ADIPONECTIN AS A RAPID-ACTING ANTIDEPRESSANT: AN INVESTIGATION OF ITS MECHANISMS OF ACTION

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Adiponectin as a rapid-acting antidepressant: an investigation of its mechanisms of action

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

The emotional, cognitive, and physiological perturbations caused by major depressive disorder affects millions of people every year, placing it as the leading cause of disability across the world. The delayed therapeutic onset and low remission rates of current antidepressants pose a great challenge for disease treatment. A better understanding of the underlying neuropathology of depression involving the hippocampus and media prefrontal cortex and the discovery of novel rapid-acting antidepressants paved the way for the development of new and more effective pharmacological interventions. Adiponectin is an adipocyte-secreted hormone active in the regulation of the body's energy metabolism and has been suggested as a linking factor between the metabolic state and the brain activity. It modulates the hippocampal structural and functional plasticity, which are two of the main mechanisms involved with the rapid-antidepressant response. Based on that, this research aims to characterize the adiponectin's potential rapid-acting antidepressant effects, investigate whether adiponectin acutely improves hippocampal synaptic and structural plasticity, and determine if the activation of the ventral hippocampus to medial prefrontal cortex (vHipp-mPFC) pathway modulates depression. We showed that, at the 1 h time point, the activation of the adiponectin signaling system is anxiogenic and, when targeting the hippocampus, adiponectin induced an acute depression-like response. Moreover, those effects were paralleled by reduced hippocampal synaptic and structural plasticity in response to acute adiponectin administration. Based on unpublished data in Dr. Yau's Lab and a literature discussion of the sub-chronic effects of serotonergic drugs, these results might implicate adiponectin as a chronic rather than an acute antidepressant. On a parallel experiment, we demonstrated that the activation of the vHipp-mPFC pathway modulates depression-like behavior independent of increased hippocampal proliferation, suggesting this pathway activation might be a separate mechanism implicated in depression relief. In summary, this research increases the understanding of the adiponectin signaling system in mood modulation as well as implicates the vHipp-mPFC pathway as a potential circuit associated with depression relief.

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CHAPTER 1: INTRODUCTION

Major depressive disorder is a debilitating disease with notorious social and economic impact. It is the worldwide leading cause of disability, accounting for more than 10% of the 777 million years lived with disability in 2010 [1]. In 2015, 4.4% of the global population was estimated to suffer from depression [2]. Antidepressant monotherapy is the first-line treatment, with approximately two-thirds of the patients being treated with selective serotonin reuptake inhibitors (SSRI) [3]. Nonetheless, roughly 20% to 40% remain resistant to available pharmacological interventions, whose delayed therapeutic onset takes 4-12 weeks to start increasing symptoms relief [4]. With suicide being a major concern in depressive disorders [5], accounting for nearly 1.5% of all deaths worldwide [2], there is a great need for more efficacious treatments.

The development of novel antidepressant treatments has been fostered by a better understanding of the underlying neurophysiological alterations present in depression. As in the latest report of the ENIGMA consortium (Enhancing NeuroImaging Genetics through Meta-Analysis) involving 43 countries and 1400 scientists, patients with major depressive disorder present lower hippocampal and orbitofrontal cortex volumes [6], suggesting these two interconnected regions as the cornerstone for mood regulation. Such volumetric alterations are likely a consequence of downregulated genes and proteins associated with synaptic function, resulting in decreased spine density characteristically seen in patients with depressive disorders [7–9]. Not only volumetric but also white-mater changes are associated with depression, suggesting that loss of connectivity between the prefrontal cortex and the hippocampus underlies depression [10,11]. Indeed, the ventral hippocampus (vHipp) sends projections to the medial prefrontal cortex (mPFC), a pathway whose functional connectivity is decreased in both depressed patients [12] and animal models of depression [13]. Not surprisingly, depression remission is associated with recovery of such structural and functional deficits [14–16].

The discovery that low doses of Ketamine (0.5 mg/kg; i.e., not at the typical anesthetic dose [17]) can produce antidepressant effects within two hours, even for treatment-resistant patients [18], paved the way for the development of a new and more effective class of rapid-acting treatments. Ketamine has been proven to be a reliable rapid-acting antidepressant, with effects being confirmed across many different clinical trials [17,19], as recently reviewed by Shin and collaborators [20]. Interestingly, the vHipp-mPFC pathway was recently shown to mediate the ketamine's long-lasting antidepressant effects in rodents [21]. Currently, there are fifteen ongoing clinical trials on the use of Ketamine or its derivate (e.g., Esketamine) for depression treatment (www.clinicaltrials.gov). However, the translation of such

astonishing results into the clinical practice has been hindered by the fact that Ketamine induces dissociative side effects, including nausea, dizziness, and blood pressure elevation [17], which restricts its administration to hospital environments. Moreover, Ketamine is also a recreational drug whose abuse is associated with the development of a dysfunctional bladder syndrome [22]. Nonetheless, Ketamine jump-started the understanding and development of rapid-acting antidepressants, and significant achievements in the field were made possible by understanding Ketamine's mechanisms of action.

Adiponectin is an adipocyte-secreted hormone acting on several tissues [23–25]. It exerts its biological effects mostly by binding to its homologous receptors, adiponectin receptor (AdipoR) 1 and 2, which were shown to regulate the energy metabolism and fatty acid oxidation in the periphery [26–28]. Adiponectin receptors are also present in central nervous system structures, such as the hypothalamus [25], mPFC [29], and hippocampus [30,31]. Due to its ability to cross the blood-brain barrier (BBB), adiponectin modulates the central metabolic system (i.e. the hypothalamus) [25,32] and other limbic structures highly relevant for behavior and mood regulation. In the hippocampus, specifically, adiponectin has been shown to stimulate structural and synaptic plasticity [33], two of the mechanisms involved with the rapid-antidepressant response. Such physiological effects naturally raised the question of whether adiponectin would have rapid antidepressant properties. Nonetheless, this remains unanswered and the mechanisms of action mediating such potential effects are still hypothetical.

Based on this assumption, this research aimed to:

1. Characterize the adiponectin's potential rapid-acting antidepressant effects
2. Investigate whether adiponectin acutely improves hippocampal synaptic and structural plasticity.
3. Determine if the activation of the vHipp-mPFC pathway modulates depression.

To accomplish such objectives, different sets of experiments were carried out to experimentally investigate the systemic and central effects of adiponectin over depressive-like behavior in health and chronically stressed mice (an experimental strategy used to induce a depressive phenotype). Moreover, *in vitro* experiments were carried out to elucidate the effect of adiponectin over structural and functional hippocampal plasticity. Lastly, a different set of experiments intended to reveal the relevance of a pathway connecting the ventral hippocampus to the mPFC in antidepressant responses. For the sake of clarity, such experiments were organized in two different chapters (Chapters 3 and 4), each of them enclosing its Introduction, Methods, Results, and Discussion sections, following a thesis-by-publication format. In Chapters 2, the reader will find a comprehensive literature review intended to define the theoretical and factual framework supporting the current investigation. Finally, this thesis

is concluded in Chapter 5, where the main findings are discussed considering the objectives and their implication to the field.

CHAPTER 2: LITERATURE REVIEW

In this and the next chapters, the reader will find a detailed literature review elucidating the underlying theory on which this research is based. However, it is important to bear in mind that, despite our advanced knowledge of the neurobiology of depression, no known mechanism can so far account for all aspects of the disease [34]. Therefore, favoring the existence of strong clinical and experimental evidence, I choose to delineate this research based on a neurodevelopmental conception of depression, which emphasizes the relevance of environmental factors in triggering secondary physiological alterations associated with the disease development. In this model, altered hypothalamic-pituitary-adrenal (HPA) axis activity due to traumatic events or chronic stress exposure putatively leads to glucocorticoid-induced neuropathology that eventually results in depression [35]. The neuropathology was, in turn, discussed in light of the neurotrophic model of depression [36,37], which has yielded fruitful results both in explaining and exploring new rapid-acting antidepressant agents.

1. Neurodevelopmental model of depression

Early-life environmental adversity is a long-known risk factor for the development of several chronic disorders in adulthood, including depression [38]. In a longitudinal study, early-life emotional neglect was the only independent predictor of adult depression onset or recurrence [39]. Such correlation is confirmed by a meta-analysis, where emotional abuse and neglect are found to be the early-life adverse events most strongly associated with depression in adulthood [40]. The increased risk for depression development is already expressive in childhood and adolescence, as suggested by a more recent meta-analysis [41]. As reported in the Adverse Childhood Experience (ACE) study, experiencing four or more different categories of early-life adverse events increased by 4.6 times the chance of developing depression in adulthood, and by 12.2 times the chances of attempting suicide [38].

Although known to possesses a transgenerational component, depression heritability is estimated to be at approximately 35% [34]. Gene-environment interactions are therefore likely to be moderating the individual's response to environmental adversity in terms of depression development [42]. The extent of such interactions, however, is still controversial. In the largest meta-analysis to study the interaction of 5-HTTLPR genotype (a polymorphism in the serotonin transporter gene SLC6A4) and stress exposure, for instance, there was no evidence supporting a gene-environment interaction although stress alone significantly increased the risk for current or lifetime depression development in up to 3.1 times [43]. Regarding specific adult-life stressors, job strain increases by 1.74 times the chances

of developing depression in the early future [44]. Stress, both in early and adult life, is thus one of the most important etiologic factors associated with depression.

Stress is an allostatic response of the organism in adaptation to an external or internal stimulus that demands adjusted physiological functions to keep the organism's equilibrium [45]. In the case of potentially threatening stimuli, the stress response involves the activation of the amygdala which recruits contextual and executive information from the hippocampus and mPFC, respectively, in order to identify the stimulus as a threat [46–48]. The amygdala relays efferent information to the hypothalamus, resulting in the activation of the hypothalamus-pituitary-adrenal (HPA) axis that culminates with the release of catecholamines (autonomic nervous system response) and glucocorticoids (GC) from the adrenal cortices (e.g. cortisol, as part of the endocrine response) [48]. Glucocorticoid receptors (GR) are wildly expressed across several brain regions, especially the hippocampus, whose activation by GC after crossing the blood-brain barrier (BBB) induce a negative feedback response that leads to the inhibition of the HPA axis, terminating the stress response [48]. Downregulation of GR is one of the factors hindering the negative feedback of the stress response which, in combination with other factors, can lead to a condition known as GC resistance [49]. The resulting hypercortisolism is associated with increased inflammatory response [50] and reduced neurotrophic support [51,52], conditions that have been linked with depression and several other psychiatric and neurological disorders [53] (the highly relevant interaction between GC signaling and neurotrophic support is discussed in subsection *ii*). Indeed, hypercortisolism is commonly observed in depressed patients, mostly when it is marked by melancholic symptoms [54].

Experimentally, there is sufficient evidence linking stress as a causative factor in depression development. In experimental research, exposing animals to stressors for a long period (3 to 6 weeks) is among the most commonly used method for induction of depression-like behavior, such as in the chronic unpredictable mild-stress animal model of depression [55]. Interestingly, depression-like behavior can also be induced by solely injecting corticosterone (the cortisol's correlate in other animals) for 21 days, mimicking the effects of chronic stress exposure [56]. Accordingly, patients with Cushing's syndrome, which results from the chronic exposure to high levels of glucocorticoids, present lower hippocampal volume accompanied by cognitive dysfunction and increased depressive symptoms [57]. When the syndrome is due to tumor-induced hypercortisolism, both cognitive and depressive symptoms are expressively recovered after surgery [57]. However, would then be reasonable to conceive depression solely as a hypercortisolemic disease? Very unlikely, given the lack of evidence for the use of antiglucocorticoids for depression treatment [58]. Would, then, trauma and/or chronic stress lead to pathophysiological alterations that eventually result in depression?

2. Hippocampal pathophysiology in depression

As mentioned in the previous subsection, GC are capable of crossing the BBB, and GR are accordingly distributed throughout the entire brain, which allows GC to modulate multiple networks. The peculiarly increased expression of GR in the hippocampus, however, makes it especially vulnerable to pathological levels of GC. Indeed, decreased hippocampal volumetric changes have been consistently observed in depressed patients, as recently reported by the largest meta-analysis combining neuroimaging studies, the ENIGMA consortium [6]. The hippocampus is part of a functional system called hippocampal formation, divided into the hippocampus proper (composed of the sub-regions *Cornu Ammonis* [CA] 1, 2, and 3) the entorhinal cortex (EC), dentate gyrus (DG), and subiculum as its main regions[1]. The entorhinal cortex is where most of the afferent inputs arrive from cortical and subcortical regions to be then processed in the intrinsic hippocampal formation circuitry. Roughly, this circuitry is formed by the perforant pathway (projecting from the EC to DG and CA3 regions), the mossy fibers (projecting from the DG to CA3), and the Schaffer collaterals (projecting from CA3 to CA1) [59]. Extrinsically, the hippocampus is part of a network conventionally named as the limbic system in which structures such as the amygdala, mPFC, and hippocampus proper are reciprocally connected [60–63]. Plasticity of such intrinsic and extrinsic hippocampal connections has long been associated with cognition (especially episodic and spatial memory and navigation) and mood regulation [64–66]. On the other hand, disruption of this network has been consistently associated with cognitive impairment and mood disorders [67–69]. Hippocampal atrophy, as we shall see, can be considered a biomarker of depression and is associated with disease severity and treatment resistance.

Such hippocampal alterations were shown to be already present in 4-7 years-old, depressed children [70] and drug-naïve first-episode depressed patients, where a roughly 4% hippocampal volumetric reduction was reported in both hemispheres [71], suggesting hippocampal alterations are likely to precede depression onset. Accordingly, late-life depression was also associated with higher cortisol levels and proportionally smaller hippocampal volumes [72], placing altered hippocampal physiology as a common depression feature across the lifespan. Recently, the use of positron emission tomography (PET) of the synaptic vesicle glycoprotein 2A (SV2A) radioligand made it possible to show the first human *in vivo* evidence correlating lower hippocampal and PFC synaptic density with disease severity [8]. MDD patients were shown to have 17% lower hippocampal synaptic density compared with healthy controls and reduced intrinsic connectivity within the dorsolateral PFC [8]. The degree of structural disruption is of clinical relevance, given that the severer the hippocampal structural

[1] For the purpose of this work, the terms hippocampus and hippocampal formation will be used interchangeably.

loss is, the lower is the chance of responding to antidepressant interventions [73]. Patients with hippocampal volume 10% below the average for depressed patients have a roughly 30% chance of responding to medication [73]. Accordingly, the rapid antidepressant effects of ketamine, which is more expressive in treatment-resistant patients, were also shown to be more evident in patients with lower hippocampal volume [74].

Interestingly, the DG region appears to be especially affected in depression. Even when total hippocampal volume remained unchanged in comparison with healthy controls, depressed patients still had significantly lower DG volume [68]. Stress is known to be a recurrent disease and every relapse episode is estimated to increase the change to the next relapse in 16% [75]. This cumulative relapse burden may be associated with progressive damage incurred to the hippocampal formation. Indeed, the number of depressive episodes was shown to be specifically associated with lower DG volumes [67]. Moreover, DG atrophy might also be sensitive to antidepressant treatment, given that unmedicated patients had significantly lower DG when compared with medicated patients [76].

Can traumatic events and/or chronic stress be liked with such pathophysiological alterations that underpin depression?

i. *The neurotrophic model of stress-induced depression*

A large body of experimental evidence demonstrates increased levels of GC plays a direct role in neuronal atrophy both in the hippocampus and mPFC. In rodents, it can be demonstrated by the exogenous application of corticosterone (CORT, usually in daily subcutaneous injections), the species-specific GC in rodents [77]. In the mPFC, both behavioral stress and chronic CORT administration were shown to decrease dendritic complexity and spine density in the apical dendrites of the infralimbic (IL) mPFC neurons [78–80], which was associated with impaired fear-memory extinction [79]. In the hippocampus, the stress-induced morphological changes are two-fold, and they were noticed before the mPFC investigations. Both chronic restrain stress and chronic CORT administration lead to neuronal atrophy by specifically reducing dendritic complexity of the CA3 pyramidal cell apical dendrites, without affecting other CA regions or the DG [81,82]. Interestingly, decreased excitatory transmission by daily phenytoin treatment (antiepileptic drug) prevented such neuronal atrophy [82]. Although chronic stress did not affect DG dendritic complexity, GC are important regulators of the DG neurogenesis [83]. As firstly observed, adrenalectomy increased neurogenesis in the DG, whereas replacement of circulating GC by CORT treatment prevented such effect [84]. Later, it was shown that the stress modulation of neurogenesis was also mediated by excitatory transmission, given that the MK-801-pharmacological blockage of the NMDA receptors prevented the stress-induced decrease in neurogenesis [85]. As previously mentioned, the DG projects to the CA3 region through the mossy fibers

(densely targeting the CA3 apical dendrites), forming a system very relevant for episodic memory that is expressively impaired in chronic-stress induced depression [86]. The experimental evidence on brain effects of chronic stress, therefore, vastly resembles the clinical observations concerning hippocampal (and mPFC) atrophy observed in depressed patients.

Such direct effect of GC on regional and neuronal atrophy led to the hypothesis that stress could be impairing neurotrophic support, as neurotrophins have long been implicated in neuronal growth and survival, structural plasticity, and synaptic efficiency [87–89]. Mature neurotrophins bind to the tyrosine receptor kinase (Trk, with A, B, or C subtypes) in both pre and postsynaptic terminals, whose downstream signaling pathway involves the activation phospholipase Cγ (PLC-γ), extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K) [87,90]. The synthesis, trafficking, and secretion (both by pre- and postsynaptic terminals) of brain-derived neurotrophic factor (BDNF, which will be hereafter discussed) were shown to be modulated by neuronal activity in hippocampal neurons both *in vitro* and *in vivo* [91–94], suggesting BDNF plays an important role in activity-dependent synaptic plasticity. Indeed, BDNF is known to modulate synaptic potentiation both by increasing presynaptic transmitter release and postsynaptic receptor activity [95], which was shown (for instance) in the capacity of BDNF to convert otherwise short-term synaptic potentiation in long-term potentiation (LTP) [96], increase the frequency and amplitude of excitatory postsynaptic currents (EPSC) through postsynaptic phosphorylation of TrkB receptors [97], or evoke postsynaptic Ca^{2+} transients that led to NMDA-dependent LTP formation when paired with weak synaptic stimulation [98]. Especially, several Trk-signaling cascades are upstream to the cAMP response element binding protein (CREB), a stimulus-induced transcription factor responsible for converting the neuronal activity into the structural changes necessary to activity-associated demands [99], pointing towards the role of BNDF in structural plasticity. Not surprisingly, hence, BNDF was not only shown to increase synaptic density in cultured hippocampal neurons as a result of the ERK-signaling pathway activation [100] but also to increased hippocampal neurogenesis when infused in the DG (two weeks) in healthy animals [101].

It is striking how BDNF seemingly counteracts all stress-induced neuronal atrophy observed in clinical and experimental models of depression. Indeed, hippocampal BDNF insufficiency (specifically in the DG region) was sufficient to elicit depression-like behaviors [102]. Not surprisingly, stress severely disrupts BDNF signaling. Within the hippocampus, the DG regions appear to be especially sensitive, where *Bdnf* mRNA levels were markedly decreased following acute (2h) or chronic (7 d) restrain stress [52]. The same observation was made regarding the BDNF protein expression in the DG after chronic mild stress, which also disrupted the transcriptional regulator calcium/cyclic-AMP responsive binding protein (CREB) signaling [51]. *In vitro,* the BNDF-mediated increase in

glutamatergic postsynaptic proteins was inhibited after chronically exposing (48 h) cultured cortical cells to the GR agonist, Dexamethasone [103].

The mechanisms by which GC hinders neurotrophic support are still unclear, although current evidence supports both signaling systems are intertwined. In vivo, acute GC treatment (6 h) can activate the BDNF receptors tropomyosin receptor kinase type B (TrkB) in the hippocampus (especially in the DG region) through the genomic activity of the GR, which resulted in a neuroprotective effect in cultured hippocampal cells [104]. It was later demonstrated that the TrkB and GR indeed interact with each other (as shown by coprecipitation), which was necessary for the BDNF-induced glutamate release [105]. Remarkably, the GR-downregulation due to chronic GC exposure (a biomarker of GC resistance) resulted in lower TrkB-GR interaction, which suppressed the BDNF-induced glutamate release by reducing the BDNF-TrkB activation of downstream signaling pathways (specifically the PLC-γ) [105]. Although not completely understood, GR dynamics are likely involved with the stress-induced disruption of neurotrophic support.

Chronic stress can, therefore, elicit neuronal atrophy at least by two interconnected mechanisms, by reducing glutamatergic transmission [106] and disrupting neurotrophic support. Such coincidence of mechanisms associated with stress and BNDF led to the proposition of a neurotrophic model of stress-induced depression [36,37]. Even though GC and BDNF dynamics can be beautifully organized as causative factors in depression onset and relief (respectively), it is worthy to remind the reader that there is no single mechanism currently know capable of explaining all aspects of depression, neither is there one that could once and for all cure the disease. BDNF signaling in response to stress is very dynamic across different brain regions [107] and the association of its peripheral levels with depression is still controversial [108]. The understanding of such mechanisms, nonetheless, paved the way for increasingly more efficacious treatments.

3. Pharmacological approaches for depression treatment

i. *Conventional treatments: targeting the monoaminergic system*

The monoamine hypothesis of depression was born in the ’50s, when a small portion of hypertensive patients chronically treated with reserpine, an agent know to deplete monoamines, developed depression [109]. This finding combined with some therapeutic success of the monoamine oxidase inhibitors (MAOI) in treating depression and, later, the selective serotonin reuptake inhibitors (SSRI), set the stage for considering depression from a biochemical perspective and raised the hypothesis of it being the consequence of a deficiency in the monoamine system transmission [110,111]. Since then, the use of monoamine-based pharmacotherapy remains the first-line, recommended

treatment for moderate and severe major depressive disorder [112]. To date, there are more than 40 antidepressants available, most of them somehow targeting the monoaminergic system [113]. Although monoamine-aiming antidepressants are genuinely more efficacious than placebo [114] and are capable of counteracting the hitherto mentioned neuropathological characteristics of depression [115], they left behind a considerable body of research pointing out two major gaps in the monoamine hypothesis of depression: monoamines do not broadly account for the pathogenesis of depression, neither do they account for the mechanisms of action of monoamine-based antidepressants [36].

Concerning the first objection, it has been known that monoamine depletion predisposes only previously depressed patients to relapse, but fail to induce depression in health volunteers [116,117]. Therefore, the monoaminergic system is involved in the sustained antidepressant response but is less likely to be involved in the pathogenesis of the disease. The second objection is sustained by the observation that such pharmacological intervention increases the monoaminergic system activity within hours of administration, whereas therapeutic response requires weeks to take place [34,36]. Moreover, current response rates to pharmacotherapy are not superior to 50%, with 20-30% of patients remaining treatment-resistant after different pharmacological and augmentation approaches [4,118,119]. It suggests chronic monoaminergic upregulation secondarily triggers the systems and/or signaling pathways responsible for the therapeutic response, instead of being the direct mechanism by which disease recovery is achieved. Indeed, chronic but not acute antidepressant treatment with monoaminergic drugs led to increased hippocampal BDNF, TrkB, and CREB mRNA expression [120,121], which markedly agree with the time course for those antidepressants action. Likewise, BDNF deficiency in heterozygous BDNF null mice [122], as well as the specific BDNF knock-out in the DG (Adachi, Barrot, Autry, Theobald, & Monteggia, 2008), were shown to hinder antidepressant responses to common monoaminergic drugs.

This accumulated body of evidence naturally raises the question of whether directly targeting a system more closely associated with the neurotrophic signaling would result in faster antidepressant response. Remarkably, a single BDNF infusion into the DG elicited antidepressant effects within 3 days, which lasted over ten days and were comparable to chronic monoaminergic treatment [123], opening the field for the investigation and development of a new class of antidepressants.

ii. *Rapid-acting antidepressants: targeting the glutamatergic system*

At exacts 20 years ago, the first double-blind, controlled trial revealed a single intravenous infusion of a subanesthetic dosage of ketamine (0.5 mg/kg over 40 min), a non-selective NMDA receptor antagonist, significantly improved depressive symptoms within 3 days, with effects lasting for up to 2 weeks [124]. Such fast and long-lasting antidepressant response was later confirmed in

treatment-resistant patients, where 71% of the patients achieved responsiveness (>50% of symptom improvement) after ketamine infusion [125]. Since its seminal discovery as a rapid-acting antidepressant, ketamine has been intensively investigated both in clinical and experimental settings [90,126,127], culminating with the recent FDA approval of intranasal ketamine (Esketamine) for treatment-resistant depression [128].

Observations of the ketamine-induced increase in glutamate response at subanesthetic dosages came a few years earlier to its breakthrough. Systemic ketamine injection at subanesthetic dosages was shown to acutely increase extracellular glutamate concentration in rats' PFC over 100 min [129]. However, such observation was initially linked as one of the mechanisms associated with the ketamine-induced spatial memory deficit. Leveraged by the clinical findings, experimental research soon linked the acute ketamine-induced glutamate burst with the activity-dependent activation of neurotrophic signaling [130]. Mediated by AMPA receptors activation, ketamine (10 mg, i.p.) acutely (30 min) increased the expression of synaptic proteins in the mPFC accompanied by increased synaptic efficiency and spinogenesis (24 h) [131], which resulted in the recovery of neuronal atrophy and depressive phenotype in chronically stressed mice [132]. Such effects were reproduced by selectively blocking the NMDA receptor subunit 2B (NR2B), and were dependent on the Akt- and ERK1/2-induced activation of the mammalian target of rapamycin (mTOR) pathway, which was not observed after acute SSRI treatment [131]. Ketamine's effect was shown to extend to 1 week after a single injection and to be absent in BNDF or TrkB knockout mice [133]. The acute effects (30 min) were shown to be dependent on the rapid increase of BDNF translation (but not transcription), whereas the long-lasting effect (24 h) relied on subsequent synaptic plasticity associated with protein synthesis [133]. The relevance of AMPA receptors in activating such process was later elucidated by showing that ketamine stimulates BDNF release by the activation of AMPA receptors and, subsequently, L-type voltage-dependent calcium channels (VDCC) [134]. Finally, BDNF was confirmed as a key factor in translating NMDAr blockage into rapid antidepressant effects when Memantine, a diverse although equally potent NMDAr antagonist that does not increase BDNF signaling, failed to induce rapid-acting antidepressant effects [135]. Indeed, different agents with rapid-acting antidepressant properties also require BDNF release, strongly suggesting it and its downstream signaling cascade as a convergent mechanisms for rapid-onset antidepressant response [90].

The different relevance of the hippocampus and mPFC in depression pathogenesis and treatment is still under debate. Although rapid antidepressant effects have increasingly been restricted to mPFC neuroplasticity [136], the hippocampus neuroplasticity remains as a key factor in depression pathogenesis [137]. Increased neurogenesis, for instance, was shown to buffer the stress-induced brain atrophy and protect against depression [138]. It is thus conceivable that stress-induced neuronal atrophy

may disrupt a hippocampal-to-mPFC network. Remarkably, ketamine was shown to increase BDNF signaling [139,140] and induce sustained hippocampal activation, specifically in the ventral portion [141]. Surprisingly, inhibition of the ventral hippocampus by the time of ketamine administration selectively blocked its long-lasting (7 d) antidepressant effect, leaving intact its rapid-acting antidepressant properties [21]. Taken together, such evidence suggests that although the mPFC was shown to be necessary for the acute and long-lasting antidepressant effect of a single dose of ketamine [136], it is very likely that "secondary" effects in the hippocampus would eventually restore such hippocampus-mPFC network as a necessary step for depression remission.

4. Frontotemporal connectivity

The vHipp has long been recognized as a source of afferent inputs to the mPFC [63,142,143]**.** Specifically, the ventral CA1 and subiculum regions send unidirectional and ipsilateral excitatory projections that predominantly innervate the prelimbic (PL) and infralimbic (IL) mPFC sub-regions [142,144]. It is estimated that roughly 93% of the vHipp projections innervate mPFC pyramidal neurons, with the GABAergic neurons accounting for the remaining 7% [144]. Specifically, corticocortical and cortico-amygdala pyramidal neurons are targeted in the IL (in the cortical layers 2/3 and 5), whereas mostly corticocortical neurons are targeted in the PL (mainly in cortical layer 5) [145]. Among the GABAergic neurons, the parvalbumin (PV+)-, somatostatin (STT+)-, and vasoactive intestinal peptide (VIT+)-expressing interneurons are the major GABAergic-targeted populations of mPFC interneurons [146]. Such vHipp projections were shown to elicit NMDA-dependent synaptic plasticity upon mPFC neurons through BDNF-dependent pathways, modulating different classes of behavior and cognition relevant to psychiatric disorders [69,144,147,148].

Alterations in this pathway are associated with aspects of schizophrenia [149], autism [149], anxiety [150], stress-related disorders [145], and depression [21]. Concerning the relevance of this network for depression, specifically, the integrity of the vHipp was shown to be essential for the sustained antidepressant effects of ketamine. As previously stated, bilateral disconnection both vHipp and mPFC blocked ketamine's sustained but not acute antidepressant effect, whereas both optogenetic and chemogenetic activation of the vHipp-targeted neurons in the mPFC resembled ketamine's antidepressant response [21]. Moreover, ketamine induced a sustained neuronal activation of the vHipp (as shown by increased ΔFosB signaling), which mediated the ketamine's prophylactic effects in stress-induced depression [141]. Likewise, clinical observations showed that hippocampal metabolism is not affected until two weeks and four applications of ketamine in depressed subjects [151], which

contributes to an understanding of the hippocampus as being involved in the long-lasting antidepressant effects of ketamine.

As one can see, not only volumetric but also white-mater changes are associated with depression, suggesting loss of connectivity between the prefrontal cortex and the hippocampus underlies depression [10,11]. This suggests that potential new antidepressants should be able to modulate and eventually restore connectivity between relevant brain systems, especially the vHipp-mPFC pathway, to elicit a full and long-lasting recovery.

5. Adiponectin

As previously stated at the beginning of this chapter, stress is an allostatic response that elicits physiological adaptations to support the required behaviors needed to cope with a potentially threatening stimulus [45], the so-called "fight or flight" response. Stress and metabolism are, therefore, intrinsically connected. It is conceivable then that pathological levels of stress would eventually lead to metabolic dysfunctions. Indeed, acute, severe stress is linked with anorexigenic effects and weight loss, whereas chronic stress is linked with orexigenic effects and increased adiposity partially induced by hypercortisolism [152]. Chronic stress, specifically, is strongly associated with metabolic dysfunctions, including obesity and metabolic syndrome [153,154]. Although the mechanisms by which chronic stress disrupts the metabolic systems are still under debate, it is important to bear in mind that the adipose tissue functions as an endocrine organ secreting a plentitude of factors involved with metabolic regulation. Dysregulation of such factors, being it the consequence of genetic predisposition, poor lifestyle choices, or environmental conditions (e.g. stress) eventually result in metabolic disorders [155]. Noteworthy, metabolic disorders are very often comorbid with neurological conditions, including cognitive impairments and mood disorders [156]. The capacity of such adipocyte factors (i.e. adipokines) to cross the BBB and their broad receptor expression across different brain systems place them as the mediating link between peripheral metabolism and brain function [157].

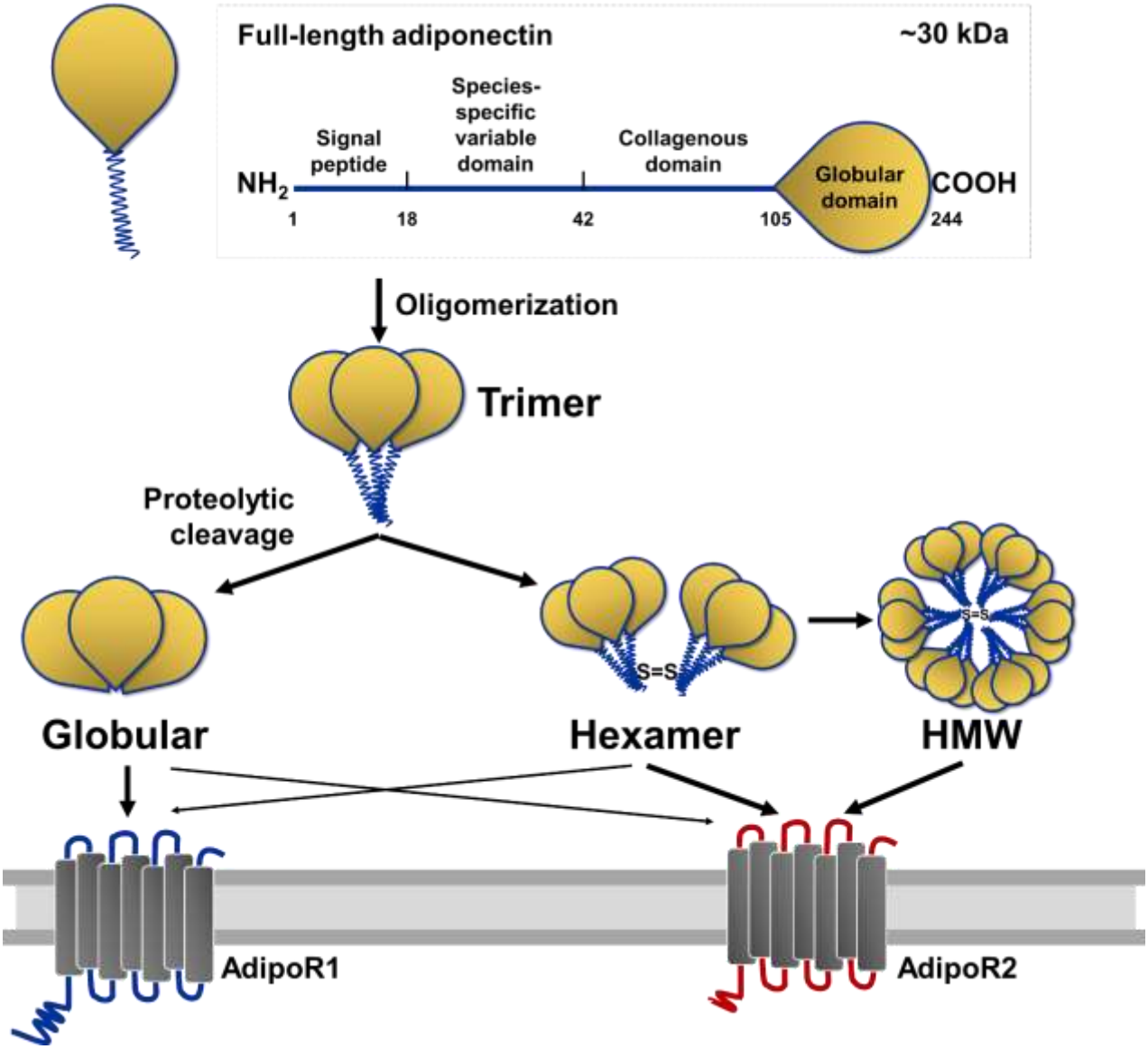
Adiponectin is one of such adipokines that, since its isolation in the '90s, has been implicated in the organism's energetic metabolism [158,159]. It has the peculiarity of being the most abundant plasma protein, circulating in up to three-fold higher concentrations than other regulatory factors, and whose circulatory levels are inversely proportional to the adipocyte size [160,161]. Lower circulating adiponectin levels are hence a hallmark of increased adiposity-associated diseases, such as obesity and diabetes [27,162,163]. Noteworthy, these conditions are usually comorbid with depression [164], which has been consistently associated with lower peripheral adiponectin levels as compared with healthy controls [165,166].

Likewise, evidence suggests pharmacological and non-pharmacological antidepressants are associated with restored adiponectin levels [33]. Adiponectin is, therefore, a representative factor involved with depression and is also permeable to the BBB. Nonetheless, would adiponectin signaling interact with the glutamatergic system and potentially induce a rapid antidepressant response?

i. *Adiponectin signaling*

Adiponectin synthesis is controlled by a multitude of transcriptional factors, including PPAR-γ, and undergoes several post-transcriptional and post-translational steps involving different chaperones before its secretion [167]. Adiponectin synthesis and secretion are inversely proportional to the visceral adipose depot [168] and by the adipocyte size [169], although the mechanisms for such negative modulation remain elusive. Structurally, the 244-aminoacid protein consists of a globular domain, a collagen-like domain, a species-specific domain, and a signal peptide (e.g. full-length form) [159,161]. The full-length adiponectin undergoes oligomerization and circulates as a trimer, hexamer, and high-molecular-weight multimers (HMW), whereas trimers can be cleaved and circulate as globular adiponectin (Figure 1) [33,159]. Although different tissues are more sensitive to specific isoforms [28], both full-length and globular adiponectin improve glucose metabolism as an insulin sensitizer and through increased fatty-acid oxidation in the liver and skeletal muscle, which counteracts hyperglycemia in obese, type and type 2 animal models of diabetes [26–28,170]. Along with its main role as an insulin sensitizer, adiponectin has been implicated in several other functions (fully reviewed in [161]), including a role as an anti-atherosclerotic agent by stimulating angiogenesis [171].

Figure 1. Adiponectin



Full-length adiponectin (~30 kDa) consists of a globular domain, a collagenous domain, a species-specific domain, and a signal peptide. Oligomerization facilitates the formation of the trimer, hexamer, and high-molecular-weight (HMW) adiponectin. Full-length adiponectin can undergo proteolytic cleavage, whose proteolytic fragment corresponds to the globular adiponectin. AdipoR1 has a greater affinity for the globular form, whereas AdipoR2 has a moderate affinity for both globular and full-length forms [33].

Adiponectin exerts its physiological functions through the activation of two identified receptors AdipoR1 and AdipoR2 [172]. Whereas AdipoR2 has a moderate affinity for both adiponectin isoforms, AdipoR1 has a higher affinity for the globular adiponectin and a lower affinity for the full-length adiponectin [172]. Both receptors interact with an adaptor protein containing a pleckstrin homology domain, a phosphotyrosine domain, and a leucine zipper motif referred as APPL1 [173], whose downstream signaling cascade results in the phosphorylation of the (AMP)-activated protein kinase

(AMPK), resulting in reduced gluconeogenesis and increased fatty-acid oxidation [174]. Moreover, the adiponectin receptors activation also increases the expression and activity of the peroxisome proliferator-activated receptor alpha (PPARα) transcription factor as one of the main mechanisms involved with increased fatty acid oxidation and energy expenditure [173,174]. Finally, APPL1 also interacts with the insulin receptor substrate (IRS)-1/2, increasing insulin signaling [173].

Through AdipoR1/2-APPL1 interactions, adiponectin has also been shown to activate pathways and secondary messenger in peripheral tissues that have been involved in the aforementioned neurotrophic support. In muscle, adiponectin was shown to increase intracellular Ca^{2+} by stimulating its release from intracellular reserve pools [175] and by inducing extracellular Ca^{2+} influx [176]. As part of its anti-atherosclerotic properties, adiponectin induced angiogenesis through activation of the PI3K-Akt pathway [171]. On the other hand, adiponectin increased phosphorylation of the CaMKII and p38-MAPK, resulting in increased expression of vascular endothelial growth factor-C (VEGF-C) and lymphangiogenesis [177]. Such adiponectin's proliferative effects have been previously observed in different tissues, where AdipoR1/2-induced activation of the Src/Ras/ERK1/2 pathway was identified as the main signaling pathway mediating this mechanism [178]. As reviewed previously, increased intracellular Ca^{2+}, CaMKII, and, specifically, the PI3K-Akt and Ras-ERK pathways are known pathways mediating the BDNF neurotrophic and, consequently, antidepressant effects. Naturally, such observation led to the driving question of this research: would adiponectin have similar rapid-acting antidepressant properties by acting in the central nervous system?

ii. *Changes in peripheral adiponectin levels in patients with depression*[2]

An association among peripheral adiponectin levels and MDD has been suggested in different populations and health conditions (Table 1). A study consisting of cross-sectional (n = 575) and longitudinal (n = 262) analyses have shown that current episode of MDD, symptom severity, and history of depression in middle-aged women were all linked to the low adiponectin levels over a 5-year follow-up [166]. In depressed women, the adiponectin levels were sharply reduced by 25% and remained low over a 24-h period when measured hourly [179]. The correlations between low adiponectin levels and depression severity were also shown in men [180] and elderly patients [181]. This adiponectin-depression relationship was briefly summarized by a recent meta-analysis, illustrating a significant

[2] This and the following topics (5.2 to 7) were published as a review paper in Formolo, D.A., Lee, T.H. & Yau, S. Increasing Adiponergic System Activity as a Potential Treatment for Depressive Disorders. Mol Neurobiol 56, 7966–7976 (2019). https://doi.org/10.1007/s12035-019-01644-3

decrease in the adiponectin levels in depressed patients compared to controls, in both males and females [165].

Adiponectin is also known as an anti-inflammatory cytokine. Metabolic disorders and cardiovascular diseases are marked with altered adiponectin levels [182,183]. Coincidentally, depression is often comorbid with these disease states [184]. A large cross-sectional study (n = 1,227) reported a correlating trend (p = 0.09) of the reduced adiponectin levels in early-stage T2DM and the severity of depression [185]. This association was later confirmed by two other studies, in which both high molecular weight to total adiponectin ratio [163] and total adiponectin concentrations [164] were correlated to the severity of depression in T2DM, but not in T1DM. In ischemic stroke patients, lower adiponectin levels at admission increased three times the risk of developing post-stroke depression [186]. In hepatitis C patients, higher adiponectin levels were associated with a lower incidence of MDD [187].

Interestingly, in consonance with the heterogeneity of depressive disorders, peripheral adiponectin levels might not be a ubiquitous biomarker for all depressive states. In a study sampling subjects in another life stage, primarily adolescents aged 11 to 18 years old, comparable adiponectin levels were found among depressed and healthy controls [188]. Additionally, adiponectin levels increased along with the pregnancy and the postpartum period with no correlation with the incidence of depressive symptoms [189]. This idea is also illustrated in rodent studies using different depressed animal models. In depressed mice models induced by chronic unpredictable mild stress [190] or chronic corticosterone administration in drinking water [191,192], the peripheral levels of adiponectin were not reduced. On the other hand, the depressed mice model induced by chronic social defeat stress significantly reduced peripheral adiponectin levels, which was inversely correlated to the increased severity of depressive behavior [30,193]. These differences in rodent studies are likely due to the variations in inducing depressive-like behaviors.

In summary, the evidence so far has suggested that decreased peripheral adiponectin levels can potentially be linked to major depressive disorder and depression co-morbid with some metabolic and cardiovascular disorders.

Table 1. Changes in peripheral adiponectin levels in patients with depression

Authors (year) (ref)	Study design	Population		Associations	
		Sex (n)[#]	Associated condition	Depression indices	Adiponectin levels
Everson-Rose et al. (2018) (39)	Cross-sectional	♀ (575)	-	Current depression Symptom severity History of depression	↓
Cizza et al. (2010) (40)	Case-control	♀ (23)	-	History of depression	↓

				Cumulative duration of depression	
Leo et al. (2006) (41)	Case-control	♀ ♂ (32)†	-	Current depression Symptom severity	↓
Diniz et al. (2012) (42)	Case-control	♀ (37) ♂ (10)	Elderly subjects	Current depression Symptom severity	↓
Laake et al. (2014) (47)	Cross-sectional	♀ (793)‡ ♂ (976)	T2DM	Current depression	↓ (trend p = *0.09*)
Herder et al. (2017) (48)	Cross-sectional	♀ (55) ♂ (84)	T1DM	Symptom severity	No association
		♀ (97) ♂ (198)	T2DM	Symptom severity	↓
Herder et al. (2018) (38)	Cross-sectional	♀ (227) ♂ (162)	T1DM	Symptom severity	No association
		♀ (88) ♂ (116)	T2DM	Symptom severity	↓
Yang et al. (2018) (49)	Cohort	♀ (117) ♂ (138)	Ischemic stroke	Poststroke depression at 3 months	↓ at baseline
Fábregas et al. (2016) (50)	Cohort	♀ (26) ♂ (24)	Hepatitis C	MDD at 3 months	↓ at baseline
Tunçel et al. (2016) (51)	Case-control	♀ (23) ♂ (7)	Adolescents (11-18 y.o.)	Current depression	No association
Rebelo et al. (2016) (52)	Cohort	♀ (177)	Pregnant women	Perinatal depression	No association

[#] *Total n* number for cross-sectional and cohort studies, *n* number of *Cases* for case-control design.
† Individual numbers of males and females are not reported.
‡ From the total sample of 1,769 subjects (male and female), 1,227 were included in the analysis.

iii. *Central and peripheral modulations of the adiponergic pathway on antidepressant effects*

The effects of antidepressant treatments over peripheral adiponectin levels are controversial in clinical studies. A short treatment period of 4 to 5 weeks by several classes of antidepressant drugs did not largely affect [188,194,195], but with chances of reducing [196], adiponectin levels in depressive patients. On the other hand, MDD-remitted patients who had undergone selective serotonin reuptake inhibitor (SSRI) or serotonin-norepinephrine reuptake inhibitor (SNRI) treatments for at least six months showed increased levels of adiponectin and decreased levels of tumor necrosis factor alpha (TNF-α) when compared to healthy, matched controls [197]. Nonetheless, the improvements in depressive symptoms after long-term non-pharmacological, behavioral-cognitive therapy for T1DM and T2DM with comorbid depression and distress were not associated with increased adiponectin levels in the 12-months follow-up [198]. The fact that adiponectin is the most abundant plasma protein

could explain these divergences, given that it may hinder the detection of subtle changes, leaving only substantial alteration in the peripheral adiponectin levels as statistically detectable.

From another perspective, peripheral manipulation of adiponectin levels appears to be effective in eliciting an antidepressant effect in rodents. Rosiglitazone is a known effective antidiabetic agent, selectively agonizing the peroxisome proliferator-activated receptor gamma (PPARγ), an upstream positive regulator of adiponectin [199]. Rosiglitazone cannot bypass the blood-brain barrier. Systemic administration of rosiglitazone resulted in adiponectin-dependent antidepressant response in mice [193]. Moreover, triple systemic administrations of rosiglitazone within 24 hours could significantly increase peripheral adiponectin levels, which was necessary and sufficient to elicit an antidepressant response in non-depressed mice [193].

Besides, rodent studies have not only demonstrated the necessity of adiponectin in exercise- and environment-induced antidepressant effects [31,192,200] but also hinted on the fact that increased adiponectin level in the central nervous system is a potential biomarker associated with the referred antidepressant effect. Particularly, 14 days of voluntary wheel running induced antidepressant effects in wild-type mice with increased adiponectin concentrations in the hippocampal DG, but not in the serum (60). Similarly, environmental enrichment prevented anxiety and depression-like states in chronically-stressed mice with a four-fold increase in the cerebrospinal adiponectin levels, whereas plasma adiponectin levels remained unchanged [192]. These animal studies shed light on the possible roles of the adiponectin signaling system in inducing antidepressant effects [201,202].

Importantly, direct activation of the central adiponergic pathway showed acute antidepressant effects. Confined activation of the central adiponergic pathway by overexpressing adiponectin [31] or i.c.v. infusion of recombinant adiponectin consistently elicited antidepressant responses [30,192,200]. Strikingly, these animal studies also showed that the adiponectin-induced antidepressant response is observable within hours. A single i.c.v. infusion of adiponectin resulted in a rapid antidepressant response within 30 min [30], 2 h [192], and 24 h [200] in both depressed and non-depressed mice.

AdipoRon is an orally active molecule that selectively agonizes the AdipoR1 and AdipoR2 [203]. As adiponectin, it exerts antidiabetic [203], anti-inflammatory [204], and cardiovascular protective properties [205]. AdipoRon can also bypass the blood-brain barrier [191] and act on brain regions like the hippocampus [206] and the ventral tegmental area [207]. Congruently, a single systemic injection of AdipoRon (0.5, 1, or 5 mg/kg) resulted in a transient antidepressant response within 1 h in several animal models of depression [191]. Altogether, this data indicate that targeting adiponectin receptors and activating the adiponergic pathway are potential strategies for rapid-acting antidepressant drugs.

6. Potential mechanisms of the antidepressant effects of adiponectin

i. *Effects of adiponectin on neurogenesis*

In the adult mammalian brain, the sub-granular zone of the hippocampal DG contains a reservoir of neural stem cells. Granule neurons are continuously generated from these progenitors via adult hippocampal neurogenesis [208–213], which can integrate into the existing neural circuit [214–217]. Conventionally, depression is closely related to brain structure integrity [11], increased cellular stress [218], and increased dendritic and spine atrophy [218]. It was further postulated that adult hippocampal neurogenesis could antagonize stress and depression [219]; concurrently, antidepressant drugs are effective in promoting adult hippocampal neurogenesis [220–223]. Given so, the endeavor to reveal the role of adiponectin in structural plasticity were made thereafter.

Current opinion towards adiponectin is far more than an adipocyte-secreted endocrine hormone, but a neurotrophic factor, such that disruption of the adiponectin signaling pathway in the hippocampus impairs neurogenesis and cognitive functions [31,224,225]. The neurotrophic effect of adiponectin was first demonstrated in adult hippocampal stem cells, which expressed both AdipoR1 and AdipoR2 [226]. Adiponectin promoted proliferation, but not differentiation nor survival, *in vitro* via the p38 mitogen-activated protein kinases (MAPK)/glycogen synthase kinase (GSK)-3β/β-catenin signaling pathway [226]. An adiponectin null mutant had reduced cell proliferation, differentiation, and survival in the hippocampus [224], whereas infusing adiponectin [224] or overexpressing adiponectin [31] in the mouse brain could promote cell proliferation in the hippocampal DG.

Physical exercise promotes adult neurogenesis in the hippocampus [227,228]. It induces the release of neurotrophic factors such as the brain-derived neurotrophic factor (BDNF) [229,230] and the insulin-like growth factor-1 (IGF-1) [231]. Rodents perform better in spatial recognition [232,233] and have better executive functions [234] after exercise. In the study dissecting the role of adiponectin in exercise-induced antidepressant effect, the exercise-induced adult hippocampal neurogenesis was abolished in adiponectin-deficient mice [31]. The role of adiponectin as a mediator in exercise-promoted adult hippocampal neurogenesis is re-confirmed using streptozotocin to induce diabetes in adiponectin-deficient mice. Exercise could restore impaired hippocampal neurogenesis in wild-type diabetic mice, but not in adiponectin-deficient diabetic mice [225]. The neurogenic effects are possibly mediated by activating the AdipoR1/APPL1/AMPK pathway as shown by Yau and colleagues [31].

ii. *Effects of adiponectin on dendritic complexity and spinogenesis*

Synaptic connections between neurons are predominantly tied up by dendritic spines. Spinogenesis is precisely regulated in response to stress, which consequently promotes the rewiring

of the neural network [235]. Depression is associated with dendritic spine pathology in the hippocampus [236–238]. Reciprocally, spinogenesis is often dysregulated in chronically stressed animals [239,240]. Antidepressants can reverse spine and dendrites atrophy in animal models of depression [241,242], leading to the idea that dendritic and spine atrophy could contribute to symptoms of depression [106,243,244]. Therefore, unraveling the role of adiponectin in spinogenesis can shed light on depression.

In this regard, adiponectin promotes dendritic growth, arborization, and spine remodeling in the hippocampal DG [224]. Adiponectin null mutants had a reduced dendritic length, branching, and spine density of granule neurons, particularly in granule neurons generated during embryonic development [224], whereas i.c.v. infusion of adiponectin for a week promoted spinogenesis and dendritic complexity in adult-born granule neurons [224]. Moreover, upregulating the AdipoR1/Nogo-66 receptor 1 (NgR1) signaling pathway by an adiponectin homolog, osmotin, could also enhance neurite outgrowth and synaptic complexity in the hippocampus in an Alzheimer's disease mouse model [245].

Adult hippocampal neurogenesis is impaired by stress and depression, whereas multiple rodent studies have demonstrated the neurogenic and antidepressant effects of adiponectin. The accumulated evidence has suggested that enhanced structural plasticity may be a critical factor in the adiponectin antidepressant properties.

iii. *Effects of adiponectin on synaptic plasticity*

Altered synaptic integrity underlies the structural changes, specifically reduced white matter integrity [11] and the mean hippocampal volume [246], reported in MDD patients. MDD patients have fewer spines in the PFC as well as reduced expression of genes participating in synaptic plasticity [7]. Such disturbance in synaptic integrity could deter synaptic transmission. Long-term potentiation (LTP) and long-term depression (LTD) are standard evaluations of synaptic plasticity [247]. Chronic stress, a conventionally accepted risk factor for depression [248], impairs hippocampal LTP [15,249] and facilitates LTD [250] in various stress-induced depressed rodent models.

Conversely, chronic treatment with standard antidepressants prevents stress-induced hippocampal LTD [250] and stress-induced disturbances in synaptic proteins, such as PSD-95 and synapsin I [251]. Considerably, a single dose of ketamine induces a fast antidepressant response and restore the LTP and NMDAR-dependent excitatory postsynaptic current in depressed mice [15]. Altogether, it indicates that altered synaptic plasticity plays a significant role in the depression pathophysiology and, concurrently, represents a potential target for rapid-acting antidepressants.

Given the rapid antidepressant effect induced by the activation of adiponectin pathways, adiponectin-mediated synaptic plasticity is envisioned (Table 2). At present, the activation of

adiponectin receptors arguably promotes synaptic plasticity. Indeed, i.c.v. adiponectin infusion increased LTP and prevented LTD in the DG [252]. However, incubation of acute hippocampal slices with AdipoRon further dampened LTP in the *Cornu Ammonis* 1 (CA1) [253]. Factors affecting the adiponectin receptor-mediated synaptic transmission are not completely understood. The differential expressions of AdipoR1 and AdipoR2 across several brain structures [30] may indicate that they play different roles in synaptic transmission. AdipoRon could increase extinction learning with a decrease in DG neurons' intrinsic excitability through an AdipoR2-dependent mechanism [206]. Congruently, ventral tegmental area (VTA) infusion of AdipoRon prevented stress-induced anxiety-like behavior with a reduction in dopaminergic neurons activity, which was mediated by AdipoR1-dependent activation [207].

Convergent evidence has pinpointed the role of adiponectin receptors in regulating synaptic plasticity. Further investigations on the mechanisms of actions will ultimately demonstrate the adiponectin signaling pathway can be a novel, potent treatment target for depression and its related symptoms, although this conjecture remains to be explored.

Table 2. Effects of adiponectin on synaptic plasticity

Authors (year) (Ref)	Subjects (age)	Methods	Site	Electrophysiological findings	Behavioral outcomes
Weisz et al. (2017) (22)	Adult and young mice (C57BL/6J)	Extracellular recording and whole-cell patch clamping	CA1	↓ Paired-pulse ratio ↓ Long-term potentiation ↓ AMPA/NMDA ratio (only in adult mice)	N/A
Sun et al. (2018) (29)	Adult mice (C57BL/6J)	*In vivo* single-unit electrophysiological extracellular recording	VTA	↓ Population activity[#] ↓ Average spontaneous firing rate	Reduced the expression of anxiety-like behaviors
Zhang et al. (2017) (30)	Adult mice (C57BL/6J)	Whole-cell patch clamping	DG	↓ Number of action potential ↑ Rheobase current ↑ Negative resting membrane potential	Improved the contextual fear memory extinction
Pousti et al. (2018) (21)	Adult rats (Wistar)	*In vivo* extracellular recording	DG	↑ Long-term potentiation ↑ Paired-pulse ratio ↑ Baseline Prevented long-term depression	N/A

[#] The number of spontaneously active neurons recorded per track.

7. Conclusion and Perspectives

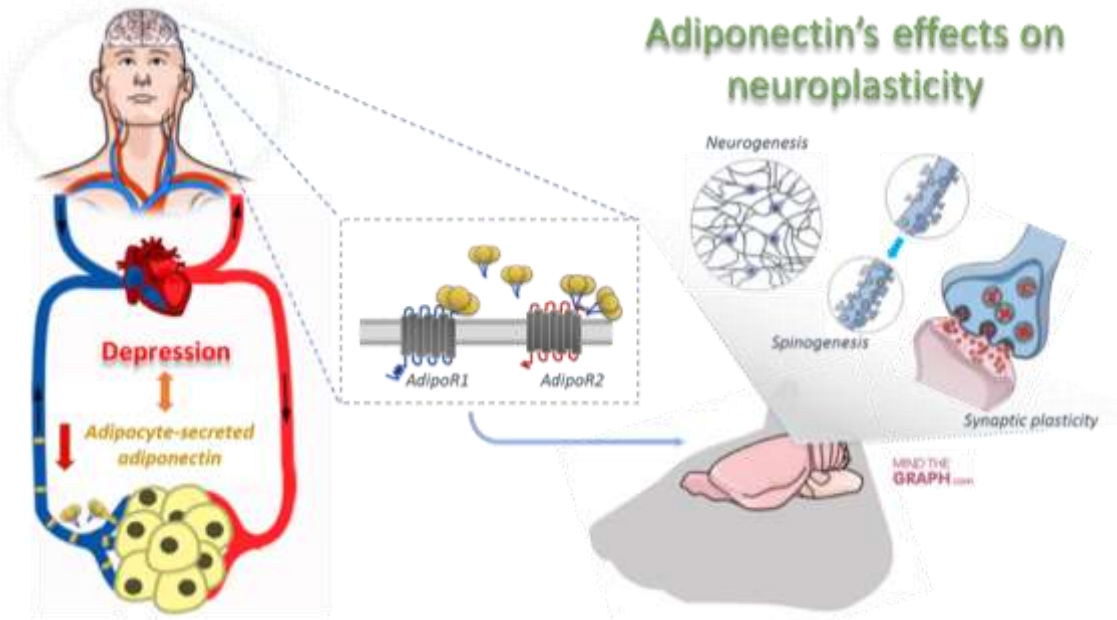
Activation of the adiponergic pathway has been shown to induce a fast antidepressant response in depressed rodent models, either by systemic administration of its selective receptor agonist AdipoRon, or by infusion of recombinant adiponectin directly into the brain. Over and above that, it

mediates physical exercise and environmental-induced antidepressant response, likely due to its neurotrophic properties over cell proliferation and spinogenesis. Finally, its effects on fear memory extinction and anxiety-like behaviors rely on AdipoR1/2-dependent modulation of synaptic plasticity and neuronal excitability. Based on that, we suggest these structural and functional neuroplasticity events that take place after adiponectin pathways activation are among the key modulators of its antidepressant effect (Figure 2).

This aligns with the current systemic conceptualization of depression in terms of its neuroplasticity changes [218,243,254] that, when counteracted, can result in rapid and long-lasting antidepressant responses. Nevertheless, adiponectin is also a metabolic regulator with insulin-sensitizing, anti-inflammatory, and cardioprotective properties, bridging the so long reported correlation between depression and metabolic disorders. It is tempting to think, hence, that targeting the adiponectin signaling system may not only induce a rapid antidepressant effect but also regulate the metabolic dysfunction commonly associated with depression.

Even though experimental studies have just started unraveling the adiponectin mechanisms of action in neuroplasticity, and some antagonisms remain to be explained, the adiponectin signaling system stands as a promising antidepressant target with fast response, low side effects, and capable of regulating possible underlying metabolic dysfunctions comorbid to depression.

Figure 2. Proposed beneficial effects of adiponectin in depression.



Whereas a large body of clinical research has been implicating low peripheral adiponectin levels with depression, the experimental activation of the central adiponergic pathway has been linked to an antidepressant response in rodents. Here, we suggest the increased structural and functional plasticity (e.g., neurogenesis, synaptogenesis, and synaptic plasticity) resulted from the activation of such pathway are the fundamental mechanisms underlying this response.

CHAPTER 3: AN INVESTIGATION OF THE POTENTIAL RAPID-ANTIDEPRESSANT PROPERTIES OF ADIPONECTIN

1. Introduction

Depression is characterized by sad or irritable mood accompanied by autonomic and cognitive changes that substantially affect the individual's functionality [255]. In approximately 50% of the cases, depression and anxiety are comorbid and the lifetime prevalence of depression comorbid with anxiety can reach 75% [256,257]. Novel antidepressant therapeutics are, therefore, commonly investigated in terms of their efficacy in both depression and anxiety paradigms. Objectively speaking, it is not possible to infer whether an animal is depressed/anxious or not, since the emotional component of the disease is inherently subjective and can only be assessed in terms of the subject's report [255]. Experimental research, thus, can only infer the potential therapeutic effects of certain drugs based on how they affect ethological components [258,259]. In rodents, the time the animal spend struggling to scape in unescapable situations is sensible to antidepressant drugs [260,261], whereas the animal's tendency to explore new although potentially threatening environments is sensible to anxiolytics [262,263]. Different behavioral paradigms exploring these ethological components can be used to investigate potential novel antidepressants and/or anxiolytics. On the other hand, different aspects of the disease can also be modeled in animals. Chronic stress exposure, as an example, is a reliable animal model of depression given that it reproduces in rodents the pathophysiological characteristics of the disease (face validity), with a similar etiology (construct validity), which is reversible with antidepressant treatments (predictive validity) [261].

As previously reviewed, adiponectin mediated the antidepressant effects of enriched environment and physical exercise [31,192]. Haploinsufficiency of adiponectin increased mice susceptibility to chronic-stress induced depression-like behaviors [30]. On the other hand, intracerebroventricular (i.c.v.) infusion with recombinant adiponectin, both full-length and globular forms, induced a rapid antidepressant effect in healthy and obese mice [30], whereas the PPARγ-induced peripheral adiponectin overexpression resulted in antidepressant and anxiolytic effects in healthy animals within 24 h [193]. AdipoRon, the AdipoR1/2 selective agonist, showed similar effectiveness in inducing a rapid antidepressant response 1 h after systemic administration (5 mg/kg, i.p.) [191]. Moreover, AdipoRon was shown to cross the BBB, with central concentrations peaking in 30 min and sharply reducing after 1 h [191]. In the brain, both AdipoRon and full-length adiponectin had a similar effect on influencing synaptic plasticity (i.e. a reduction in neuronal excitability) [206,207]. In this regard,

the central effect of the adiponectin signaling system activation is still controversial, with some reporting its negative effect in reducing LTP in the CA1 subregion [253], suppressing neuronal excitability in the DG [206] and ventral tegmental area [207], whereas others showing its opposite effects on promoting synaptic plasticity in the DG [264], and neuronal excitability in the dorsal raphe nucleus [191], as well as rescuing CA1 LTP impairment in an animal model of Alzheimer's disease [265].

Taking these findings into consideration, we observed that adiponectin has been better characterized as an antidepressant after chronic administration (through the application of its agonist, AdipoRon, unpublished data in Yau lab), whereas the involvement of hippocampal functional and structural plasticity in such process remains elusive. Based on that, we aimed: i) to characterize the adiponectin's potential rapid-acting antidepressant effects, and ii) to investigate whether adiponectin acutely modulate hippocampal synaptic and structural plasticity.

2. Material and methods

i. *Animals*

C57BL/6J male mice (4-8 weeks old) were provided and kept in the Centralized Animal Facility (CAF) of the Hong Kong Polytechnic University. Animals were group-housed under a 12-h light-dark cycle with food and water available ad libitum, unless specified. All the procedures were approved by the Animal Subjects Ethics and the student licensed to conduct experiments.

ii. *Chemicals*

AdipoRon was purchased from ApexBio (Houston, USA) and freshly prepared in saline for administration (5 mg/kg in 1.25% DMSO, Sigma-Aldrich, USA) from a stock solution (40 mg/ml in DMSO) stored at 4ºC. Full-length recombinant mouse adiponectin (trimer) was a gift from Prof. Aimin Xu, University of Hong Kong, and stored in a stock solution (1.5 mg/ml in PBS) at -80ºC. Corticosterone (Sigma-Aldrich, USA) was freshly prepared in saline (20 mg/kg in 0.5% DMSO and 0.5% Tween-80, Sigma-Aldrich, USA) under 40 min sonication from a stock solution (400 mg/ml in DMSO) stored at 4ºC.

iii. *Chronic corticosterone administration*

Even though the etiology of depressive disorder is not completely understood, there is a clear link between chronic stress-induced levels of glucocorticoids and depression [1,266]. To experimentally reproduced such conditions, chronic exposure to corticosterone in rodents has been a successful model for reproducing several aspects of stress-induced depression [261], including behavioral despair [56] and

anhedonia [267]. In our laboratory, adult mice were injected with corticosterone (20 mg/kg, subcutaneous) over 21 days, between 1 and 3 pm, daily, whereas control animals were treated with vehicle (saline). This protocol has been used previously developed and validated for the induction of a depressive phenotype in mice [56,268,269].

iv. *Behavioral tests*

All behavioral tests were carried out from 12 to 5 pm (unless specified) and animals could habituate to the testing environment for 2 h before experiments. The testing apparatus was cleaned with an ethanol solution between animals.

v. *Sucrose-preference test*

The loss of interest for previously pleasurable things, anhedonia, was measured through the sucrose preference test (SPT). The SPT is a taste-preference test, where the rodents' natural preference for sweetened solutions is tested through their average intake of a sucrose solution over time in comparison with tap water [270]. To do so, animals were singly housed at 6 pm with each cage containing two identical water bottles, one containing normal tap water and the other a sucrose solution (2% in normal tap water). In the next morning, positions of the water bottles were swapped to avoid any side-preference bias. The test was finished at 6 pm the following day and the animals were returned to their home cages. Both water and sucrose bottles were weighed before and after the test and the total intake measured in milligrams, whereas the sucrose preference was calculated as the percentage of sucrose consumption compared with the total intake (water + sucrose). The SPT has been used to evaluate anhedonia-like behavior in rodents [260,270] and the protocol here implemented was sensitive in detecting anhedonia in chronic-stress induced animal models of depression [191].

vi. *Forced-swim test*

The forced swim test (FST) is a classical protocol to evaluate behavioral despair and is a sensitive test for screening potential antidepressant treatments [261]. Animals were placed in a cylindrical water tank (23-25ºC) in an unescapable condition for 6 min. The test was video recorded in a bright-light room. An experienced researcher, blinded for the groupings, scored the amount of time spent immobile for the last 4 min as a measure of the animal's behavioral despair. Immobility was considered the complete absence of movement apart from those necessary for floating. A second, independent researcher scored a random batch of animals for ensuring reliability in the scoring across experiments. Researchers' scores differed less than 5%. The immobility time in the FST is inversely

proportional to antidepressant effects [261,271], and this protocol was previously implemented for the screening of rapid-antidepressant drugs [133].

vii. *Tail-suspension test*

As the FST, the tail suspension test (TST) intends to evaluate behavioral despair, and has been equally effective in the screening of antidepressants [272,273]. Animals were suspended by the tail and held on a bar with adhesive tape, 40 cm above the floor, for 6 min while video recorded in a bright-light room. Following the same scoring procedures as in the FST, the time spent immobile during the last 4 min was manually recorded. The TST has also been implemented for the screening of potential rapid antidepressants [30].

viii. *Open-field test*

The open-field test (OFT) measures the expression of the rodent's natural exploratory behavior towards unfamiliar although potentially threatening environments (approach-avoidance paradigm), like an unprotected open space, as well as locomotor activity [274]. In this test, animals were individually placed in a squared box (40 cm x 40 cm) enclosed with 40-cm high walls, randomly facing one of the corners, in a dim-light room. Animals could freely explore the environment for 5 min. The test was recorded and videos were later analyzed through automated tracking systems (ANY-maze©, Stoelting Co., USA). The total distance traveled (m) during the 5-min session and the average speed (m/s) were used as proportional indexes of the animal's locomotor activity, whereas the number of entries, time spent (s), and distance traveled in the open-field center area (a 15-cm^2 field on the apparatus center region) were recorded as inversely proportional indexes of anxiety-like behavior [262]. The OFT is a reliable way of measuring anxiety-like behavior and to be sensitive to anxiolytic treatments [262].

ix. *Light-dark box test*

As in the OFT, the light/dark box (LDB) test is based on the approach-avoidance paradigm, where the rodent is given the option to freely explore a bright, open compartment (potentially aversive) or an enclosed, dark compartment (safe, unexposed are) [275]. Here, the apparatus consisted of a squared box (40 cm x 40 cm) divided into two equally sized compartments by a black Plexiglas wall with a retractable door that, when open, allowed the animal to transit between compartments. One compartment was enclosed by transparent Plexiglas walls (40 cm), whereas the other was enclosed by black Plexiglas walls (40 cm) and equally covered to be completely protected and dark. Animals were individually placed in the dark compartment with the retractable door closed. Once the door was open, the test was initiated and the animal was let to freely explore the apparatus for 5 min. The recorded

videos were later analyzed through an automated tracking system (ANY-maze©, Stoelting, USA). The latency to the first entry (s), number of entries, time spent (s), and distance traveled in the open, bright compartment were recorded as inversely proportional indexes of anxiety-like behavior [276]. The LDB has been chosen as a reliable test for the investigation of both anxiogenic and anxiolytic pharmacological properties [262,276]

x. *Tissue preparation*

Transcardial perfusion was performed under deep anesthesia (isoflurane, 1-3% vaporizer, Abbot Laboratories, USA) with 0.9% saline followed by 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) in 0.01 M phosphate buffer saline (PBS). Brains were isolated and post-fixed in 4% PFA overnight (at 4 ^{o}C), followed by 30% sucrose solution (in 0.01 M PBS) until they sunk. Thirty-micron coronal brain slices (1-in-6 series) were obtained with a vibratome (Leica VT1200S, Germany) and stored in cryoprotectant solution at 4 ^{o}C.

xi. *Immunohistochemical staining*

Free-floating brain slices were washed in 0.01 M PBS and retrieved in citric acid buffer (pH 6.0) at 95 ^{o}C for 10 min. For Ki-67 staining [a marker of cellular proliferation [277]], brain slices were incubated with rabbit polyclonal anti-Ki-67 primary antibody (1:1000, Abcam, UK) overnight and with biotin-conjugated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, USA) for 2 h in 5% blocking solution containing normal goat serum (in room temperature). Brains were rinsed in 0.01 M PBS before, between, and after antibodies incubation. Staining was visualized through the peroxidase method after being incubated with avidin-biotin complex (ABC system, Vector Laboratories, USA) for 2 h and, after rinsing in 0.01 M PBS, diaminobenzidine (DAB kits, Sigma-Aldrich, USA) for 10 min. Brain slices were mounted in gelatin-coated microscope slides, dehydrated, and coverslipped for analysis. For c-Fos [a cellular marker of neuronal activation [278]] and NeuN [a specific neuronal marker [279]] immunofluorescence co-labeling, brain sections were rinsed (0.01 M PBS) and incubated overnight with mouse monoclonal anti-c-Fos (1:200, Abcam, UK) and rabbit monoclonal anti-NeuN (1:1000, Abcam, UK) primary antibodies in 5% blocking solution containing normal goat serum, in room temperature. On the next day, slices were rinsed (0.01 M PBS) and incubated with goat anti-mouse (Alexa Fluor 488, Thermo Fisher Scientific, USA) and goat anti-rabbit (Alexa Fluor 568, Thermo Fisher Scientific, USA) in 5% blocking solution containing normal goat serum for 3 h in room temperature. After rinsing (0.01 M PBS), slices were mounted in gelatin-coated microscope slides with fluorescence mounting medium (Dako, Denmark) for future analysis. These protocols were followed as previously done [225,280].

xii. *Quantification of Ki-67$^+$ and c-Fos$^+$ cells*

The number of positive cells in four sample sections of the dorsal (1.34 to 2.54 mm anterior to the Bregma) and ventral (2.54 to 3.40 mm anterior to the Bregma) portions of the DG were averaged for each animal and multiplied by the estimated number of 30-µm section available for the dorsal (40 sections) and ventral (33 sections) portions of the DG [281]. Ki-67$^+$ cells were identified within two-cell diameters of the subgranular zone (SGZ) using the Nikon series Eclipse H600L microscope, whereas c-Fos/NeuN$^+$ co-labeling was identified within the granule cell layer using a Zeiss LSM 800 confocal microscope. The number of positive cells was counted by a researcher blinded to the sample treatments.

xiii. *Stereotaxic surgery*

Adult mice (8 weeks old) were subjected to stereotactic surgery for bilateral cannula implantation and evaluation of direct adiponectin infusion into the DG (2 mm anterior, 1.7 mm lateral, and 1.7 mm ventral to the Bregma) (Paxinos and Franklin, 2002). Animals were deeply anesthetized with a Ketamine/Xylazine mixture (100 mg/kg to 10 mg/kg, respectively) and held by ear bars in the stereotaxic apparatus (Stoelting, USA). Eye ointment was used to maintain ocular lubrication and room the temperature was kept warm (25 ^{o}C) to avoid anesthesia-induced hypothermia. The skull was exposed and adjusted in a flat position after being cleaned with hydrogen peroxide and iodine solutions. Holes on the skull were bilaterally drilled on the proper coordinates and guide cannulas (1.7 mm, 26-gauge, Plastic One, USA) were then inserted. Cannulas were held in place with dental cement after a micro jewelry screw was inserted on the skull frontal region to increase cement adherence. Animals were single housed in transparent cages and let fully recover before being transferred back to the animal holding room.

xiv. *Brain infusion*

Nine days after cannulation, intra-DG adiponectin infusion was performed in awake, unrestrained mice in their home cages 30 min before behavioral analysis. To do so, two 33-gauge injection cannulas (2.0 mm, Plastic One, USA) connected to two 5-µl syringes (Hamilton Company, USA) were inserted into the guide cannulas attached to the animals' head. The 5-µl syringes containing the solution to be infused were coupled to an automated pump (PHD Ultra, Harvard Apparatus, USA) programmed to inject a total volume of 0.25 µl/site at a rate of 0.1 µl/min. Injection cannulas were left in place for an additional 5 min before withdrawal to prevent fluid reperfusion, as previously described [206].

xv. *Electrophysiology*

For the investigation of the effects of adiponectin on synaptic plasticity, acute transverse hippocampal slices were obtained using a vibratome (Leica VT1200, Germany) in cooled (4^{o}C), oxygenated (95% O2/5% CO2) normal artificial cerebrospinal fluid (nACSF) containing (in mM) 125 NaCl, 2.5 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_3$, 2 CaCl2, 1 $MgCl_2$, and 10 dextrose. Fresh slices were transferred to a recovery chamber containing warm (35 ^{o}C), oxygenated nACSF for at least 2 h before experiments. Using a multielectrode array system (Alpha MED64, AutoMate Scientific, USA), slices were placed in a perfusion chamber and constantly perfused with oxygenated nACSF at a 2-ml/min rate. The electrode grid was positioned under the DG region and the medial perforant pathway was stimulated, whereas field excitatory postsynaptic potentials (fEPSP) were recorded in the medial molecular layer [282]. Stimulation intensity was set to evoke 50% of maximum EPSP, delivered at 0.06 Hz. For the investigation of LTP, tetanic high-frequency stimulation (HFS) consisting of four trains of 50 pulses at 100 Hz, 30 sec apart, was used to induce LTP [283]. HFS-LTP was induced 20 min after a stable baseline had been recorded, keeping the recordings for an additional 60 min after LTP induction. Bicuculline methiodide (BIC, 5 μM, Sigma Aldrich, USA) was used throughout the whole experiment to avoid interference of gamma-aminobutyric acid receptor type A (GABAa) [284]. The effect of 50 or 500 nM adiponectin (trimer) on the induction of LTP was investigated by perfusing slices with adiponectin for 10 min before the HFS, whereas control slices were perfused only with nACSF and BIC.

For the investigation of adiponectin effects over NMDA-isolated fEPSP, slices were prepared for fEPSP recordings as previously described, with additional pharmacological manipulations for the isolation of the NMDA-receptors mediated fEPSP. Specifically, after a 10-min stable baseline recording in nACSF, slices were perfused with low-Mg (0.1 mM) ACSF together with Picrotoxin (100 μM, Sigma Aldrich, USA) and NBQX (0.5 μM, Tocris, UK) for $GABA_A$ and non-NMDA receptors blockade, respectively, throughout the remaining of the experiment for the isolation of the NMDA-mediated fEPSP [285,286]. After 30 min of stable NMDA-mediated fEPSP response, adiponectin (500 nM) was added to the perfusion solution and the recordings kept constant for the next 60 min.

xvi. *Cell culture*

Primary rat hippocampal neurons were cultured on 18-mm coverslips at high-density (1.4^5) in Neurobasal medium combined with 2% B27 and 0.25% L-glutamine (Invitrogen, USA). Cultured neurons were transfected with green fluorescent protein (GFP) on the 12th day in vitro (DIV) and incubated with adiponectin (50nM), AdipoRon (50 nM), or PBS (Control) for 1 h on the 17th DIV [287].

After the treatment, cells were fixed with paraformaldehyde (4% in sucrose) and the coverslips were mounted in microscope slides for confocal imaging.

xvii. *Spine analysis*

Using a confocal microscope (Leica DMi8, Germany), one to three secondary apical dendrites with similar width from ten to twenty cells per condition were imaged with 63x magnification (1024x1024 DPI), scan speed 6, and averaged two times using Z-stacks at an optimal level. Spine density was manually counted and classified as mushroom, stubby, thin, or filopodia-like spines using the MetaMorph software (Molecular Devices, USA) from 60-70-µm dendrites, based on the size of the head comparatively to its length and neck size [288]. Mushroom spines were classified as having a head/neck ratio ≥ 1.5 µm, stubby spines as having a head/neck and length/neck ratios ≤ 1 µm, thin spines as having a head/neck ration between 1 (inclusive) and 1.5 µm, and a length/neck ration between 1.5 and 3 µm (inclusive), and filopodia-like spines as having a head/neck ration < 1.2 µm and a length/neck ration > 3 µm [287,289]. The spines that did not fall under any category were still included in the total. The total number of spines and from each category was divided by the dendrite length and multiplied by 10, generating the spine density per 10 µm.

xviii. *Statistical analysis*

Data analysis and graph plots were done using GraphPad Prism 6 (GraphPad Software, USA). Data distribution was analyzed by the Shapiro-Wilk test. Once normal distribution was confirmed, data were analyzed by unpaired Student's *t*-test when comparing two independent groups or One-Way ANOVA for three or more independent groups, with Fisher's LSD post-hoc test for multiple comparisons. The different recording sections of the NMDA-mediated fEPSP, specifically, were compared using One-Way ANOVA with repeated measures. Outliers were checked with Grubbs' test, excluded from statistical analysis, and informed in the figure captions. Non-normally distributed data were not identified. Statistically significant results were considered when the *p* values were lower or equal to 0.05.

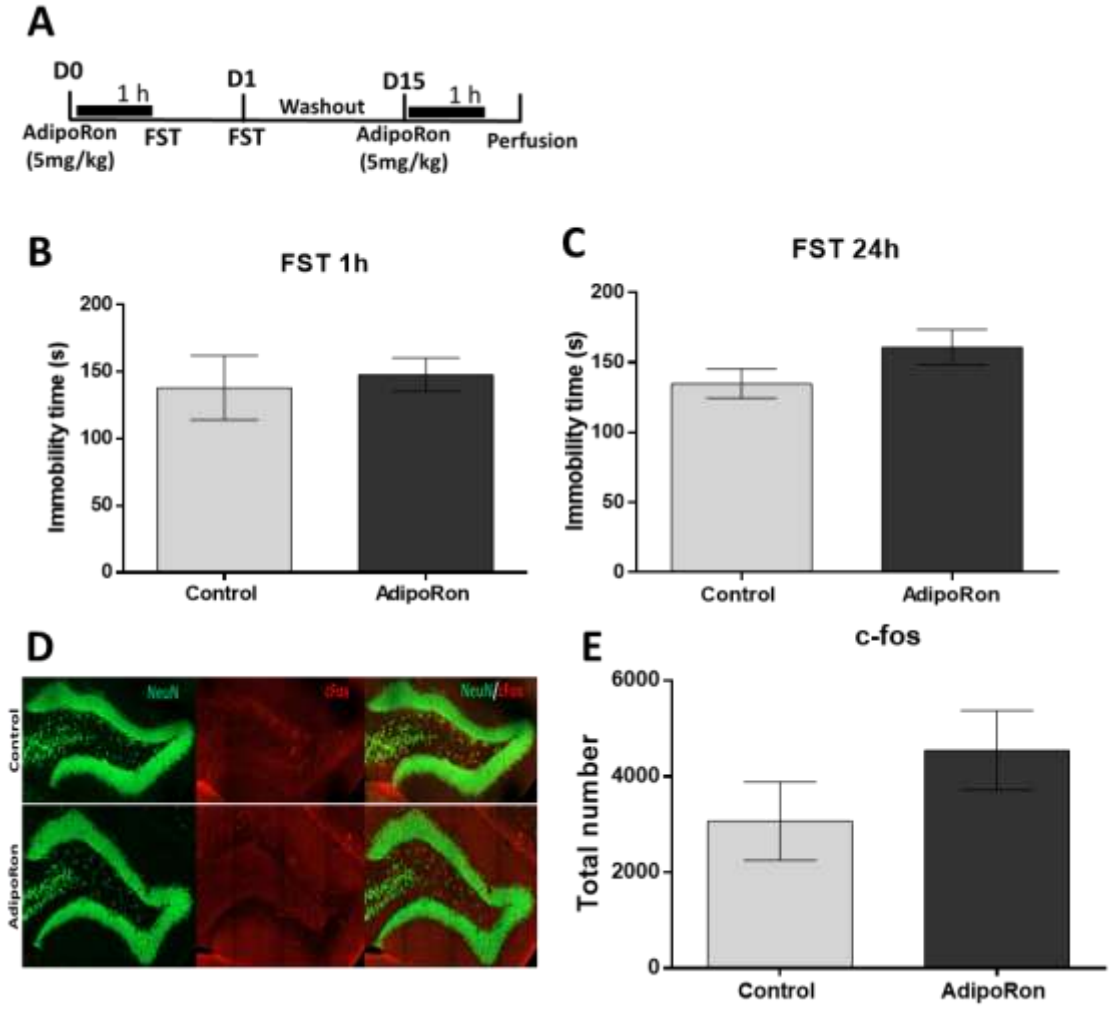
3. Results

i. *Acute AdipoRon treatment does not affect depression-like behavior in healthy mice*

Adult mice were treated with AdipoRon (5 mg/kg, intraperitoneal [i.p.]) or saline (Control) and submitted to the FST 1 and 24 h after treatment. After a two-week washout interval, animals were treated again and sacrificed for immunohistochemistry (Figure 3A). AdipoRon treatment did not affect

depression-like behavior in the FST at the 1-h (Figure 3B) or 24-h time point (Figure 3C), neither did it affect the DG neuronal activation according to the c-Fos expression levels (Figure 3D and E, unpaired *t*-test: $p = 0.25$).

Figure 3. AdipoRon administration (5 mg/kg, i.p.) does not acutely affect the expression of depression-like behavior nor DG neuronal activation in healthy mice



(A) Experimental timeline. (B-C) Immobility time in the FST of animals treated with saline (Control, n = 5) or AdipoRon (n = 8) 1 h and 24 h after AdipoRon treatment. (D) Representative image of the hippocampal DG depicting c-Fos+ and NeuN+ co-labeled cells. (E) Comparison of the total number of c-Fos+ cells in the hippocampal DG 1 h after treatment (n = 5/group). Unpaired *t*-test. Bars representing mean ± SEM.

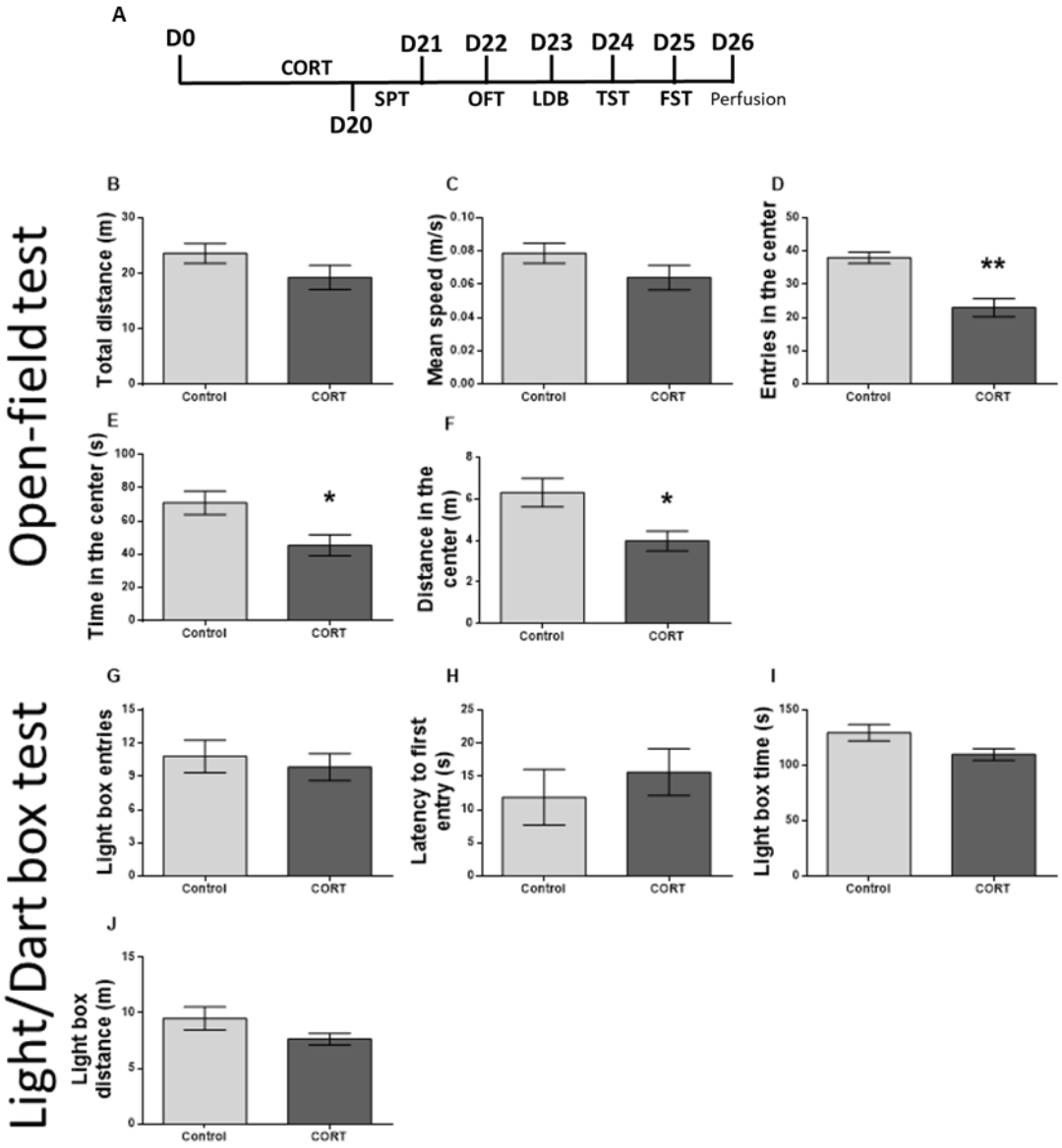
ii. *Behavioral characterization of an animal model of depression induced by chronic corticosterone administration*

Since AdipoRon had no detectable effect in healthy mice, we reasoned the activation of the adiponectin signaling system would rather elicit therapeutic effects in pathological conditions.

Therefore, the stress model of depression induced by chronic corticosterone administration was characterized. Mice were subjected to chronic corticosterone administration and then subjected to a battery of behavioral tests for the assessment of anxiety- and depression-like behaviors. The mice were then sacrificed on the following day for immunohistochemistry analysis (Figure 4A).

In the OFT, animals had no detectable difference in the locomotor activity (Figure 4B-C), although anxiety-like behavior was present as demonstrated by a reduced number of entries (Figure 4D, $p < 0.01$), time spend (Figure 4E, $p = 0.02$), and distance traveled in the apparatus central area (Figure 4F, $p = 0.01$). In the LDB, there was a non-significant trend towards reduced exposure to the enlightened compartment of the apparatus (Figure 4I $p = 0.05$ and 4J $p = 0.12$), in agreement with what had been observed in the OFT.

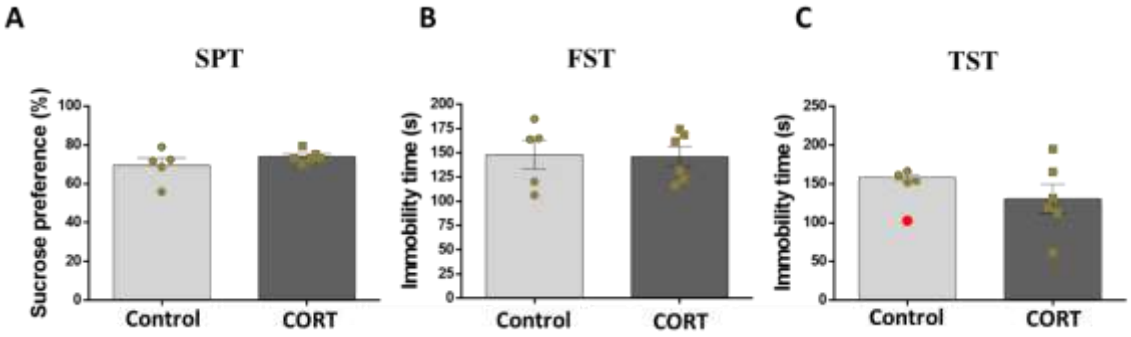
Figure 4. Anxiety-like behaviors in the animal model of depression induced by chronic-corticosterone administration



(A) Experimental timeline. (B-F) OFT results. (G-J) LDB test. n = 5-6/group. * $p < 0.05$, ** $p < 0.01$. Unpaired *t*-test. Bars representing mean ± SEM.

Concerning depression-like behavior, neither the SPT (Figure 5A), FST (Figure 5B), nor TST (Figure 5C) could detect behavioral alterations.

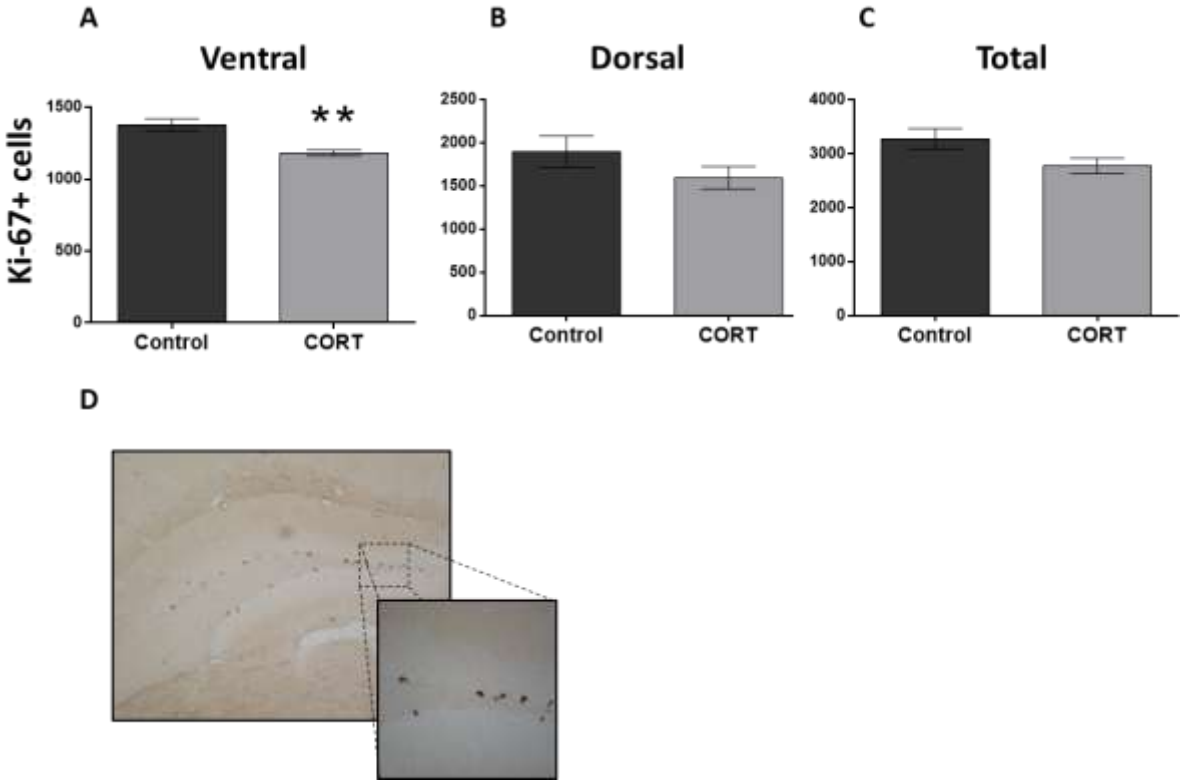
Figure 5. Depression-like behaviors in the animal model of depression induced by chronic-corticosterone administration



(A) SPT results. (B) FST results. (C) TST results. The subject marked in red in the TST represents an outlier (Grubb's test, $p < 0.05$) and was not included in the analysis. n = 5-6/group. Unpaired *t*-test. Bars representing mean ± SEM.

Fewer Ki-67 positive cells were observed in the ventral portion of the hippocampal DG (Figure 6A, $p < 0.01$), suggesting cell proliferation specifically in the ventral region was affected by the corticosterone treatment.

Figure 6. Cell proliferation in the animal model of depression induced by chronic corticosterone administration



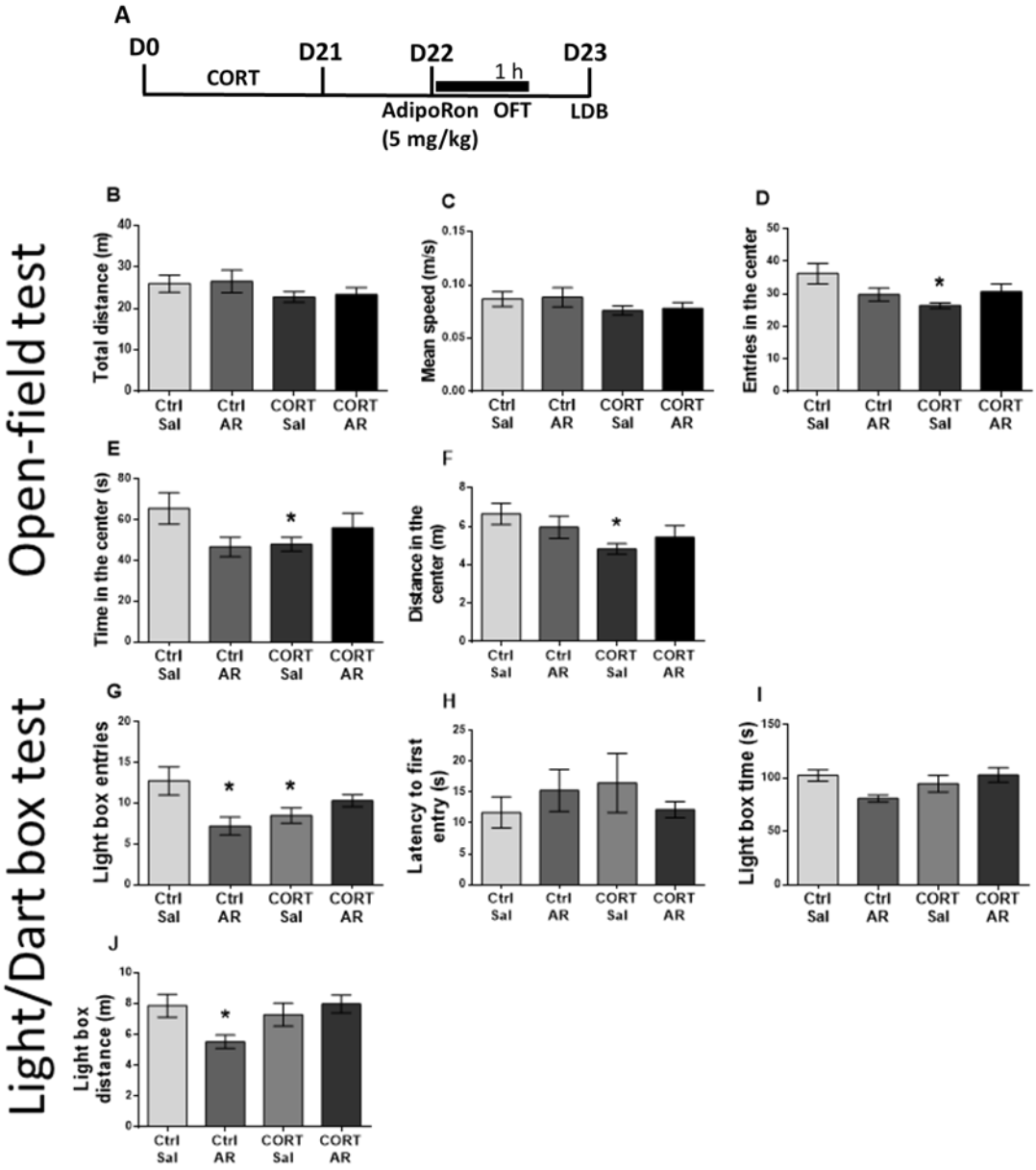
Comparison of the ventral (A), dorsal (B), and total (C) number of Ki-67+ cells of saline (Control, n = 3) and corticosterone-treated mice (CORT, n = 4). (D) Representative image of the hippocampal DG depicting the expression of Ki-67+ cells. ** $p < 0.01$. Unpaired *t*-test. Bars representing mean ± SEM.

iii. *AdipoRon is anxiogenic in healthy but potentially anxiolytic in animals chronically treated with corticosterone*

We aimed to investigate whether an increase in the adiponectin signaling system activity would improve anxiety-like behaviors. Mice were subjected to chronic corticosterone administration and then treated with AdipoRon (5 mg/kg, i.p.) or saline and tested in the OFT and LDB 1 h and 24 h later, respectively (Figure 7A).

As expected, chronic corticosterone treatment induced an anxiogenic phenotype, as shown by a reduced number of entries, time spent, and distance traveled in the OFT-center area (Figure 7D-F) and the number of entries of the LDB bright compartment (Figure 7G) of corticosterone-treated animals (CORT Sal, $p < 0.05$ compared with control animals [Ctrl Sal]). On the other hand, animals co-treated with corticosterone and AdipoRon (CORT AR) did not affect anxiety-like behavior when compared to control non-treated mice (Ctrl Sal, $p > 0.05$), implying that AdipoRon had an anxiolytic effect in corticosterone-treated animals. Surprisingly, AdipoRon was anxiogenic in animals not exposed to chronic corticosterone (Ctrl AR), as demonstrated by a trend towards a reduction in the number of entries (Figure 7D, $p = 0.09$) and time spent in the OFT-center area (Figure 7E, $p = 0.07$), when compared with animals treated with saline (Ctrl Sal). This effect lasted for 24 h, as shown by a decreased number of entries (Figure 6G, $p < 0.01$) and distance traveled (Figure 6J, $p = 0.04$) in the enlightened compartment of the LDB. Although the anxiogenic phenotype was marked by a trend in the OFT results, this trend appeared to be constant across different parameters and on a second behavioral test (LDB). Moreover, ongoing experiments in our laboratory with higher dosages of AdipoRon (20 mg/kg) confirm the AdipoRon anxiogenic effect in healthy animals (unpublished data).

Figure 7. AdipoRon is anxiogenic in healthy but potentially anxiolytic in chronically stressed animals



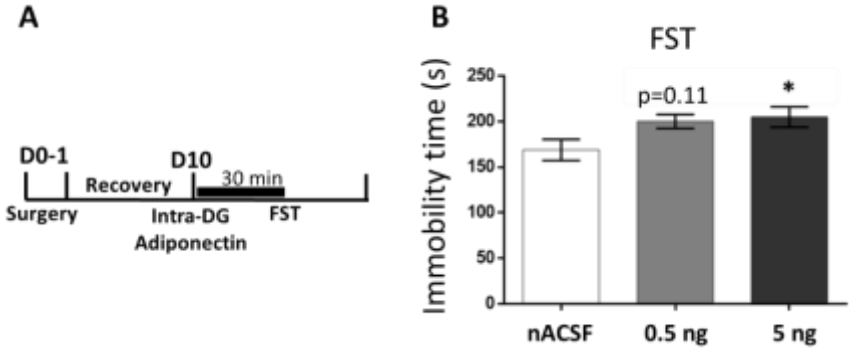
(A) Experimental timeline. Thirty-six animals were either injected with corticosterone (CORT) or saline (Ctrl) and then treated either with AdipoRon (Ctrl AR and CORT AR) or saline (Ctrl Sal and CORT Sal) previous to OFT and LDB evaluation. n = 6-11 per group. (B-F) OFT results. (G-J) LDB results; * $p < 0.05$ Vs Ctrl Sal. One-Way ANOVA test with Fisher's LSD post-hoc. Bars representing mean ± SEM.

iv. *Intra-DG adiponectin acutely induces a depressive phenotype in healthy mice*

Next, we investigated the direct effect of adiponectin in the hippocampus in healthy conditions. Adult mice were subjected to stereotactic surgery for bilateral cannula implantation targeting the DG region. Animals could recover for 9 days after surgery, being handled daily for the last 7 days. On the tenth day, 30 min after bilateral infusion of nACSF or full-length adiponectin (0.5 ng or 5.0 ng),

animals were submitted to the FST (Figure 8A). Acute intra-DG adiponectin infusion increased depression-like behavior in a dose-dependent manner in healthy animals (Figure 8B, 0.5 ng p = 0.11; 5 ng p = 0.04), when compared with controls.

Figure 8. Intra-DG adiponectin infusion acutely induces depression-like behavior in healthy animals



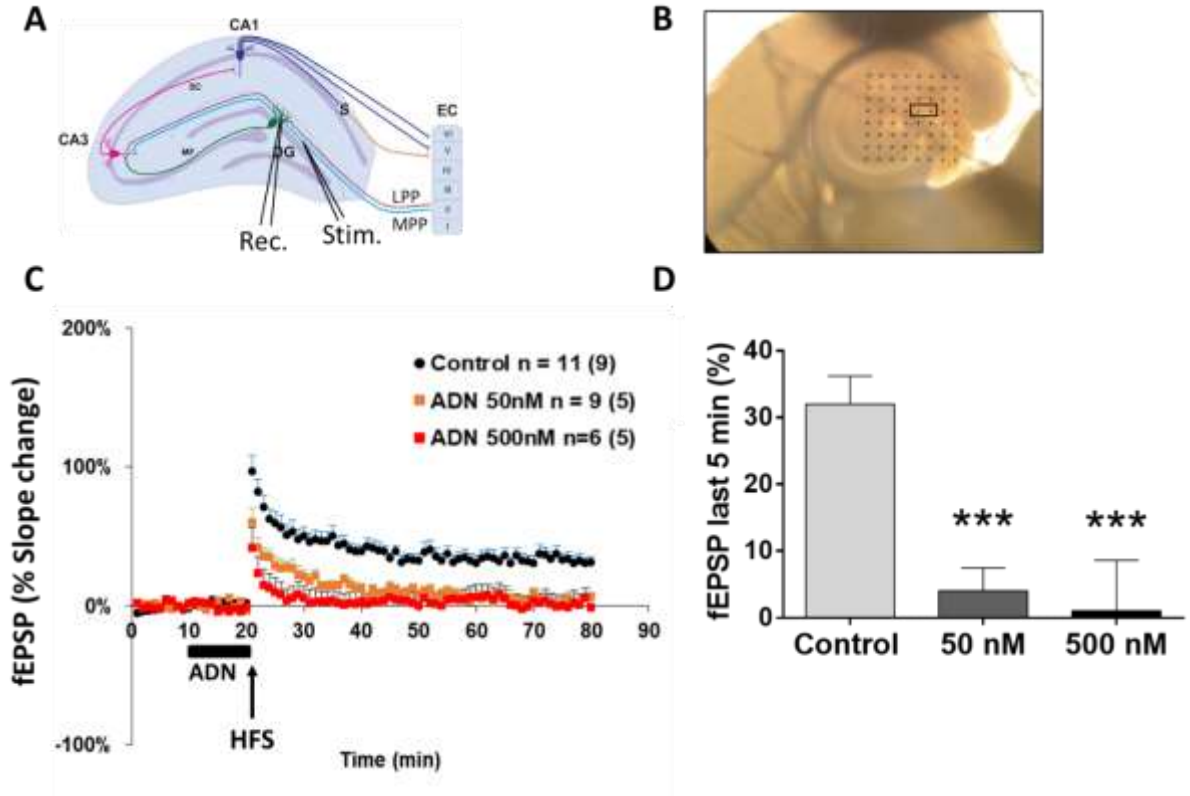
(A) Experimental timeline. (B) Immobility time in the FST of controls (nACSF, n = 7), 0.5-ng (n = 3), and 5-ng (n = 4) adiponectin-infused animals. * $p < 0.05$ Vs nACSF. One-Way ANOVA with Fisher's LSD post-hoc. Bars representing mean ± SEM.

Taken together, these data suggest that acutely systemic activation of the adiponectin signaling system elicits anxiety-like behavior, whereas local activation targeting the DG elicits depression-like behavior in healthy animals.

v. *Adiponectin inhibits LTP formation in the DG in healthy animals without affecting NMDA-mediated fEPSP*

Hippocampal plasticity has long been implicated in the modulation of mood and behavior. Based on the relevance of the hippocampus on the adiponectin's depressogenic properties in healthy animals, the adiponectin's effect over hippocampal synaptic plasticity was investigated. Field excitatory postsynaptic potentials (fEPSP) of the medial perforant path in the hippocampal DG (Figure 9A) were recorded in using a multielectrode array system (Figure 9B) in response to tetanic high-frequency stimulation (HFS) to investigate the acute effect of adiponectin (50 and 500 nM) on LTP formation (Figure 9C). BIC (5 µM) was perfused throughout the whole experiment to avoid interference of GABAa receptors. Adiponectin blocked the LTP induction in a concentration-dependent manner (Figure 9C), as demonstrated by a significant reduction in the percentage of fEPSP-slope change in the last 5 min of evoked EPSPs (Figure 3D, $p < 0.001$ for both concentrations) when compared with control slices.

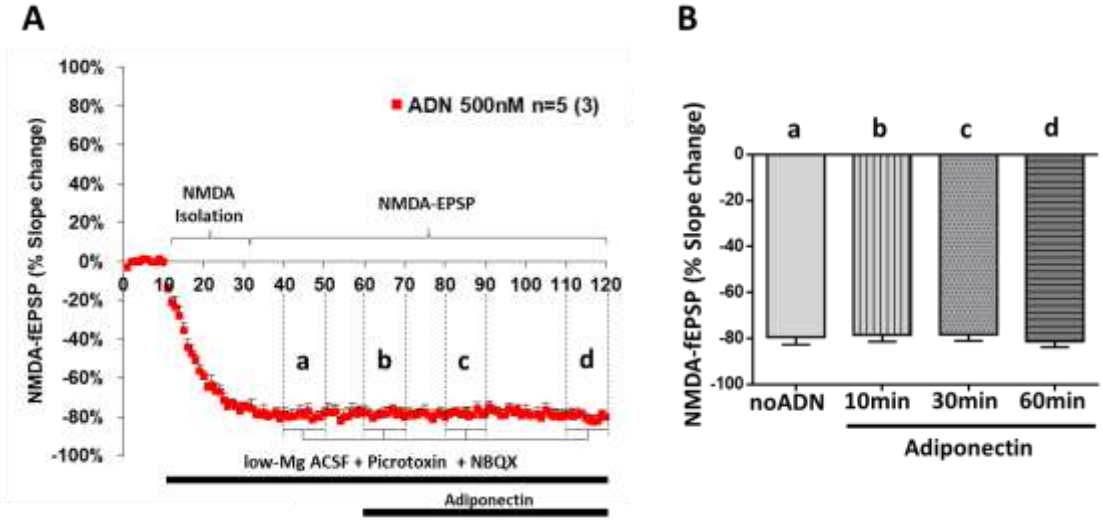
Figure 9. Adiponectin inhibits the LTP formation in the DG



(A) Schematic representation of the targeted medial perforant pathway (MPP, blue line) (adapted from [290]). (B) Representative image of a transverse hippocampal slice on the multielectrode array system (black square depicting the selected stimulation and recording electrodes). (C) Percentage of fEPSP-slope change after HFS-induced LTP over time. The black bar represents the adiponectin (ADN) perfusion period before HFS (arrow). BIC (5 µM) was perfused throughout the whole experiment to avoid interference of GABAa receptors. n stands for the number of slices recorded from the given number of animals, in brackets. (D) Percentage of fEPSP-slope change in the last 5 min of the recording. *** $p < 0.001$ Vs Control. One-Way ANOVA with Fisher's LSD post-hoc. Bars representing mean + SEM.

NMDA receptors are fundamental for hippocampal LTP induction [283,291,292]. Therefore, we questioned whether adiponectin inhibits LTP induction in the DG by modulating NMDA receptor function. NMDA-mediated fEPSP was isolated by perfusing low-Mg (0.1 mM) ACSF together with Picrotoxin (100 µM) and NBQX (0.5 µM) for $GABA_A$ and non-NMDA receptors blockade, respectively. After 30 min of stable NMDA-mediated fEPSP response, adiponectin (500 nM) was added to the perfusion solution and the recordings kept constant for the next 60 min (Figure 10A). Adiponectin did not affect the NMDA-mediated fEPSP over 60 minutes (Figure 10A, sections b, c, and d), when compared with the fEPSP response in the absence of adiponectin (Figure 10A, section a; and Figure 10B, $p > 0.05$ for all time points). This data suggest NMDA receptors are not involved in the adiponectin inhibition of LTP formation.

Figure 10. Adiponectin does not affect the NMDA-mediated fEPSP in the DG

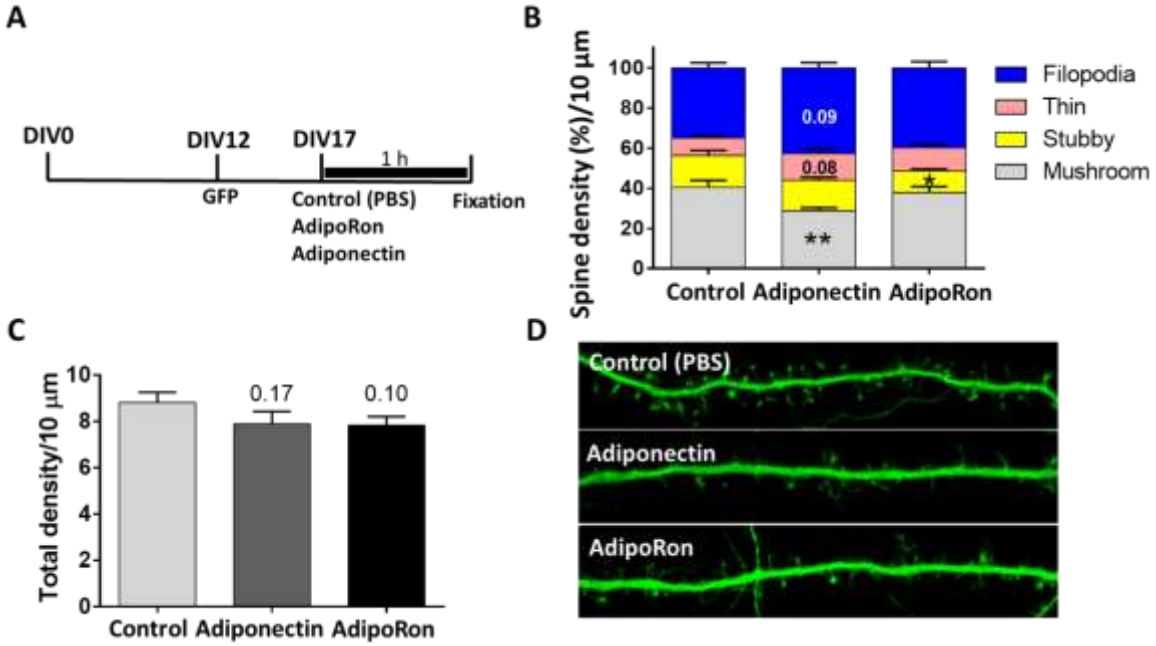


(A) Percentage of fEPSP-slope change after NMDA receptor isolation over time. Bars represent the perfusion time-course of referred drugs. 'a', 'b', 'c', and 'd' represent the sections used for the analysis of slope change (%) after adiponectin perfusion. n stands for the number of slices recorded from the given number of animals, in brackets. (C) Percentage of the fEPSP-slope change at different time points after adiponectin perfusion (b, c, and d), compared with a recording period without adiponectin (noADN, section a). One-Way ANOVA with repeated measures. Bars representing mean + SEM.

vi. *Adiponectin and AdipoRon disrupt hippocampal dendritic spine morphology*

Changes in dendritic spines can contribute to synaptic plasticity [293]. Therefore, we investigated whether the inhibitory effects of adiponectin over DG synaptic plasticity were related to disrupted spine morphology and density. Using the primary culture of rat hippocampal neurons, we observed that 1 h incubation of either recombinant trimeric adiponectin or AdipoRon (50 nM) (Figure 11A) disrupted hippocampal spine morphology (Figure 11B). Adiponectin reduced the percentage of mushroom spines (Figure 11B, $p = 0.01$) and tended to increase the percentage of thin and filopodia-like spines (Figure 11B, $p = 0.08$ and $p = 0.09$, respectively) in comparison with control. On the other hand, AdipoRon significantly decreased the percentage of stubby spines (Figure 11B, $p < 0.05$). Neither adiponectin nor AdipoRon affected the overall spine density (Figure 11C, adiponectin $p = 0.17$; AdipoRon $p = 0.10$) when compared to control (PBS).

Figure 11. Adiponectin and AdipoRon disrupt dendritic spine morphology in primary cultured rat hippocampal neurons



(A) Experimental timeline. (B) Percentage of spine morphology density for control (PBS), adiponectin, and AdipoRon (50 nM) treatments. (C) Total spine density. (D) Representative images of second-order dendrites from Control (n = 22 dendrites from 13 cells), adiponectin (n = 13 dendrites from 8 cells), and AdipoRon (n = 16 dendrites from 9 cells) treated cells. * $p < 0.05$, ** $p = 0.01$. One-Way ANOVA with Fisher's LSD post-hoc. Bars representing mean + SEM.

4. Discussion

Adiponectin has previously been found to have rapid-acting antidepressant properties [30,191], although it had not been directly investigated and the potential mechanism underlying such effect remained elusive. Based on that, we aimed to investigate whether adiponectin was able to induce rapid-antidepressant responses and if such effects were associated with altered hippocampal plasticity. Opposite to what was expected, acute activation of the adiponectin signaling system induced anxiogenic and, when restricted to the DG region using the trimeric form of adiponectin, depressogenic effects, which were likely mediated by the adiponectin-induced impaired structural and functional plasticity in the hippocampus. Although controversial at a first sight, these effects are remarkably similar to those observed in response to acute serotonergic antidepressant drugs [294–296].

Specifically, AdipoRon was anxiogenic in healthy mice, as shown by reduced exposure to the aversive regions of the OFT and LDB (i.e., the unprotected open areas of both tests). Increased anxiety is a common side effect of serotonergic drugs during the first few weeks of antidepressant treatment. In experimental conditions, acute SSRI administration is commonly shown to induce anxiety-like

behaviors [295,297]. The mechanism for such anxiogenic effect was recently narrowed to the short-term activation of the dorsal raphe serotonergic system [296]. Through chemogenetic manipulation of the dorsal raphe serotonergic neurons, Urban and collaborators [296] demonstrated that their short but not chronic activation induced anxiogenic effects. Remarkably, AdipoRon was shown to directly and acutely modulate dorsal raphe nucleus serotoninergic neurons by increasing their firing frequency, whereas chronic AdipoRon treatment resulted in increased serotonin release in stressed mice [191]. Therefore, adiponectin directly modulates the central serotonergic system.

Apart from inducing anxiety as a side effect, sub-chronic fluoxetine administration (5 d, i.p.), a prototypical SSRI, was shown to block LTP formation in the DG [298] and to induced depression-like behavior (3 d, i.c.v.) [299]. Both chronic fluoxetine (4 w) [300] and acute electroconvulsive therapy (an alternative to otherwise treatment-resistant depression with fast onset antidepressant properties) converted mature granule cell neurons to a rather immature phenotype [301], which was associated to a marked reduction in foot shook-induced c-Fos activation in the DG [300]. Our *in vitro* experiments have shown that adiponectin acutely blocked LTP formation in the DG and reduced the density of functional spines in the primary hippocampal cells, whereas *in vivo* intra-DG adiponectin infusion induced depression. Such similarity of effects between acute adiponectin and sub-chronic serotonergic drug administration poses the question of whether, instead of a rapid-acting antidepressant response mediated by the glutamatergic system activation, adiponectin would rather induce depression relief through the chronic modulation of the serotonergic system.

AdipoR1 was shown to be highly expressed in the dorsal raphe nucleus serotonergic neurons [302]. Selective knock-out of the AdipoR1 in these neurons reduced the tryptophan 5-hydroxylase 2 (TPH2) protein levels (required for the endogenous synthesis of serotonin) and serotonin production in the dorsal raphe, inducing a depressogenic phenotype in both male and female mice [302]. Surprisingly, chronic (14 d) overexpression of hippocampal PPRAα, one of the main downstream targets of the adiponectin receptors, was shown to induce an antidepressant response in healthy and chronically stressed mice by recovering BNDF signaling and neurogenesis [303]. Noteworthy, the selective knockdown of hippocampal PPRAα completely blocked the fluoxetine antidepressant effects [303]. This piece of evidence adds to the hypothesis that the adiponectin signaling system activation share remarkably similar antidepressant properties with serotonergic antidepressants.

Unpublished data in the Yau Lab of ongoing experiments are in line with this perspective. AdipoRon at a higher dosage (20 mg/kg, i.p.) induced a fast antidepressant response in the FST at 2 h time point, although it was anxiogenic when mice were subsequently tested in the OFT and LDB 2 h after AdipoRon treatment. On the other hand, chronic AdipoRon administration over 7 days was capable of inducing antidepressant effects in the SPT, FST, and novelty-suppressed feeding [a

behavioral paradigm sensible to chronic but not acute serotonergic drugs [260,304]], and to reduce anxiety as evaluated in the LDB. These effects were not associated with increased hippocampal neurogenesis but, surprisingly, chronically treated mice displayed reduced DG cFos expression. As previously exposed, the dematuration of granule cell neurons is a characteristic of chronic fluoxetine administration, which similarly led to reduced DG cFos expression [300]. Noteworthy, after 14 days of daily AdipoRon administration using this higher dosage, the antidepressant effect remained evident and was then accompanied by increased neurogenesis, which is a biomarker and relevant mediator of chronic serotonergic antidepressant administration [305].

The mechanisms mediating the acute effects of serotonergic drugs and adiponectin over hippocampal structural and synaptic plasticity, however, might not necessarily be the same. Through the adiponectin receptors' interaction with the APPL1, adiponectin activates the p38-MAPK signaling pathway, which in the periphery has been shown to mediate the adiponectin-induced angiogenesis and osteogenesis [173,177,306]. Noteworthy, central p38-MAPK modulation is involved with hippocampal LTD [307] and its inhibition is associated with improved hippocampal LTP [308]. Notwithstanding, this pathway along with ERK1/2 signaling also mediates the adiponectin-induced hippocampal cellular proliferation [178,226]. Therefore, signaling cascades that in the short term deprive hippocampal functional plasticity may alternatively improve structural plasticity (i.e., neurogenesis) in the long term.

Our experiment also suggested acute AdipoRon treatment at a low dosage of 5 mg/kg is potentially anxiolytic in chronically stressed mice. This effect can be reasonably interpreted considering the adiponectin anti-inflammatory effects and its role in regulating the HPA axis and GC response. AdipoRon treatment (although chronically administered) normalized the increased hippocampal pro-inflammatory profile induced by chronic stress [191]. In parallel, adiponectin haploinsufficiency impaired the glucocorticoid-induced negative feedback over the hypothalamus-pituitary-adrenal axis (HPA) [30], suggesting adiponectin could be involved in modulating glucocorticoid levels. Indeed, adiponectin was capable of reducing corticosterone production in a short time, likely through the activation of the adiponectin receptors located in the adrenal glands [309]. Therefore, adiponectin can potentially counteract the pro-inflammatory and hypercortisolemia profile induced by chronic stress, resulting in acute anxiolytic effects in such pathological conditions.

Finally, it is relevant to ponder about the acute antidepressant properties of adiponectin in the previous publications, which oriented this thesis main hypothesis. Although behavioral despair paradigms (e.g. FST and TST) have very good predictive validity (i.e. the reliability of the test in truly reflecting a clinical antidepressant response) [261], their major drawback is that they are not reliable in detecting the time-window of antidepressant effect for serotonergic drugs. In the laboratory, these

behavioral tests are responsive to acute SSRIs treatment [302], whereas clinical mood improvement requires long-term administration [260]. In this case, the novelty suppressed feeding test is recommended, given its sensitivity to chronic but not acute SSRIs treatment [304]. Therefore, if adiponectin works as a serotonergic compound, the acute antidepressant effects observed in the FST and TST after systemic or central adiponectin upregulation in the previous publications [30,191,193,200] might be misleading.

In conclusion, our results using 5 mg/kg AdipoRon disagree with previous publications using the same dosage and indicating of adiponectin as having rapid-antidepressant properties. At the same time, it offers a new interpretation of the reduced hippocampal plasticity after adiponectin administration, observed here and corroborated by previous findings in the literature, when interpreted in light of the similarity between acute adiponectin and serotonergic drug. Combined with the adiponectin's capacity to directly modulate the dorsal raphe nucleus, we raise the question of whether it exerts its antidepressant properties through the modulation of the serotonergic system after chronic or sub-chronic administration.

CHAPTER 4 – THE INVOLVEMENT OF THE vHipp-mPFC PATHWAY IN MODULATING DEPRESSION-LIKE BEHAVIORS

1. Introduction

The medial prefrontal cortex (mPFC) and hippocampus are key brain structures involved in depression pathogenesis and relief [6,8,35]. Whereas the former was implicated in short-term antidepressant response [136], the latter was necessary for antidepressant maintenance [21]. It is thus likely that pathways integrating the hippocampus with the mPFC are implicated in depression.

Impaired white-matter integrity has been consistently found in depressed patients. As recently meta-analyzed, depressed patients present reduced global white-matter integrity when compared with healthy controls [11], and specific impairments in frontal lobe fibers were also reported [10]. Resting-state functional magnetic resonance allows for the investigation of abnormal regional homogeneity across brain areas, which is a functional evidence of intrinsic neuronal activity synchronization. This recent meta-analysis shows that first-episode drug-naïve patients present increased left-hippocampus regional homogeneity and decreased left-orbitofrontal cortex regional homogeneity when compared with healthy control subjects [310]. This is consistent with a commonly observed decreased activation of prefrontal cortex structures and increased activity of limbic areas including the hippocampus, amygdala, and hypothalamus in depressed patients [115]. These studies point towards an imbalanced hippocampal-PFC functional connectivity in depression. Indeed, both depressed and schizophrenia patients demonstrated impaired motor memory consolidation that relies on such functional connectivity [12]. Noteworthy, this imbalance between temporal and frontal lobe activity is corrected with antidepressant treatment in depressed patients [115].

Hippocampal monosynaptic projections to the mPFC almost exclusively originate in the ventral hippocampus (vHipp) and project to the ventral portion of the mPFC (infralimbic [IL] and prelimbic [PL] regions), whereas the dorsal region of the mPFC (anterior cingulate) projects to the dorsal hippocampus [311]. The longitudinal specialization of the hippocampus can be generally divided in the posterior hippocampus (correspondent to the dorsal portion in rodents), specialized in cognitive functions, and the anterior hippocampus (correspondent to the ventral portion in rodents), involved with mood and emotional regulation [312]. Likewise, the PFC specialization can also be broadly divided into the dorsolateral portion, associated with cognitive and executive functions, and the ventromedial portion, involved with affective functions [313]. In agreement with that, the ventral hippocampal

projection to the mPFC has been implicated in mood modulation, whereas the dorsolateral PFC projection to the dorsal hippocampus has been involved with cognitive function [311].

As previously reviewed in Chapter 2, the ventral *Cornu Ammonis* 1 (CA1) subregion is the source of hippocampal projections to the mPFC, including the PL and IL subregions [142,144], which is here addressed as the vHipp-mPFC pathway. Experimental manipulation of this pathway has mostly focused on behavioral changes associated with schizophrenia [149], sociability [144,146], anxiety [150], and fear-related disorders [314,315]. Remarkably, despite the clinical relevance that has been attributed to this pathway [12,69,316,317], very little is known regarding its relevance in modulating the depressive phenotype from an experimental perspective. Brain imaging confirmed reduced functional connectivity in the vHipp-mPFC pathway in mice with truncated Disrupted-in-Schizophrenia 1 (DISC1) gene [13], a genetic risk factor involved with psychiatric disorders that were shown to increase depression-like behavior [318]. Chronic stress has been shown to impair HFS-induced facilitation of vHipp synaptic inputs to the mPFC [317,319], which is likely to be one of the underlying mechanisms associated with stress-induced depression. On the other hand, the bilateral disconnection between the vHipp and mPFC blocked the ketamine's long-term antidepressant effect, whereas activation of this pathway was able to induce antidepressant effects [21]. Moreover, ketamine was suggested to induce ΔFosB expression in the ventral hippocampus [141], a marker of cumulative neuronal activation associated with long-lasting adaptive changes in the brain [320], suggesting that sustained vHipp activation may be one of the mechanisms underlying antidepressant effects.

Physical exercise is a natural antidepressant that was shown to be as effective as pharmacotherapy [321] and to reduce relapse rates in the maintenance phase [322]. During exercising and recovery, the body releases a plethora of peripheral factors with central activity (i.e. exerkines) that eventually counteracts the neuropathological features associated with impaired mood regulation [156,231]. Increased neurogenesis in the hippocampal dentate gyrus (DG) region is one of the main mechanisms associated with the physical exercise benefits in the brain [323]. Adult newborn neurons originate from neural stem cells (NSC) located in the DG subgranular zone (SGZ) go through several week-long differentiation and maturational steps until they integrate the granule cell layer as mature granule neurons [324,325]. Running, specifically, is one of the strongest factors inducing neurogenesis, including cellular proliferation, survival, and differentiation [227,323]. Thirteen days of voluntary wheel running increased DG cellular proliferation nearly twice as much as learning, swimming, or enriched environment [227]. Such exercise-induced neurogenesis has been shown to support hippocampal-dependent tasks [326,327] and to be a key component of the physical exercise antidepressant properties [31].

Based on these findings, we aimed to investigate whether chemogenetic-driven chronic activation of the vHipp-mPFC pathway would modulate rodents' depressive phenotype and if such

process would be associated with increased cellular proliferation in the DG. Moreover, we also questioned if such pathway would mediate the antidepressant properties of physical exercise by chemogenetically inhibiting this pathway in voluntary wheel running exercise.

2. Material and methods

i. *Animals*

Animals were provided and housed as previously described in Chapter 4. For the experiment involving voluntary wheel running, specifically, animals were housed in two mice per cage. All other housing procedures remained the same.

ii. *Chemicals*

Clozapine N-oxide (CNO, ApexBio, Houston, USA) was freshly prepared in sterile saline for administration (0.3 mg/kg in 0.15% DMSO) from a stock solution (20 mg/ml in DMSO) stored at 20ºC negative.

iii. *Microinjection*

The transgene expression of engineered proteins capable of modulating neuronal activity in response to design drugs otherwise inert (i.e. chemogenetics) has made possible the manipulation of specific circuits [328]. Here, we used the Cre-loxP system to drive the transgene Cre-mediated specific expression of double-floxed (DIO) coupled proteins known as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) [328]. Stereotaxic surgery was performed as previously described (Chapter 4), with the following adaptations. A 5-µl syringe (Hamilton Company, USA) connected to an automated pump (11 Elite Nanomite, Harvard Apparatus, USA) was used for bilateral microinjections of 0.5 µl of virus per site (0.15 µl/min) in the mPFC region (1.9 mm posterior, 0.4 mm lateral, and 2.8 mm ventral to Bregma) and vHipp (3.0 mm anterior, 3.3 mm lateral, and 3.6 mm ventral to Bregma) (Paxinos and Franklin, 2002). The needle was left on site for an additional 2.5 min to avoid solution reperfusion. Due to viral diffusion, the mPFC included the IL and PL regions. A retrograde adenovirus expressing the Cre recombinase under the PGK promoter (AAV-pgk-Cre, titer 1×10^{13} vg/ml, Addgene, USA) was bilaterally injected in the mPFC. The human muscarinic Gq-coupled receptor 3 (hM3Dq) and Gi-coupled receptor 4 (hM4Di) were used to drive neuronal activation and inactivation, respectively [296,328–330]. Under the human Synapsin promoter (hSyn), adenovirus expressing either hM3Dq (AAV-hSyn-DIO-hM3Dq-mCherry, titer 4.7×10^{12} vg/ml, Addgene, USA) or hM4Di (AAV-hSyn-DIO-hM4Di-mCherry, titer 5.3×10^{12} vg/ml, Addgene, USA)

was bilaterally injected in the vHipp. For control animals, adenovirus expression mCherry only (AAV-hSyn-DIO- mCherry, titer 3.2×10^{12} vg/ml, Addgene, USA) was injected in the vHipp. After injections, the head skin was sutured and the animals returned to their home cages (unless specified) after fully recovered from anesthesia. Animals were let recover for 10 d before experiments started. Since DREADDs do not present constitutive activity, a low dose of CNO (0.3 mg/kg, i.p.), an otherwise inert drug capable of crossing the blood-brain barrier [331], was used to activate the receptors.

iv. *Voluntary wheel running*

After microinjection, animals were housed in two mice per cage to avoid social isolation-induced stress [332]. Eight days after surgery, a blocked wireless running wheel (Low Profile Running Wheel for Mouse, Med Associates Inc., USA) connected to a computer was included in each cage for a 2-days adaptation period. After completing a 10-days recovery period from surgery, running wheels were unblocked for 14 days and then blocked again during the behavioral assessment. Non-running control mice had their running wheels blocked during the entire period, making running impossible while maintaining the environmental conditions of running and non-running mice the same. The number of wheel revolutions per hour was automatically converted to kilometers per hour (km/h) using the wheel manufacturer software (Wheel Manager, Med Associates Inc., USA). Since animals were housed in two per cage, the distance traveled per day was divided by two and expressed in kilometers. This protocol was adapted from Suk Yu Yau et al. (2014) where it increased neurogenesis and had antidepressant-like properties in mice.

v. *Behavioral tests*

The behavioral assessment was carried out following the same procedures described in Chapter for, including the sucrose-preference test (SPT), open-field test (OFT), and forced-swim test (FST) protocols.

vi. *Y-maze test*

The Y-maze test (YMT) is an ethological spatial recognition memory test that is dependent on hippocampal integrity [333]. It relies on the rodent's natural preference to explore a novel environment rather than a familiar one, guaranteed that the animal has intact hippocampal-dependent spatial memory to recall one of the environments had been previously explored [334,335]. The opaque acrylic apparatus is composed of three 40-cm long arms, interconnected by a central platform in a Y-shape format, with 10-cm high walls, elevated 1.5 m from the floor. The test was divided into two phases. In the recognition phase, the mouse was placed in one of the arms (registered as the "starting arm" for

that specific animal) and let explore one of the other arms (the "familiar arm") for 10 min while the third arm remained closed by a retractile door. After a 4-h interphase interval, in the recall phase, the animal was placed in the starting arm and allowed to freely explore the familiar and "novel" (previously closed) arms for 5 min while video recording. The time countdown during the recall phase started when the animal entered the central platform, and an animal was considered exploring an arm when the four paws were inside it. The entire test was performed in dim light and, for both recognition and recall phases, the apparatus' floor was covered with wood shaving bedding. The time spent exploring the novel (Tn) and familiar (Tf) arms was recorded by an experienced researcher blind to the treatments. The exploration ration was calculated as the exploration time of either familiar or novel arms concerning total time spent in both the familiar and novel arms ($Ratio = \frac{Tn\ or\ Tf}{Tn+Tf}$). The exploration ratios between familiar and novel arms were compared within groups, and a significantly higher exploration ratio of the novel arm is expected in intact spatial memory recognition. The exploration index was calculated as the time spent in the novel arm subtracted by the time in the familiar arm in relation to the total time spent in both arms ($Index = \frac{Tn-Tf}{Tn+Tf}$). The exploration index was compared in between groups to evaluate the effect of treatment on the spatial recognition memory.

vii. *Tissue preparation*

The animals were sacrificed and the brain tissue prepared for immunohistochemical analysis as described in Chapter 4.

viii. *Immunohistochemical staining*

Immunohistochemistry staining was performed as described in the previous chapter.

ix. *Quantification of Ki-67 and c-Fos positive cells and confirmation of viral expression*

Ki-67 positive cells were quantified as described in chapter 4. The c-Fos expression was identified within the granule cell layer using a Zeiss LSM 800 confocal microscope. Images were processed to remove the background in Image J (National Institute of Health, USA) and the total cell count per area from three dorsal (from Bregma −1.34 mm to −2.54 mm) and three ventral (Bregma −2.54 mm to −3.40 mm) unilateral hippocampal slices were obtained. Injection location and viral expression were confirmed by the observation of mCherry expression in unstained ventral hippocampal slices using Zeiss LSM 800 confocal microscope.

x. *Statistical analysis*

The statistical analysis followed the same conditions established in the previous chapter, with the following tests included. Within-group analysis for the YMT ratio was performed using paired samples *t*-test. In-between group analysis of two groups with non-normal distribution was performed using the Mann-Whitney test. Other statistical conditions and significance remained the same.

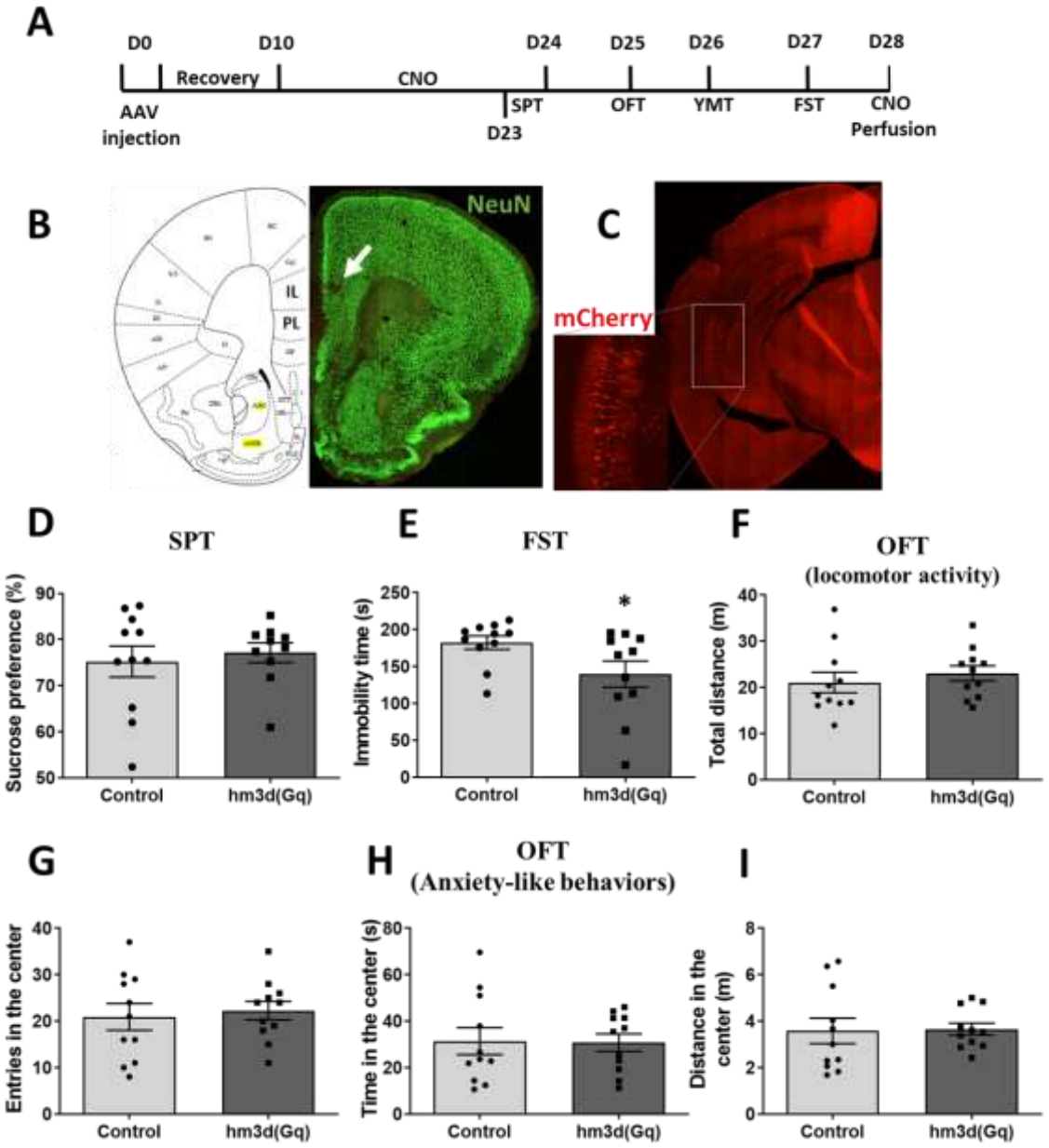
3. Results

xi. *Chronic activation of the vHipp-mPFC pathway induces an antidepressant-like effect*

As previously addressed, the relay of vHipp information to the mPFC is extensively involved in mood regulation [21,69]. Genetic approaches allowing the manipulating of specific groups of neurons have made possible the understanding of behavior and pathology from on the circuit level [336]. Here, we aimed to investigate whether the chronic activation of the vHipp-mPFC pathway by chemogenetics could induce antidepressant effects and if it would be associated with increased DG neurogenesis. Therefore, a retrograde adenovirus expressing the Cre recombinase was microinjected into the mPFC, whereas adenovirus driving the expression of the hM3Dq receptor was microinjected into the vHipp. Animals were let recover for 10 days to allow viral expression. After such recovery period, animals started receiving CNO injections (0.3 mg/kg, i.p.) for fourteen consecutive days (between 2-4 pm) and were then subjected to a battery of behavioral tests for the evaluation of depression-like behaviors (SPT and FST), anxiety-like behavior (OFT) and spatial recognition memory (YMT) (Figure 12A). One day after the last behavioral test, animals were again treated with CNO and perfused for the immunohistochemical analysis of DG neuronal activation (through cFos expression), cellular proliferation (Ki-67), and viral expression (mCherry). Chronic CNO administration was previously shown to continuously and effectively drive neuronal activity in DREADD expressing neurons over 3 weeks [296] and the 0.3 mg/kg CNO dosage was shown to effectively activated hippocampal neurons expressing the hM3Dq DREADD in the absence of seizures [331].

As shown in Figure 12, pictures A and B, vHipp neurons projecting to the mPFC were successfully infected. Chronic activation of this pathway did not affect anhedonia, as measured in the SPT (Figure 12D, $p = 0.63$), although an antidepressant-like effect was observed in the FST, as seen by a significant reduction in the immobility time (Figure 12E, $p = 0.02$), without locomotor activity changes (Figure 12F, $p = 0.47$). Anxiety-like behaviors were not affected, as measured by the number of entries (Figure 12G, $p = 0.69$), time spent (Figure 12H, $p = 0.92$), and distance traveled (Figure 12I, $p = 0.90$) in the OFT center area.

Figure 12. Chronic activation of the vHipp-mPFC pathway induces an antidepressant-like effect

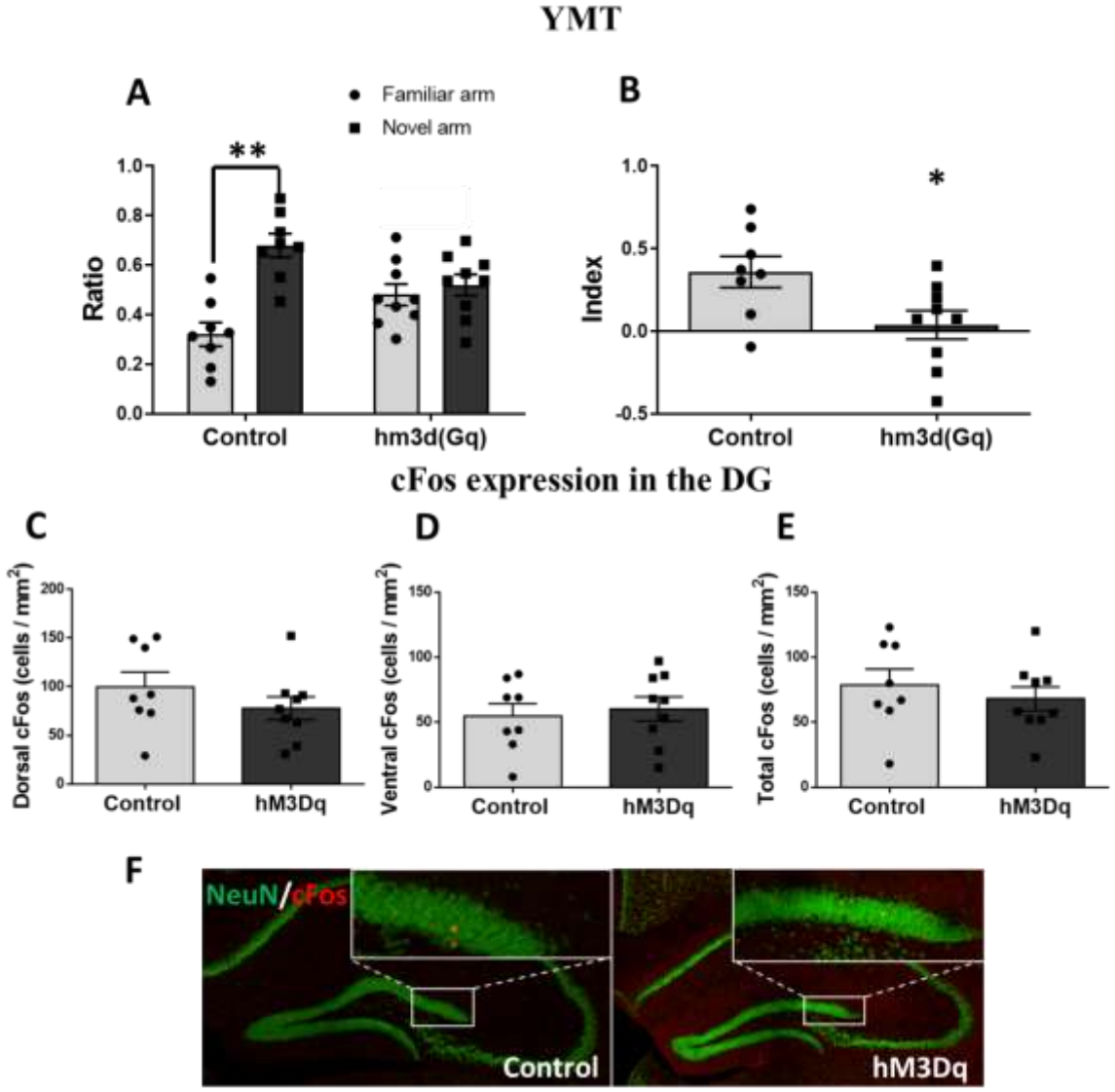


(A) Experimental timeline. Mice were injected with adenovirus expressing hM3Dq-mCherry or mCherry only (Control). Clozapine N-oxide (CNO) was injected i.p. (0.3 mg/kg) daily. (B) Representative image of the injection site in the mPFC (white arrow). (C) Expression of hM3Dq-mCherry in vHipp neurons projecting to the mPFC. (D) SPT results. (E) FST results. (F) Locomotor activity results in the OFT. (G-I) Anxiety-like behaviors in the OFT. n = 11/group. * $p < 0.05$. Unpaired *t*-test. Bars representing mean ± SEM.

On the other hand, chronic activation of this pathway impaired the animal's short-term spatial memory, as demonstrated by an impaired discrimination ratio between the familiar and novel arms in the YMT (Figure 13A, Control $p = 0.006$; hM3Dq $p = 0.66$), resulting in a significantly lower exploration index of hM3Dq-expressing mice compared with controls (Figure 13B, $p = 0.02$). However, impaired spatial memory recognition was not associated with decreased neuronal activation

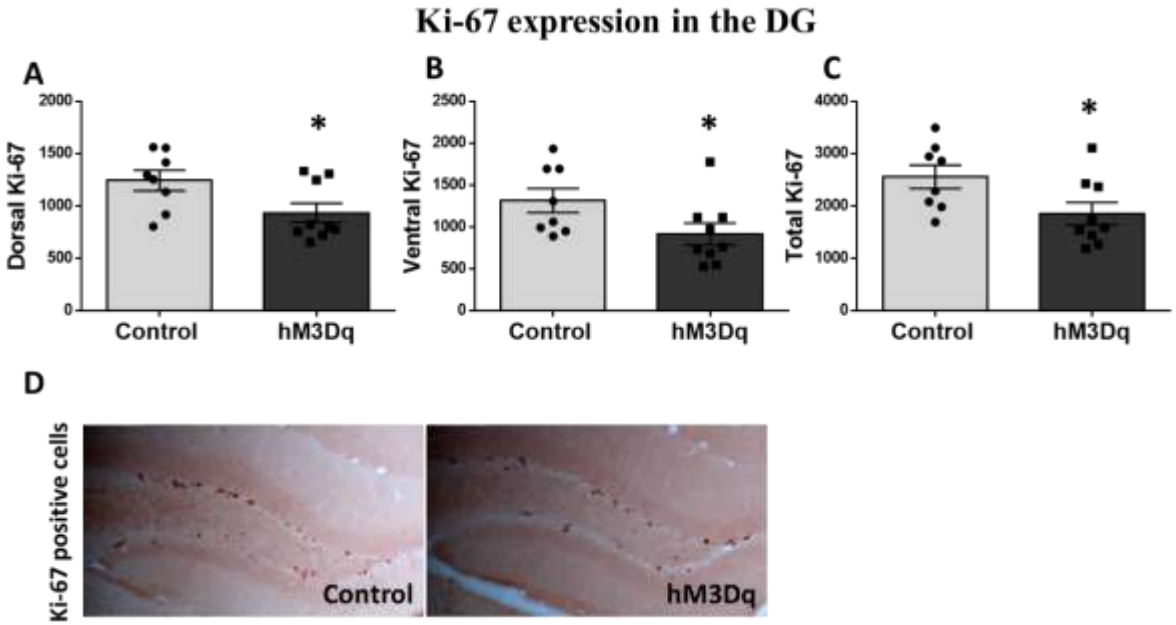
in the DG in response to vHipp-mPFC activation. As observed in Figure 13, neither dorsal (Figure 13C, $p = 0.26$), ventral (Figure 13D, $p = 0.67$), nor total (Figure 13E, $p = 0.48$) DG cFos expression were affected 1 h after CNO. Such effect was associated though with decreased cell proliferation, as observed by fewer Ki-67 positive cells in the dorsal (Figure 14A, $p = 0.03$), ventral (Figure 14B, $p = 0.05$), and total DG (Figure 14C, $p = 0.03$) in hM3Dq-expressing mice compared with controls.

Figure 13. Chronic activation of the vHipp-mPFC pathway impairs short-term spatial memory



(A) Exploration ratio (paired *t*-test) and (B) index (independent *t*-test) of the novel and familiar arms 4 h after the recognition phase. n = 11/group. (C) Dorsal, (D) ventral, and (E) total DG cFos expression. (F) Representative image of DG cFos expression. n = 8-9/group. ** $p < 0.01$. Bars representing mean ± SEM.

Figure 14. Chronic activation of the vHipp-mPFC reduces cellular proliferation in the DG



(A) Dorsal, (B) ventral, and (C) total DG Ki-67 expression. (D) Representative image of DG Ki-67 expression. n = 8-9/group. * $p \leq 0.05$. Bars representing mean ± SEM.

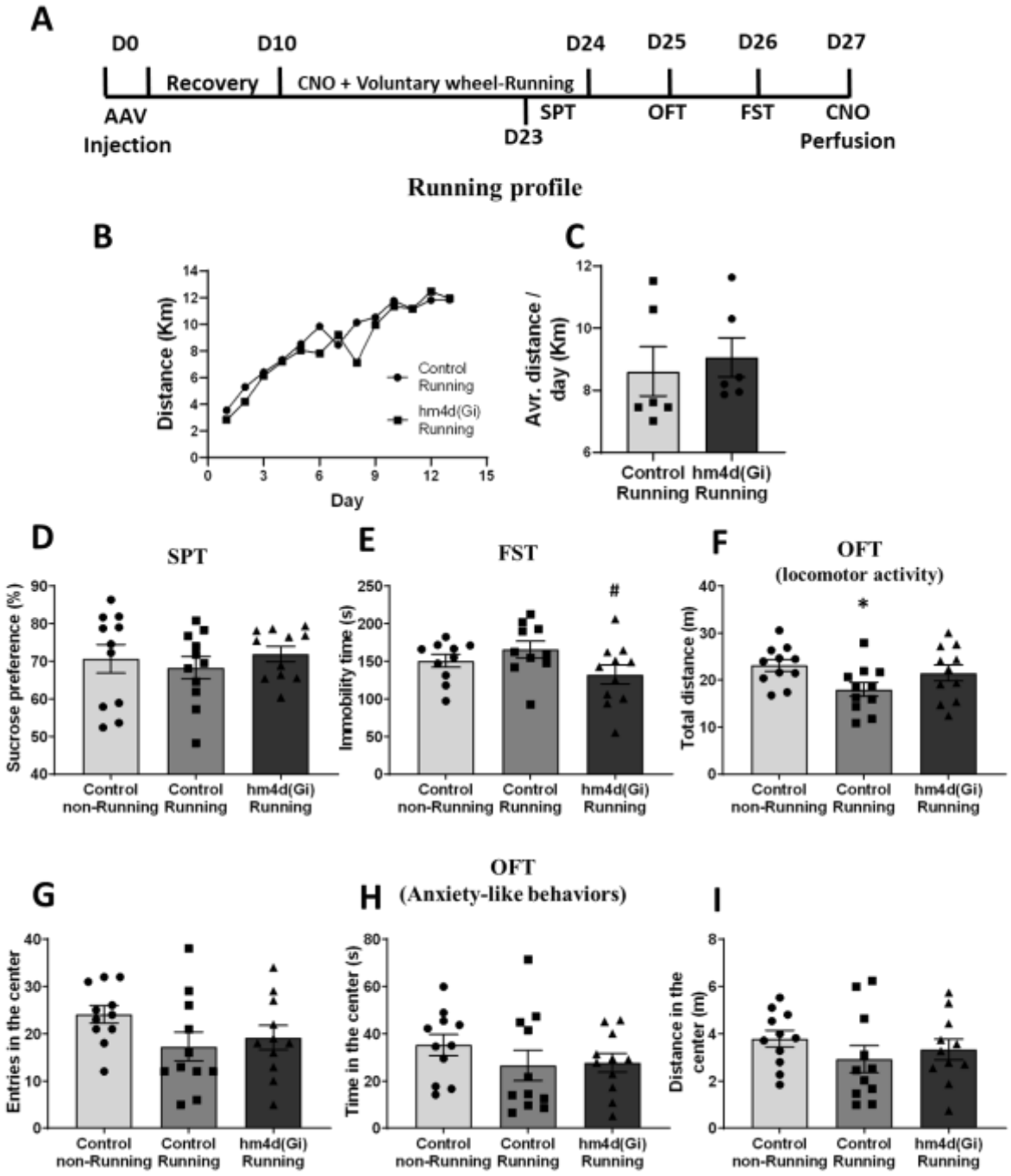
xii. *Inactivation of the vHipp-mPFC pathway protects against a physical exercise-associated reduction in locomotor activity*

Since the chronic vHipp-mPFC pathway activation could elicit an antidepressant-like effect, we next investigated whether it would be required for the expression of the physical exercise-associated antidepressant properties. The human muscarinic receptors 4 (hM4Di) was used for driving neuronal inactivation, whereas control animals were injecting with mCherry-expressing adenovirus only. Animals were housed at two per cage and, after 8 days of recovery from the adenovirus injection, a wireless running wheel was introduced in each cage for wheel habituation. Non-running control animals (which were also injected with mCherry-expressing adenovirus) had their running wheels blocked, impeding exercise while maintaining the same environmental conditions between running and non-running mice. After 2 days of wheel habituation, animals could run voluntarily in their home cages for 14 days while receiving daily CNO injections (0.3 mg/kg, i.p.), whereas non-running control animals received CNO injection only. Depression- and anxiety-like behaviors were investigated in the fourteenth day onwards, with all wheels blocked (Figure 15A).

As observed in Figure 15A, running distance started at less than 4 km per day and steadily increased up to 12 km/day towards the end of the protocol. The average distance traveled was of 9.14 km/day for control animals and 8.52 km/day for vHipp-mPFC inactivated animals. However, there was no statistically significant difference between groups (Figure 15B, p = 0.24). Considering

depression-like behaviors, there was no difference in the SPT (Figure 15D, $p = 0.68$), and a trend in the FST (Figure 15E, $p = 0.11$). A post-hoc analysis revealed that hM4Di-expressing animals had a significantly lower immobility time when compared with control running mice ($p = 0.04$). Although this might be an indicator of antidepressant-like activity, the interpretation of this result is questionable since running *per se* tended to decrease locomotor activity, as observed in the OFT (Figure 15F, $p = 0.06$). A post-hoc analysis revealed that control running mice traveled a significantly lower distance in the OFT when compared with non-running controls ($p = 0.02$). Interestingly, the vHipp-mPFC pathway inhibition protected mice from the physical exercise-associated decrease in locomotor activity, since hM4Di-expressing mice did not differ in the OFT distance traveled compared with non-running controls ($p = 0.45$). The expression of anxiety-like behaviors, on the other hand, was not affected. The number of entries (Figure 15G, $p = 0.16$), time spent (Figure 15H, $p = 0.42$), or distance travelled (Figure 15I, $p = 0.42$) in the OFT center area were not significantly affected.

Figure 15. Inactivation of the vHipp-mPFC pathway protects against a physical exercise-associated reduction in locomotor activity



(A) Experimental timeline. Mice were injected with adenovirus expressing hM4Di-mCherry or mCherry only (Control running and non-running). Clozapine N-oxide (CNO) was injected i.p. (0.3 mg/kg) daily while voluntary wheel running was allowed in the home cage. Control non-Running animals had their running wheels blocked (B) Daily distance traveled per mice over 14 days. (C) Averaged distance traveled per day over the 14-days running period. Data points represent the information of a single wheel for every two mice. Non-normal distribution compared by the Mann-Whitney test. (D) SPT results. (E) FST results. (F) Locomotor activity results in the OFT. (G-I) Anxiety-like behaviors in the OFT. n = 11/group. * $p < 0.05$ compared with Control non-Running, # $p < 0.05$ compared with Control Running. One-Way ANOVA test with Fisher's LSD post-hoc. Bars representing mean ± SEM.

4. Discussion

Impaired functional connectivity between vHipp and the mPFC has been observed in depressed patients [12], and the bilateral disconnection of these structures was shown to impair the long-lasting antidepressant effects of ketamine [21]. Here, we showed that the chronic activation of the vHipp-mPFC pathway could induce an antidepressant-like effect. Although it was not possible to confirm the involvement of such pathway in the physical exercise-induced antidepressant properties, we observed that the inhibition of this pathway protected against the excessive running-induced decrease in locomotor activity.

The chemogenetic activation of the vHipp projections to the mPFC for 14 days significantly reduced mice immobility time in the FST. It was previously shown by others that the acute chemogenetic or optogenetic activation of the vHipp-mPFC pathway induced antidepressant-like effects in the FST [21]. Carreno et al. (2016) used a similar chemogenetic approach with hM3Dq DREEAD to drive neuronal activation, treating the animals with CNO (0.5 mg/kg) 30 min before the behavioral test, whereas optogenetic activation of this pathway took 10 min, being the last 6 min during the behavioral test. Since CNO (both 0.3 mg/kg and 0.5 mg/kg) was shown to increase hippocampal activation of hM3Dq expressing mice for over 9 h [331], this previous result could not discard the possibility that the observed behavior was a transient effect of the ongoing pathway activation, which could be elicited by increased locomotor activity that has been also involved with these projections [146]. Our results expand the previous findings by showing that, when chronically activated for 14 days, this pathway induced a sustainable antidepressant-like effect in the FST 3 days after the last activation day, which was not associated with the altered locomotor activity. Such aspect, however, was not associated with increase cellular proliferation in the DG. It was previously shown that some antidepressant proprieties of chronic antidepressant administration are not mediated by hippocampal neurogenesis. Specifically, depressed mice with X-irradiation abolished hippocampal neurogenesis still presented reduced immobility time in the FST (i.e. antidepressant-like behavior) after chronic fluoxetine administration [305]. Moreover, the antidepressant effect of environmental enrichment was also shown to be neurogenesis independent [192].

Chronic activation of this pathway also led to impaired spatial memory recognition, as shown by an undistinguishable preference for the familiar and novel arms of the YMT, although DG neuronal activation was not affected by the pathway activation. The vHipp-mPFC pathway, specifically the vHipp CA1 projections to PL, is necessary for contextual fear memory learning [314,315], ventral hippocampal hyperactivity was also shown to impair different cognitive tasks. Reduced inhibitory transmission in the vHipp and consequent ventral hippocampal hyperactivity is associated with

schizophrenia's positive symptoms (e.g. delusion) and reduced cognitive flexibility [337]. Specifically, the acute inactivation of this pathway in an animal model of schizophrenia that displays a characteristic vHipp hyperactivity improved cognitive flexibility [337]. The vHipp hyperactivity is also characteristic of autism spectrum disorder. The specific increased activity of the vHipp-mPFC pathway in transgenic animal models of autism was associated with impaired social memory behavior, which could be recovered by chemogenetically decreasing this circuit activity [144]. Therefore, it is possible the impaired memory recognition here observed to be a side effect of vHipp over-activation. On the other hand, it could also be associated with reduced cellular proliferation in the hippocampal DG.

In our experiment involving chronic vHipp-mPFC silencing during voluntary wheel running, mice ran an average of 8.83 km/day over a 14-d period, with no representative difference between treated and control animals. In agreement with the previous findings [338,339], running activity steadily increased over the days and achieved a plateau around the tenth day. From the tenth day onwards, animals ran an average of 11.69 km/day. Surprisingly, running did not induce anxiolytic or antidepressant-like activity as expected [31,339], but rather reduced locomotor activity as observed in the distance traveled during the OFT. It was previously reported that locomotor activity-dependent tests such as the OFT and FST, when performed 24 h apart from the last running day, would not be affected by running-induced fatigue [339]. Accordingly, both OFT and FST were here performed more than 24 h after the last running day. Excessive running, on the other hand, was another factor reported to interfere with running-associated benefits and impair locomotor activity [338,340]. Normal C57Bl/6J mice have been shown to run an average of nearly 4 km/day [340,341]. As one can see, the average running distance displ

ayed by the mice in our experiment was at least twice as much as the distance normally observed for this strain. Such elevated running distance is similar to what is performed by animals selectively breed to express high voluntary wheel running, which ran an average of 12 km/day [340]. Remarkably, excessive runners do not benefit from running-associated therapeutic effects. As reported, although high voluntary wheel running mice had increased hippocampal cell proliferation, such neurological feature was not translated into improved cognition as it was in non-hyperactive runners [340]. Moreover, although still leading to increased neurogenesis and hippocampal BDNF levels, excessive voluntary wheel running led to decreased motor activity in the OFT and increased anxiety, while not affecting the expression of depression-like behaviors [338]. Such effects were associated with increased fecal corticosterone metabolites in runners compared with nonrunners [338]. It is thus possible that our results were influenced by excessive wheel running. Curiously, the chronic vHipp-mPFC inactivation protected the animals from the running-associated reduction in locomotor activity.

As reported in the literature, the hippocampal DG is one of the structures more strongly correlated with running activity [342]. The level of DG neuronal activation was strongly correlated with the distance an animal had run during the previous 5 h [343]. Since the DG can indirectly modulate CA1 activity through the CA3 Schaffer-collateral projections [59], we hypothesize that the running-induced DG activation resulted in CA1 hyperactivity and, more specifically, the CA1 projections to the mPFC. The selective activation of the mPFC vasoactive intestinal peptide-expressing interneurons that are targeted by ventral CA1 projections was shown to reduce locomotor activity during the social interaction test [146]. Specific components of the vHipp-mPFC pathway, therefore, could downregulate locomotor activity in excessive runners, whereas inactivation of this pathway during the running protocol was protective. This hypothesis, however, is subjective to contradiction given that the pathway inactivation did not affect voluntary wheel running levels in the home cage. Concerning this aspect, it is arguable that mice behave differently and have different activity levels in the home cage than when exposed to novel environments, such as the OFT open arena [342]. As reported by others, the activity level of voluntary wheel running did not match locomotor activity in a 3-min version of the OFT [344]. It is possible that home cage voluntary wheel running is mediated by the DG activation [343] whereas locomotor activity in a novel environment is subjected to the vHipp-mPFC components [146]. Since silencing protocol was restricted to the vHipp-mPFC pathway, only the latter aspect was affected.

In summary, the current investigation expands the understanding of the hippocampus and mPFC involvement in depression relief in the circuit level. Specifically, it shed some light on the potential involvement of the chronic activation of the vHipp-mPFC pathway in mediating antidepressant-like effects. Nonetheless, these results should be interpreted with caution in face of the complexity of this pathway. Up-to-date findings have been showing that the selective activation of different mPFC GABAergic neurons targeted by the vHipp projections is involved in opposite behavioral effects [146]. Further investigation is needed to dissect the components of this pathway that are involved with depression.

CHAPTER 5 – CONCLUSION

Depression is a pervasive disease with a high lifetime prevalence and recurrence, which makes it accountable for a big share of the global disease burden. Such scenario is worsened by the delayed-onset and low remission rates of the current antidepressants. Although more efficacious pharmacological interventions have been intensively investigated, it took half a century for a novel class of a rapid-acting antidepressant to be discovered and approved for clinical use. Motivated by this urge for better treatments, we aimed to investigate the potential rapid-acting antidepressant effects of the adipocyte-secreted hormone adiponectin and its mechanisms of action, specifically focusing on the adiponectin's acute effects over the hippocampal synaptic and structural plasticity.

Our behavioral results disagreed with previous findings. Specifically, when applying the same dosage of the adiponectin receptors agonist AdipoRon (5 mg/kg, i.p.) used by other, we observed it was anxiogenic rather than an antidepressant at the 1 h time point. Moreover, we observed that the infusion of adiponectin targeting the hippocampus increased depression-like behavior *in vivo*, which was associated with a reduction in the hippocampal synaptic and structural plasticity *in vitro*. By comparing these results with the literature of sub-chronic serotonergic antidepressant administration, we questioned whether adiponectin would act through the modulation of the serotonergic system and have chronic rather than acute antidepressant properties. Based on unpublished ongoing experiments in Dr. Yau's Lab, sub-chronic administration of AdipoRon at a higher dosage (20 mg/kg, i.p. for 7 days) resulted in antidepressant and anxiolytic results independent to adult neurogenesis. Although this perspective disagrees with our initial hypothesis, namely that adiponectin acted as a rapid antidepressant through the modulation of the hippocampal plasticity, it increases our understanding of the adiponectin's antidepressant properties. Moreover, adiponectin stands out as a pharmacological intervention based on its combined metabolic properties. In a parallel experiment, Dr. Yau has just shown that adiponectin (20 mg/kg) can reverse the cognitive and mood impairments associated with a diabetic model (Lee et al., 2020, *manuscript under revision*).

We should also bear in mind that the adiponectin signaling system is very complex. Recalling what has been reviewed in previous chapters, the adiponectin expression is regulated by at least five transcriptional factors, and undergoes several post-translational and post-transcriptional steps involving different chaperones before it is ready to be secreted. It can circulate in different oligomeric forms, each of them having different half-lives, selectively working in different tissues, and having different affinities for each adiponectin receptors. The adiponectin receptors, in turn, represent a novel class of receptors whose structure was recognized and described only a few years ago. The relevance

of all these factors need to be further investigated in order to draw solid conclusions on how and when the adiponectin signaling system can be targeted as a therapeutic intervention.

Complementary, we aimed to determine if the activation of a pathway connecting the hippocampus with the mPFC (i.e., the vHipp-mPFC pathway) would be involved in the modulation of antidepressant response. Such objective was based on the relevance of both structures for mood modulation, and on previous findings implicating loss of connectivity between these regions in depressed patients. In agreement with what we expected, chronic activation of the vHipp-mPFC pathway induced an antidepressant response in healthy mice. Interestingly, this effect was accompanied by a reduction in hippocampal cellular proliferation and an impairment in spatial memory recognition. It suggests that increased neurogenesis and the activation of the vHipp-mPFC pathway could be independent factors modulating antidepressant actions. Moreover, over activation of this pathway may be associated with side effects involving other hippocampal dependent tasks, such as spatial learning and memory.

In summary, our findings expand the understanding regarding the acute effects of adiponectin in mood modulation and hippocampal plasticity, suggesting that the acute activation of the adiponectin signaling system in healthy conditions might be associated with side effects in the short term. Moreover, we shed light over a potentially new property of the vHipp-mPFC pathway in modulating antidepressant effects, enhancing our understanding of depression from the circuit level.

CHAPTER 6 – REFERENCES

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