-anesthetic dose of ketamine [5]. Additionally, the role of APPL1 nuclear translocation in modulating histone acetylation mediating the rapid antidepressant effect is also consistent with an antidepressant study of ketamine. Ketamine triggers transient phosphorylation and nuclear export of HDAC5. The primary role of HDAC5 in the nuclear is to reduce histone acetylation, and further suppress gene transcription by targeting MEF2 promoter [288]. Despite different promoter targets, APPL1-HDAC2 and HDAC5-related nuclear exportation mechanisms share the same result in promoting histone acetylation,

which further enhances transcription of synaptic genes and protein expression, eliciting rapid antidepressant responses. Our data have shed light on the mechanisms involving histone modification in rapid antidepressant response of single-bout physical exercise.

Individuals with depression, as well as mice with depressive-like behaviors, show structural deficits in synapses, including reduced synapse density and dendritic spine formation [103, 294]. Antidepressant drugs such as ketamine and psilocybin have been reported to promote spine formation in the prefrontal cortex, thereby mitigating depressive symptoms [5, 103, 291]. These studies establish a connection between the therapeutic effects of rapid antidepressants and the enhancement of spine formation in the prefrontal cortex. Our *in vivo* imaging data found that single-bout treadmill exercise can induce new spine formation in the ACC 2-hour after exercise. Consistent with one study a single bout of motor learning in the rotarod test rapidly stimulates spinogenesis in the motor cortex of mice within 6 hours [295]. These findings suggested that single-bout physical exercise is sufficient to induce new spine formation in the cortex within a short time and new-spine function varied depending on different brain regions. Furthermore, the pharmacological inhibition of the APPL1 nuclear translocation abolished the exercise-induced new spine formation and rapid antidepressant effects (Fig 7.3), confirming that nuclear APPL1 is required for exercise to promote spinogenesis that is required for the rapid antidepressant action. However, the essential function of nuclear APPL1 in promoting spinogenesis and modulating depressive-like behavior was investigated in non-stressed mice rather than in stressed mice. Considering that the antidepressant effect of single-about physical exercise in stressed mice also existed (Fig 3), it would be intriguing to explore the specific mechanisms of nuclear APPL1's involvement using the depression model.

Overall, our results shed light on molecular mechanisms underlying the rapid antidepressant effect of single-bout physical exercise. The results have demonstrated the crucial role of APPL1 nuclear translocation in mediating exercise's effect on new spine formation and antidepressant response, indicating that interventions that trigger APPL1 nuclear translocation could be a novel target for rapid antidepressant treatments.

Chapter 8: Conclusion

Emerging clinical evidence has shown that a single-bout physical exercise can produce rapid antidepressant effects or alleviate mood immediately [153, 154], yet the neural mechanisms that underpin its therapeutic benefits are still not fully understood. Our study revealed that single-bout physical exercise produces rapid antidepressant effects through the adiponectin/AdipoR1 pathway to trigger APPL1 nuclear translocation in ACC glutamatergic neurons (Fig 8).

In Chapter 3, we validated the antidepressant behavioral effects of a single-bout physical exercise in humans and animals. For human participants, exercise at moderate to high intensity elevated positive mood states (vigor and esteem) in both symptomatic and asymptomatic groups and reduced negative mood states (anxiety, fatigue, depression, confusion, and anger) in the symptomatic group. Similarly, in the rodent model, mice from both non-stressed and stressed groups exhibited rapid onset of antidepressant responses that persisted for at least 24 hours. These findings suggest that this rapid beneficial effect of exercise is consistent in humans and mice, supporting single-bout physical exercise interventions as an effective treatment for the immediate alleviation of depressed mood. This non-pharmacological intervention could be implemented early to halt further development of depression symptoms. The results of the behavioral phenotype, presented in Chapter 3, pave the way for the investigation of the neurological mechanism of the rapid antidepressant effect of single-bout physical exercise.

Chapter 4 investigated the neural mechanisms underlying the rapid antidepressant effects of a single-bout physical exercise. Whole brain c-Fos mapping in mice identified ACC as a pivotal region implicated in the rapid antidepressant effects induced by exercise. It was also found that ACC glutamatergic neurons were rapidly and persistently activated by exercise, and this activation was essential for the rapid antidepressant response. Overall, these findings identified the specific brain region and cellular target - ACC glutamatergic neuron activation in facilitating the rapid antidepressant effects of a single-bout physical exercise.

Chapter 5 investigated the involvement of adiponectin—an exerkine implicated in mediating the antidepressant effects of chronic physical exercise. Adiponectin signaling is found to be potentially involved in activating ACC-glutamatergic neurons. An increase in adiponectin levels in the mPFC negatively correlated with levels of depression-like behavior in response to acute exercise. In addition, the adiponectin-AdipoR1 signaling was required for the rapid antidepressant effect induced by single-bout physical exercise via activating ACC-glutamatergic neurons. Overall, these findings provide mechanistic insights into the antidepressant effect of physical exercise, from identifying specific cell populations in the brain to the involvement of adiponectin-AdipoR1 signaling.

Chapter 6 revealed the key role of APPL1 in ACC glutamatergic neurons in mediating the rapid antidepressant effect of single-bout physical exercise. We found that APPL1 underwent rapid nuclear translocation from the cytoplasm in ACC-glutamatergic neurons following exercise and its nuclear translocation relies on the adiponectin-AdipoR1 signaling. These findings suggest a novel mechanism underlying the rapid antidepressant response of single-bout physical exercise and report peripheral-central crosstalk involving the adiponectin-AdipoR1 signaling to the nuclear translocation of APPL1 in ACC glutamatergic neurons.

Chapter 7 provided investigated the function of nuclear APPL1. Nuclear APPL1 is involved in histone acetylation, facilitating the transcription of synaptic genes and the expression of synaptic proteins within the ACC. Concurrently, the inhibition of APPL1 translocation prevented molecular alterations and antidepressant effects induced by acute exercise. Furthermore, our results for the first time offered in vivo and neuron-specific evidence that single-bout physical exercise induces dendritic spine formation through the APPL1 nuclear translocation in ACC glutamatergic neurons. Neural remodeling following single-bout physical exercise involves changes in histone acetylation, synaptic proteins, and new spine formation, providing the biological basis for the observed behavioral changes. Future studies will be required to further examine APPL1 nuclear translocation as a novel target in antidepressant therapy development.

In conclusion, results from all chapters elucidate the neural mechanisms underlying the rapid antidepressant effects of a single-bout physical exercise. The findings not only provide a novel cellular target that is involved in the rapid antidepressant effect of exercise but also pave the way for targeting adiponectin-AdipoR1-APPL1 signaling for developing the rapid antidepressant. This work also highlights the potential of a single-bout physical exercise as an effective therapy for immediate mood elevation.

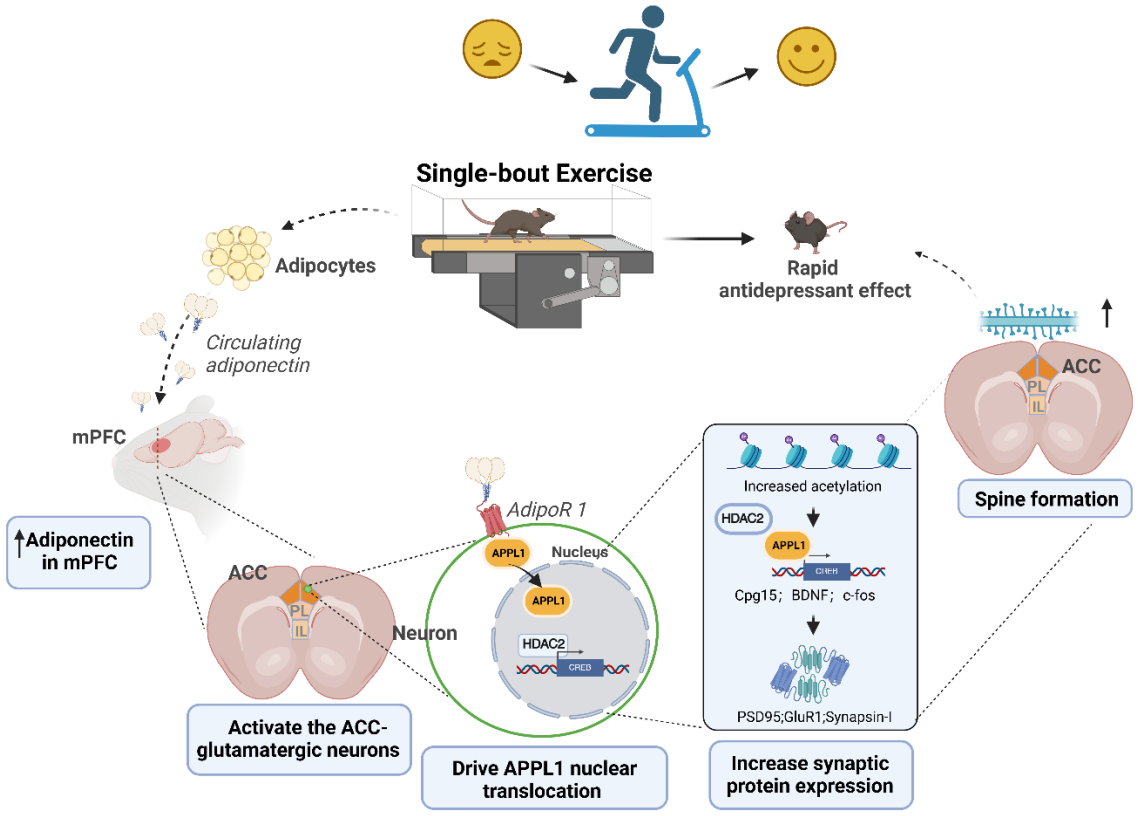

Figure 8. Schematic summary.

Single-bout treadmill exercise produces rapid antidepressants in both human and mouse models. Single-bout exercise increased adiponectin in the mPFC that binds to the AdipoR1 receptor to activate the ACC-glutamatergic neurons resulting in APPL1 nuclear translocation. Nuclear APPL1 interrupts the binding between HDAC2 and the CREB promoter to promote histone acetylation, consequently enhancing synaptic protein expression and spinogenesis.

Abbreviations: mPFC, medial prefrontal cortex; ACC, anterior cingulate cortex; PL, prelimbic cortex; IL limbic cortex; AdipoR1, adiponectin receptor 1; APPL1, adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1; HDAC2, histone deacetylases 2; CREB, cyclic AMP response element-binding; BDNF, brain-derived neural factor; Cpg 15, candidate plasticity gene 15.

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