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α-ketoglutarate (αKG) acts as a protective factor against chronic stress and has antidepressant properties by modulating BDNF levels in the brain

By

Fady Eid

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α-ketoglutarate (αKG) acts as a protective factor to chronic stress and has antidepressant properties by modulating BDNF levels in the brain.

Fady Eid

ABSTRACT

Major depression and anxiety are one of the most common mental disorders worldwide. Attempts to alleviate these mental burdens are usually through psycho-therapeutic approaches, but little focus has been placed on lifestyle modifications. Adoption of these modifications, such as physical exercise, improved dietary intake, and proper sleep, have served as a nexus between clinical treatments as well as general health promotion. A multitude of studies points to how physical exercise has positive outcomes on depression and anxiety via the induction of a neurotrophic factor known as brain-derived neurotrophic factor (BDNF). α-ketoglutarate (aKG) was recently identified as a factor that is released into the blood upon exercise. In this study, we investigated whether aKG has prophylactic and antidepressant effects in chronic social defeat stress and chronic variable stress models of depression, as well as unravel the underlying molecular mechanisms. Our work shows that aKG serves as a potential prophylactic treatment for depression in both males and females through the modulation of BDNF in specific brain regions. In males, aKG pretreatment promotes resilience to stress via the PGC1a-BDNF axis within the hippocampus. Moreover, aKG exhibited antidepressant effects in susceptible female mice by modulating BDNF levels. In the hippocampus, aKG increases BDNF levels possibly through the PGC1a-BDNF pathway. In the NAc, aKG decreases BDNF levels possibly through the FNDC5-BDNF pathway.

Keywords: α-ketoglutarate, chronic social defeat stress, chronic variable stress, BDNF, PGC1a, FNDC5

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List of Abbreviations

aKG: α-ketoglutarate

BDNF: Brain Derived Neurotrophic Factor

CBT: Cognitive behavioral therapy

CSDS: Chronic Social Defeat Stress

CVS: Chronic Variable Stress

FNDC5: fibronectin domain-containing 5

H3K9me2: Histone H3 lysine 9 dimethylation

H3K9me3: Histone H3 lysine 9 trimethylation

MDD: Major Depressive Disorder

NAc: Nucleus accumbens

OF: Open Field

PGC1a: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

SI: Social Interaction

VTA: Ventral Tegmental Area

Chapter One

Introduction

1.1. Major depressive disorder

1.1.1 Background

Major depressive disorder (MDD) is a disease marked by anhedonia, loss of social functioning, impaired cognitive function, and a myriad of other symptoms (Otte et al., 2016). It is one of the most prevalent disorders worldwide (WHO, 2021). Two out of every three individuals exhibit MDD symptoms, yet fail to realize that they are suffering from this burden. As a result, they don't seek proper medical attention (Greenberg et al., 2015). Additionally, women have almost twice the chance to be diagnosed with MDD than men (Otte et al., 2016). The impairments in social activity and communication remain prevalent even after a patient has recovered from depression (Rhebergen et al., 2010) and are tightly associated with decreased work performance and increased unemployment (Rizvi et al., 2015). The loss of productivity in individuals with MDD as well as the high cost of ineffective treatments lead to both direct and indirect costs that exhaust a country's economy (Simon, 2003; Blazer 2000).

1.1.2 Etiology and Pathophysiology

Our understanding of MDD pathophysiology remains limited. MDD is associated with both structural and functional changes in the brain (Dai et al., 2019). The establishment of vertebrate animal models that exhibit depression-like symptoms have facilitated our understanding of the molecular mechanisms that link different brain regions to social anhedonia, social avoidance, and anxiety (Heshmati and Russo, 2015). Indeed, growth factor signaling is

significantly altered in MDD patients. Brain-derived neurotrophic factor (BDNF), a neurotrophic growth factor, is highly disrupted in animal models of chronic stress (Murakami et al., 2007; Duman and Monteggia, 2006; Castrén et al., 2007) and MDD patients (Lee et al., 2007; Kim et al., 2007). BDNF is involved in diverse functions including neurogenesis, dendrite formation, and neural plasticity (Rossi et al., 2006). Alterations in BDNF expression are affected due to both genetic and environmental factors (de Paula et al., 2021). BDNF expression in response to stress is differentially altered in distinct brain region, particularly the hippocampus and the nucleus accumbens (NAc). The hippocampus is a complex part of the brain that plays key roles in memory formation and learning (Gray et al., 2013). Stress induces structural and functional changes in the hippocampus that are a direct result of alterations in BDNF expression (Gray et al, 2013). Hippocampal BDNF levels are decreased upon exposure to stress, and this decrease can mediate depression-like symptoms (Eckert et al., 2020; Nasrallah et al., 2019; Dulcot and Kabbaj, 2013; Jiang et al., 2014; Tsankova et al., 2006). Another brain region that mediates reward and satisfaction is the NAc. This brain region is a major component of the dopaminergic pathway that projects from the ventral tegmental area (VTA) (Cui et al., 2020). Increased BDNF signaling through this pathway results in susceptibility to stress, while blocking BDNF action in this brain area leads to resilience to stress (Koo et al., 2016).

1.1.3 Treatments of Depression

The treatment approach needs to be tailored to the patient's personal preferences and medical condition (Duval et al., 2006). Established psycho-therapeutic approaches are the major form of treatments for depression and anxiety. Psychosocial treatments have been gaining popularity. The most common is cognitive behavioral therapy (CBT), which leads to positive outcomes for late-life depression (Renn & Areán, 2017). Medications to treat MDD can be split

into different groups. These include the tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), and selective serotonin noradrenaline re-uptake inhibitors (SNRIs) (Institute for Quality and Efficiency in Health Care, 2015). The most common form of treatment is the prescription of antidepressants, particularly SSRIs such as fluoxetine (Penn & Tracy, 2012). These antidepressants target the same mechanistic pathway that has been established over half a century ago (Block & Nemeroff, 2014). They tend to have a slow mode of action; as a result, months of treatment may be necessary before any positive adaptations can be felt by the patients (Krishnan & Nestler, 2008). Due to the limited efficacy of antidepressants, it is integral to develop novel therapies. This is highly dependent on identifying the molecular mechanisms underlying depression. A newfound breakthrough is ketamine, a medication commonly used to induce anesthesia. Ketamine has shown promise as a putative non-invasive type of therapy that rejuvenates the aging brain (Venturino et al., 2021) and has antidepressant and prophylactic properties in animal models (Wang et al., 2011; Mastrodonato et al., 2018). Interestingly, ketamine is the fastest acting antidepressant that is being prescribed (Matveychuk et al., 2020; Dunman, 2018).

1.2. Lifestyle Modifications and MDD

1.2.1 Beneficial Effects of Diets

Psycho-therapeutic approaches are utilized to prevent depression and anxiety (Christensen et al., 2010), however few have focused on the importance of lifestyle modifications. Adoption of these modifications, such as physical exercise, improved dietary intake, and proper sleep, have served as a nexus between clinical treatments as well as general health promotion (Sarris et al., 2014).

One lifestyle modification that plays a role in treatment of and protection from depression is diet. Different types of diets affect are correlated with either improvement or worsening of depression symptoms. A high protein diet (HPD) promotes social interaction and resilience to chronic stress, by inducing hippocampal BDNF expression (Nasrallah et al., 2019). Indeed the effect of the HPD is mediated by branched-chain amino acids (BCAA), often used by athletes. BCAA reverse social avoidance behavior and promote resilience to chronic stress in a chronic social defeat stress (CSDS) model (Nasrallah et al., 2019). The positive effect of BCAA was associated with the induction of hippocampal BDNF expression through the upregulation of the exercise-regulated PGC1a/FNDC5 axis, an established mechanistic pathway for the induction of BDNF (Nasrallah et al., 2019). The amino acid methionine also promoted resilience to chronic stress and enhanced social interaction suggesting the the positive effects of HPD were not only mediated by BCAA (Bilen et al., 2020). Another type of diet, the ketogenic diet, also has positive effects on brain health. Indeed, ketogenic diets induce neurogenesis and rescue memory defects (Benjamin et al., 2017). Intake of high sugar and high fat diets on the other hand is tightly associate with depression-like symptoms (Del Rio et al., 2016). Some studies have assessed the effects of individual nutrients. The Omega 3 (n-3) fatty acid is inversely associated with symptoms of depression (Murakami et al., 2010). Vitamins such as vitamin B_{12} and minerals such as iron, zinc, and selenium were also investigated. Deficiencies in vitamin B_{12} is associated with an increased risk of depression (Penninx et al., 2000), while zinc dysregulation correlates with neurological illnesses, including depression (Petrilli et al., 2017). This work suggest the importance of diet in preventing depression-like symptoms and validates the need to understand the molecular mechanism regulated by the diet components.

1.2.2 Beneficial Effects of Exercise

Physical exercise exerts positive effects on stress and depression (Paolucci et al., 2018; Balchin et al., 2016) through established endogenous mechanisms that involve the induction of hippocampal BDNF (Sleiman et al., 2016). One established underlying mechanism that promotes the upregulation of hippocampal BDNF is the activation of the exercise-regulated PGC1a/FNDC5 pathway (Wrann et al., 2013; El Hayek et al., 2019).

Studies have shown that alterations in morphology and size of white matter within the brain were linked to depression (Taylor et al., 2004; Nobuhara et al., 2004). Exercising rats that exihibit depression-like behavior showed significant sucrose preference as compared to their sedentary controls (Xiao et al., 2017). Moreover, white matter morphology was restored and size was increased in the exercise rats as opposed to sedentary ones (Xiao et al., 2017). Another study showed that voluntary running wheel exercise has a greater impact in reversing hyperalgesia induced by social defeat stress (SDS) when compared to the established SSRI fluoxetine (Pagliusi Jr et al., 2020).

Exercise leads to the release of peripheral factors by multiple tissues including muscle, liver, and bone into the blood (Moon et al., 2016; Zhang et al., 2017). Certain exercise-induced factors are metabolites such as β-hydroxybutyrate (DBHB), lactate, and α-ketoglutarate (aKG) (Kim et al., 2013; El Hayek et al., 2019; Yuan et al., 2021). Previous studies have shown that the liver secretes DBHB into the circulation during voluntary exercise. DBHB in turn reaches the hippocampus where it induces BDNF expression by acting as an HDAC inhibitor (Sleiman et al., 2016). Additionally, DBHB decreases depressive-like behavior is mice models of stress through the promotion of BDNF and the upregulation of histone3-lysine9-β-hydroxybutyrylation (Chen et al., 2017).

Another exercise factor released during exercise by the muscles is lactate. This metabolite can cross the blood brain barrier via specific transporters and safeguards neurons from ischemic stress (Berthet et al., 2009). Lactate produced in the muscle, enters into the hippocampus and activates the histone deacetylase sirtuin 1 (SIRT1), which in turn activates upstream elements such as PGC1a and FNDC5, leading to hippocampal BDNF expression (El Hayek et al., 2019). Lactate was also seen to act as both a prophylactic factor as well as antidepressant in mouse model of chronic stress through the modulation of HDAC levels and in turn gene expression in the hippocampus (Karnib et al., 2019). Peripheral lactate administration also showed antidepressant properties in animal models of depression (Carrad et al., 2018).

Exercise induces the expression of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1a) in skeletal muscles and also leads to the formation of fibronectin domain-containing protein 5 (FNDC5), whose cleavage leads to the generation of irisin (Norheim et al., 2014). Irisin crosses the blood brain barrier and acts on the hippocampus and the VTA (Phillips et al., 2014). Irisin induces beneficial effects on neuronal function by ameliorating depressive behavior (Wang et al., 2016). exercise-induced irisin activates the hippocampal FNDC5-PGC1a axis, which leads to the amelioration of depressive-like symptoms (Wrann et al., 2015). Another study connected this axis to BDNF, demonstrating that irisin could promote BDNF expression, and therefore enhance mood (Papp et al., 2017).

1.3. α-ketoglutarate (aKG)

aKG is a tricarboxylic acid cycle intermediate that is also a source of glutamine and glutamate, stimulating protein formation and hindering protein degradation in the muscles (Wu et al., 2016). αKG is a cosubstrate for several histone demethylases that contain a Jumonji-C

domain (Tian et al., 2020). This suggests that changes in its levels can potentially have important effects on the status of gene expression in cells. Interestingly, aKG is a newly identified exercise factor. Indeed, 40 minutes of resistance exercise and voluntary exercise in 10-week-old mice induce a significant increase in aKG serum levels (Yuan et al., 2020). aKG plays important roles in the body. It prevents neuronal damage, reverses aging, maintains gut integrity, and acts as an antioxidant (He et al., 2017; He et al., 2015; Liu et al., 2018). It also has neuroprotective effects, such as reducing the accumulation of alpha-synuclein, which is a presynaptic neuronal protein known to be linked to Parkinson's disease pathogenesis (Satpute et al., 2013). A separate study showed that aKG delays age-related diseases, as well as extends lifespan (Chin et al., 2014). The findings that suggest that aKG has beneficial effects on ageing are supported by the observation that delivery of aKG promotes a healthier, longer life associated with decreased levels of systemic inflammatory cytokines (Shahmirzadi et al., 2020). Metabolic profiling of urine samples from rats exhibiting depression-like symptoms revealed a decrease in αKG as compared to control rats (Zheng et al., 2010). Indeed, patients diagnosed with MDD have decreased levels of αKG in their urine (Chen et al., 2017). This suggests that lower αKG levels may serve as a biological marker for depression.

1.4. Aim of the study

In this study, we hypothesized that aKG pretreatment may protect mice against chronic stress and may help alleviate depressive-like symptoms. Hence, we tested whether aKG can serve as prophylactic treatment for depression. We also hypothesized that, like other exercise factors, aKG can have antidepressant effects following the establishment of depressive-like behavior suggesting that it can serve as a novel treatment. We tested our hypotheses in both female and male mice in an attempt to determine if there are any sex-specific differences. For

that purpose, we used the chronic variable stress (CVS) to establish depression-like symptoms in females and the chronic social defeat stress (CSDS) to establish depression-like symptoms in males.

Chapter Two

Materials and Methods

2.1 Animal housing

For all experimental paradigms, adult male and female C57BL/6J mice were housed in cages and provided with food and water *ad libitum* and kept on a 12h light-dark cycle.

2.2 Chronic Social Defeat Stress (CSDS) model:

The CSDS model is used to mimic the symptoms of depression in male C57BL/6J mice, as previously described (Golden et al., 2011). This paradigm consists of three stages. First, CD-1 aggressor mice are screened followed by the selection of the aggressive ones. Second, the experimental is exposed to social defeat for 10 consecutive days. The defeat sessions require direct physical contact between the aggressor mouse and experimental mouse for 7 min. The experimental mouse is then transferred to the other compartment in the cage separated by a perforated plexiglass to allow for sensory interaction between the experimental and aggressor mice for the next 24h. The control mice were housed with the aggressor as well and alternated daily for 10 days, but no direct contact was allowed between the mice. The third stage involves social behavioral testing, which occurs on the 11^{th} day, followed by animal sacrifice and brain tissue collection.

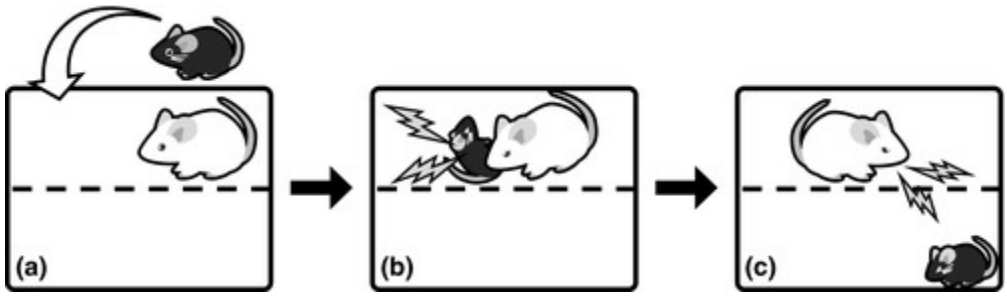


Figure 1: Illustrative schematic of CSDS model of depression (Extracted from Toyoda, 2017).

2.3 Chronic Variable Stress (CVS) model:

The CVS model is used to mimic the symptoms of depression in female C57BL/6J mice, as previously described (Rosinger et al., 2020). Animals are singly housed into separate vivariums where stressed animals endure the variable stress paradigm for 9 days while control mice are not handled until behavioral testing on the 10th day. Briefly, mice undergo one or two variable stressors, daily. When two stressors are present, there is a minimum of 2h between them. The following artillery of variable stressors were exposed to the animals: Food deprivation overnight (12h), cold water swim (5 min, or 10 min), restraint stress (30 min), tail suspension (10 min), 45^{o} cage tilt (3h), wet cage (3h), and naphthalene exposure (1h). On the first day, 300mL of water was poured into the home cage and was mixed with the bedding and left undisturbed for three hours. At night, food was removed from the cages for 12 hours. On the second day, the cages were tilted at 45^{o} and left undisturbed for 3 hours. On the third day, the mice were suspended from their tail for 10 minutes. On the fourth day, the mice were subjected to a cold water swim (18^{o}C-20^{o}C) for 5 minutes. They were then dried and placed back into their cages. Later, naphthalene was placed within the home cage enclosed in a container that cannot be accessed by the mice. On day 5, the mice were subjected to 30 minutes of restraint within a 50mL falcon tube. Day 6, 7, 8, and 9 involved a repetition of the previous stressors. Social

behavioral testing occurs on the 10th day, followed by animal sacrifice and brain tissue collection.

2.4 Social interaction test

The social interaction test is conducted one day after the last CSDS or CVS session as previously described (Kaidanovich-Beilin et al., 2011). The mice are habituated for 5 min in an apparatus composed of three interconnected chambers. Two of those chambers contain a circular wire enclosure. After completion of the habituation phase, a social stimulus C57BL/6J mouse is placed in one of the two empty wire enclosures and the experimental mouse is placed back to the central chamber. For the next 10 min, the experimental mouse is allowed to navigate freely between the chambers while its movement is recorded with a camera. The time spent in each chamber is measured by the ANY-maze program. To establish if the mouse was susceptible or resilient to stress, the social interaction (SI) ratio is calculated by dividing the time spent in the interaction zone over the time spent in the no interaction zone. The mouse is considered susceptible if the ratio is less 1 and resilient if the ratio is greater 1 (Henriques-Alves and Queiroz, 2015).

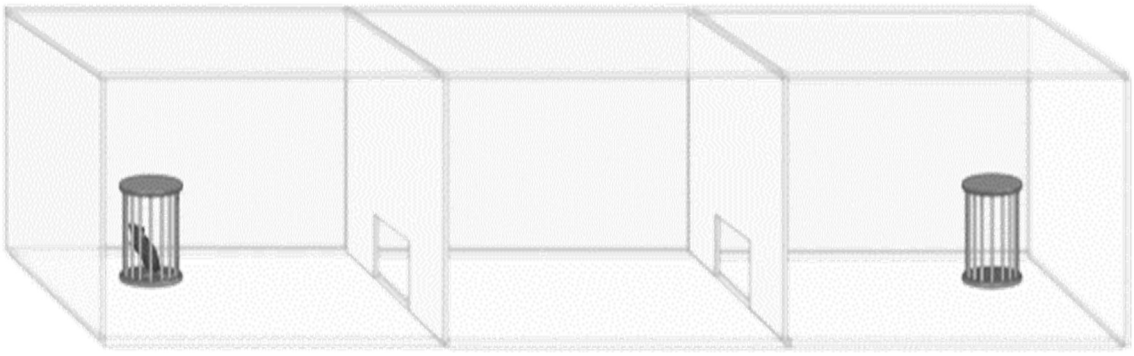


Figure 2: Schematic illustration of SI test showing the three interconnected chambers and the two circular wire enclosure, as previously described (Kaidanovich-Beilin et al., 2011).

2.5 Open Field test

The open field test is used to measure anxiety-like behaviors and general locomotor ability (Seibenhener and Wooten, 2015). Each mouse freely explores the open field for 5 min. The average distance travelled and the time will be both recorded with a camera and measured by the ANY-maze program.

2.6 aKG injections

For the pretreatment paradigm, mice either received daily intraperitoneal injections (i.p.) of saline or aKG for 5 days prior to the start of a chronic stress paradigm. For the post-treatment paradigm, mice were subjected to chronic stress and then underwent SI test (test day 1). After that, mice were split into susceptible or resilient groups. Susceptible mice received wither saline or aKG i.p injection for 14 days. After the 14 day treatment, the SI test was performed again (test day 2). The dose used for aKG is 300 mg/kg and was selected based on the finding that this dose extends the lifespan of a mouse model of Leigh syndrome and improved neurological phenotype (Lee et al., 2019).

2.7 Immunoblot Analysis

To determine FNDC5, PGC1a, BDNF, ACTIN and GAPDH relative protein levels, total cellular proteins were extracted from the hippocampi and nucleus accumbens of sacrificed mice using RIPA-B (1% SDS, 1% Triton X-100, 50 mm Tris-Cl, 500 mm NaCl, pH 7.4 and 1 mm EDTA) in the presence of MG132 (SIGMA) and protease inhibitors (SIGMA). Antibodies PGC1a (ab54481; abcam; 1:1000), FNDC5 (ab174833; abcam; 1:1000), BDNF (sc-65514; Santa Cruz Biotechnology; 1:500) ACTIN (sc-47778; Santa Cruz Biotechnology; 1:1000) and GAPDH

(14C10, Cell Signaling; 1:1000) were used. Proteins are visualized using the ChemiDoc Imaging System (BioRad) and quantified with the ImageJ software.

2.8 Statistical Analysis

2way ANOVA followed by Tukey post-hoc tests were done using GraphPad Prism (Version 9.1) to measure statistical significance. $p < 0.05$ was considered to be statistically significant.

Chapter Three

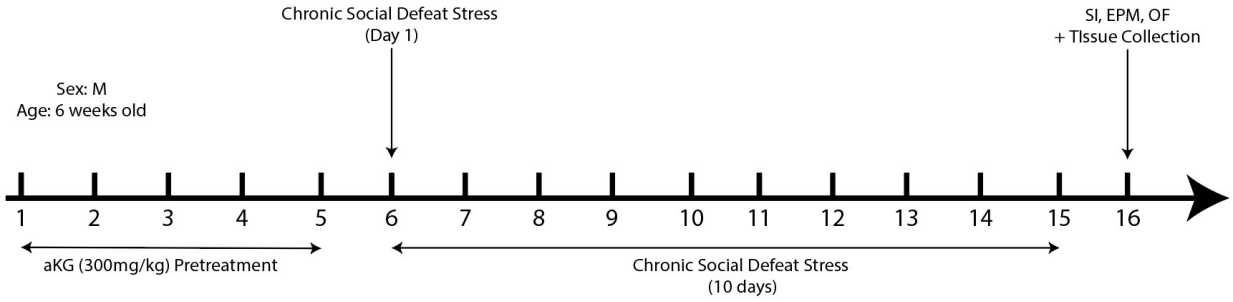
Results

3.1 aKG pretreatment promotes resilience to stress and prevents social avoidance behavior in male C57BL/6J mice

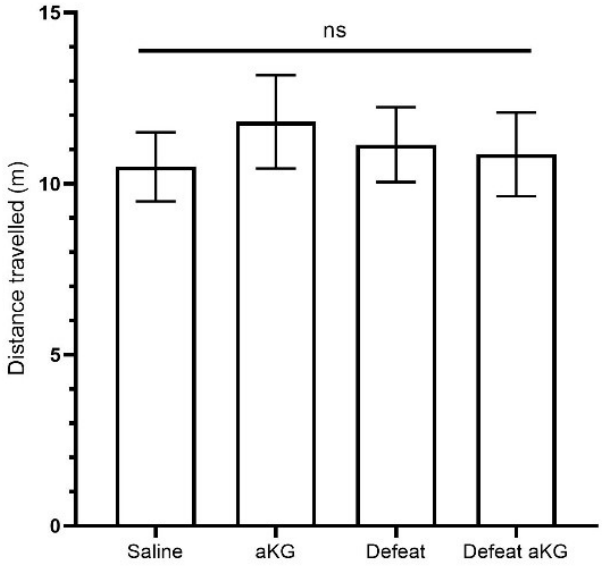
To determine whether aKG prevents susceptibility to chronic stress, 6-7-week-old male C57BL/6J mice were subjected to a CSDS paradigm, a validated model utilized to mimic depression symptoms in male mice. Experimental mice were exposed to social defeat for 10 consecutive days through direct contact with the aggressor for 7 minutes per defeat session, while the control mice only had sensory contact with the aggressor mouse (Figure 3A). On the 11^{th} day, the mice were subjected to the social interaction (SI) test. This test directly assesses susceptibility versus resilience to stress as well as social avoidance behavior by comparing the time mice spent in the interaction zone to the time spent in the non-interaction zone. The mice were divided into four groups: mice receiving saline, defeat mice receiving saline, mice receiving aKG, and defeat mice receiving aKG. aKG treatment commenced five days prior to the start of CSDS. First, locomotor activity was analyzed using the open field test, where the average distance travelled was the same for all groups (Figure 3B). This indicated that CSDS does not affect locomotor activity. The SI test revealed that aKG promotes resilience to CSDS: 20% of defeat mice receiving saline were resilient to stress, whereas 100% of defeat mice receiving aKG were resilient to stress (Figure 3C). We next assessed the distribution of the SI ratios of the different groups (Figure 3D). The dotted line represents the threshold above which individual animals are considered to be resilient to stress. As expected, The SI ratio of defeat mice receiving

saline was significantly lower than controls. In contrast, the SI ratio of defeat mice receiving aKG was significantly higher than that of defeat mice receiving saline (Figure 3D). Accordingly, defeat mice receiving saline spent significantly less time interacting with the social stimulus as compared to control animals (Figure 3E). This social avoidance phenotype was prevented by aKG pretreatment since the average time spent interacting with the social stimulus was significantly higher in the defeat aKG group as compared to the defeat saline group (Figure 3E). The converse results were observed when we assessed the no interaction time. Together, our results suggest that aKG serves as a protective and prophylactic treatment against the onset of depressive-like symptoms associated with this paradigm.

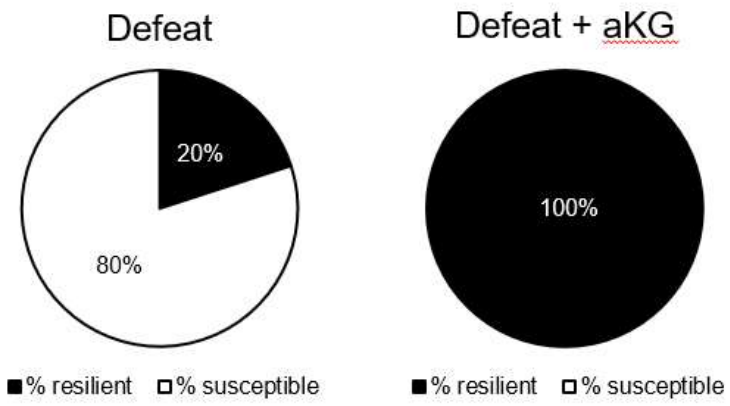
A



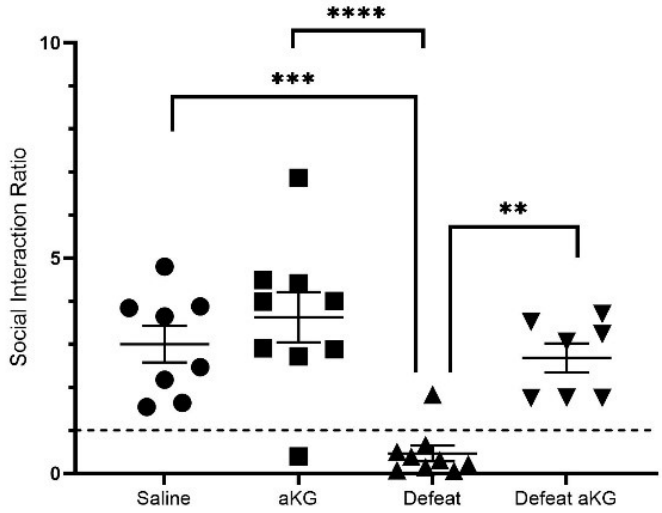
B


ns
Distance travelled (m)
15
10
5
0
Saline
aKG
Defeat
Defeat aKG

C


Defeat
20%
80%
% resilient
% susceptible
Defeat + aKG
100%
% resilient
% susceptible

D



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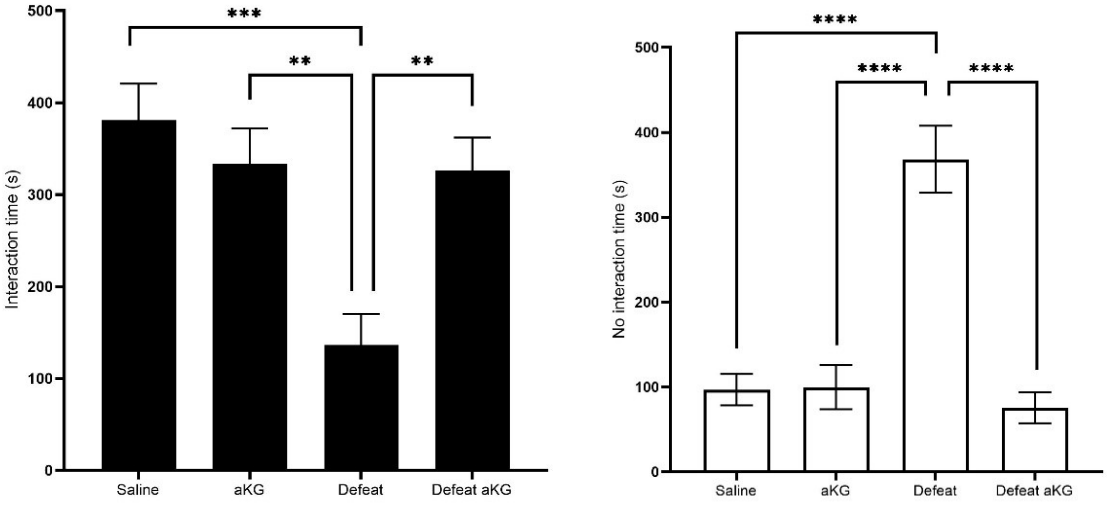


Figure 3: aKG serves as a protective factor that promotes resilience to stress and prevents social interaction deficits in mice subjected to CSDS. Statistical significance were measured by 2way ANOVA followed by Tukey's multiple comparison test.

(A) Mice were treated with aKG (300mg/kg) five days prior to the start of CSDS. The CSDS paradigm consists of ten consecutive days of defeat sessions involving direct contact between the resident (aggressor) mouse and the experimental mouse for 7 min. On day 11 of the CSDS paradigm, behavioral tests and brain tissue collection are conducted.

(B) The average distance travelled in the open field between all mice groups was unchanged, indicating that locomotor activity was not affected by aKG or defeat.

(C) aKG promotes resilience to stress. In the group of mice treated with saline and then subjected to CSDS (n=10), 20% were resilient to stress, while 80% are susceptible to stress. In the group of mice treated with aKG and then subjected to CSDS (n=10), 100% are resilient to stress, while 0% are susceptible.

(D) Scatter plot graph showing the SI ratio distribution across the different mice groups. The dotted line represents the threshold above which mice are classified as resilient to stress. ****p<.0001. Defeat: $F(1,29) = 17.18$, $p=0.0003$. Treatment: $F(1,29) = 11.50$, $p= 0.0020$. n= 7 for Saline, 8 for aKG, 10 for Defeat Saline, and 7 for Defeat aKG.

(E) Graphs show the social interaction time (left) and no interaction time (right). i.p. injections of aKG (300mg/kg, 5 days prior to CSDS) showed a significant protection against the social avoidance phenotype in defeat mice treated with saline. Interaction time: **p<.0021,***p<.0002. Interaction: $F(1,36) = 10.21$, $p=0.0029$. Defeat: $F(1,36) = 11.49$, $p= 0.0017$. n= 10 per group. No interaction time: ****p<.0001. Interaction: $F(1,36) = 30.12$, $p<0.0001$. Defeat: $F(1,36) = 21.10$, $p<0.0001$. Treatment: $F(1,36) = 28.95$, $p<0.0001$. n= 10 per group.

3.2 aKG mediates resilience to CSDS by modulating BDNF levels

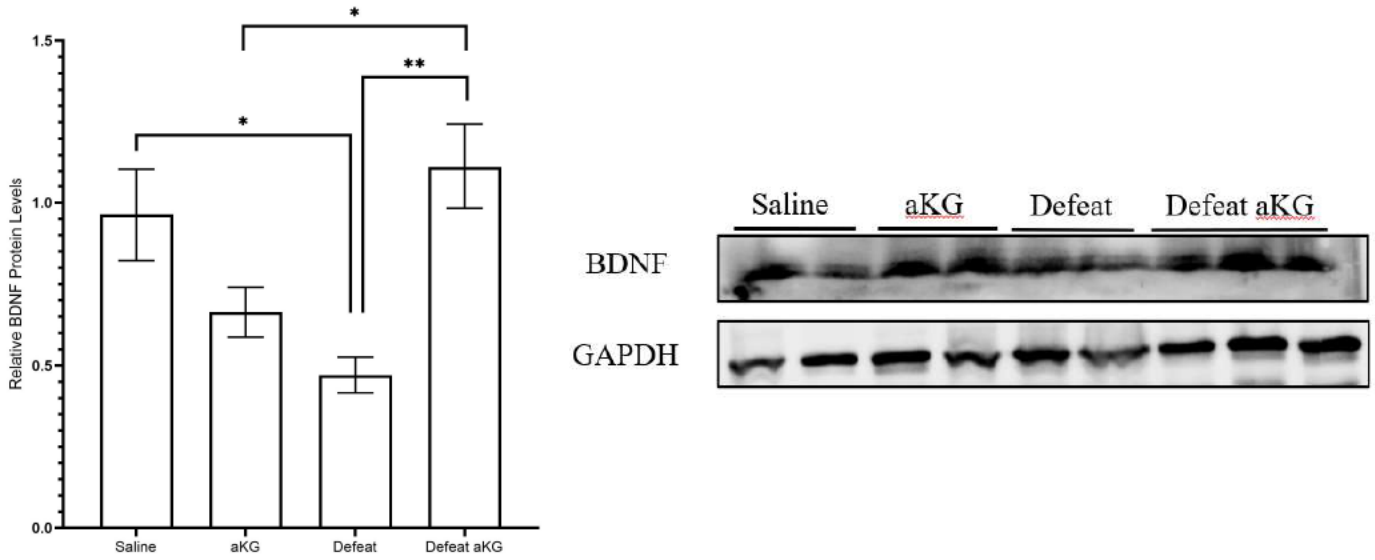
The hippocampus plays a major role in learning and memory. It is highly sensitive to stress, which induces structural and functional changes on the cellular level (Gray et al., 2013). Several studies have showed that BDNF expression is downregulated in the hippocampus in response to stress (Nasrallah et al., 2019; Eckert et al., 2020; Dulcot and Kabbaj, 2013; Jiang et al., 2014; Tsankova et al., 2006). Decreases in hippocampal BDNF expression is highly

associated with depression (Notaras et al., 2015; Yu et al., 2012). Because of its important role in psychiatric disorders, we assessed whether aKG rescues BDNF expression in the hippocampi of mice subjected to CSDS. Western blot analysis revealed a significant decrease in BDNF levels in the hippocampi of defeat mice receiving saline as compared to control. aKG pretreatment significantly increased the hippocampal BDNF protein levels in defeat mice back to control levels (Figure 4A). Since PGC1a acts as an upstream activator to BDNF and is regulated by exercise (Wrann et al., 2013; El Hayek et al., 2019), we assessed whether its levels are modulated by stress and aKG and whether the changes in its levels are correlated with those observed in BDNF levels. Interestingly, stress didn't significantly modulate hippocampal PGC1a levels (Figure 4B). However, aKG pretreatment significantly increased hippocampal PGC1a levels in defeat mice, but not control mice. Our results suggest that hippocampal BDNF is modulated by stress and that aKG pretreatment restores normal BDNF levels in part through increasing the levels of PGC1a.

Another key brain area that mediates reward and satisfaction is the NAc. The specific roles for the NAc in depression remain poorly understood, but some studies have shown that this brain region is an important mediator of depressive-like outcomes in response to CSDS (Francis et al., 2015). In contrast to the hippocampus, BDNF levels in the NAc are significantly increased in response to stress (Walsh et al., 2014). Western blot analysis revealed that BDNF levels in the NAC were significantly increased in defeat mice receiving saline, but this increase was prevented in the defeat mice receiving aKG pretreatment (Figure 4C). Interestingly, no significant changes were observed in its upstream activator PGC1a (Figure 4D). Taken together, our results are consistent with aKG pretreatment promoting resilience to CSDS by modulating

BDNF levels in specific brain regions. In the hippocampus, aKG pretreatment potentially promotes resilience via the PGC1a-BDNF signaling pathway.

A



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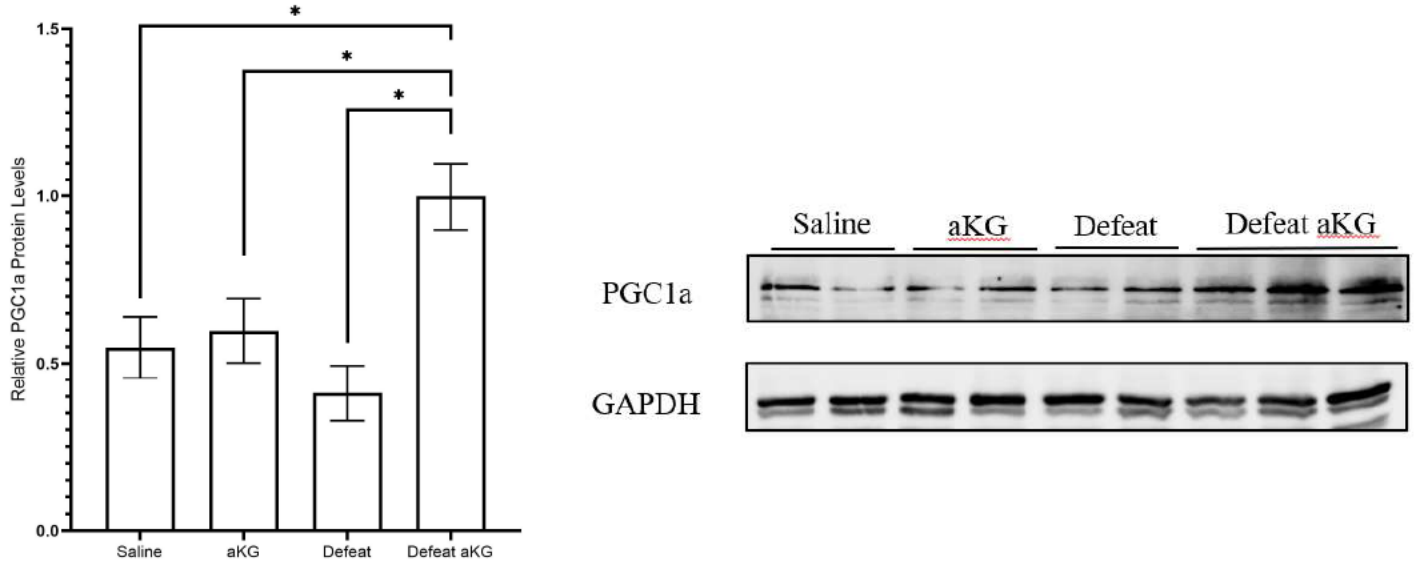

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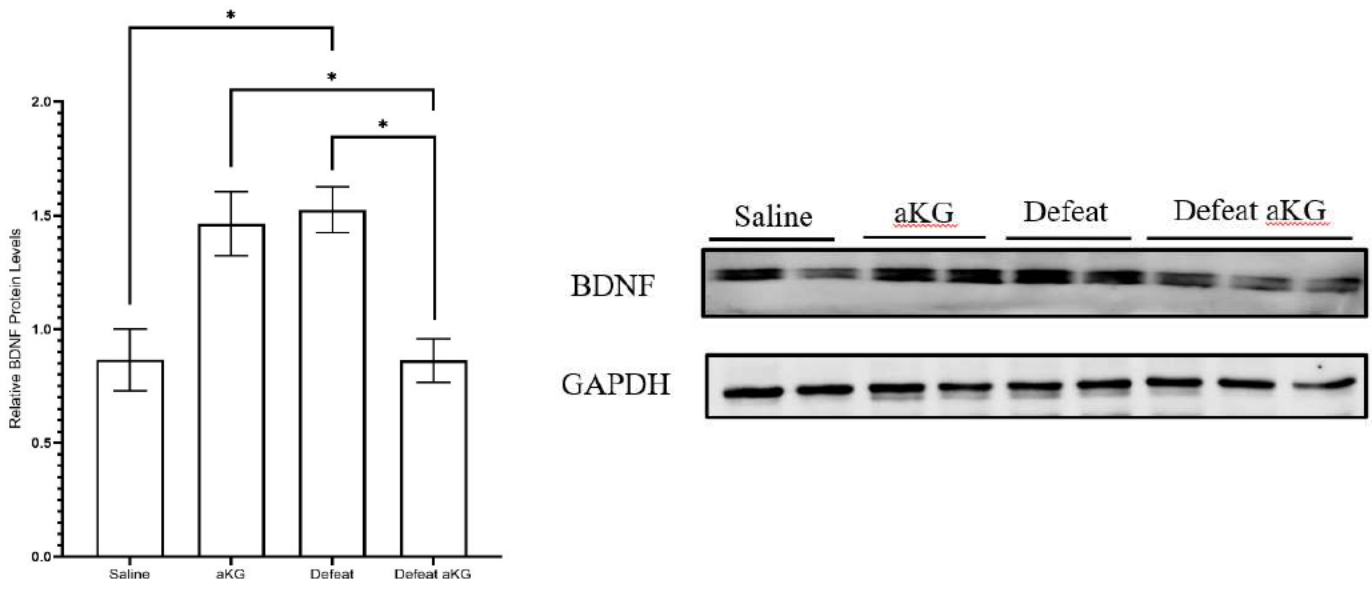


D

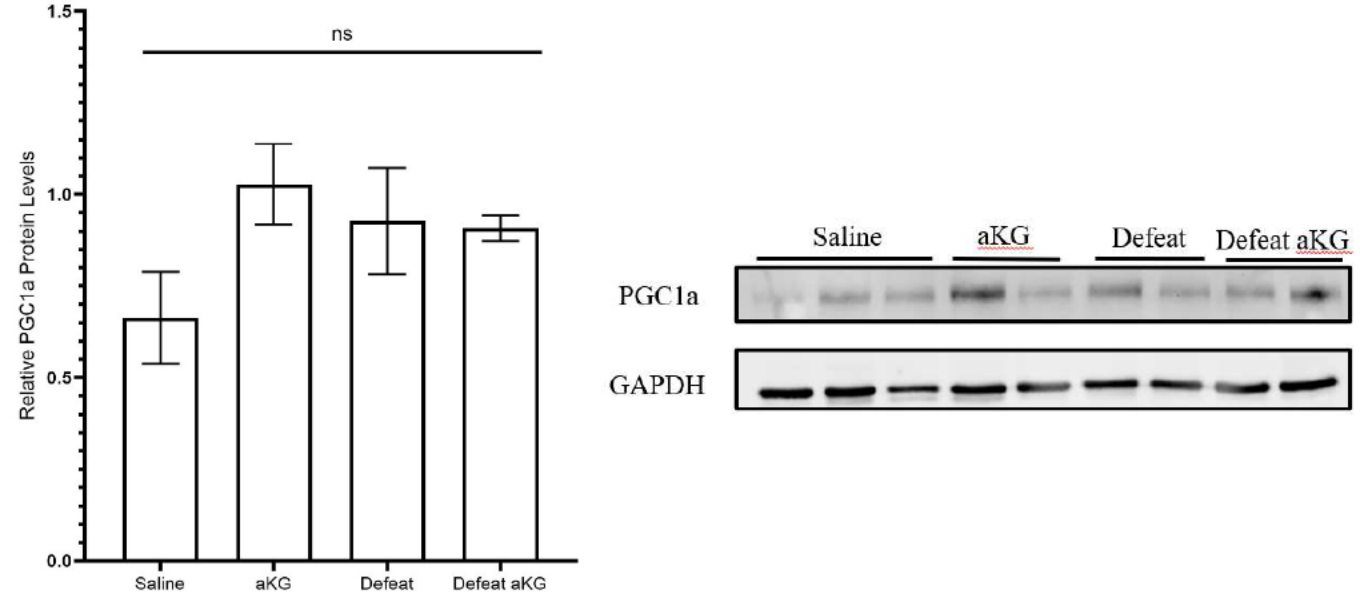


Figure 4: aKG pretreatment restores BDNF protein levels in the hippocampus and prevents its increase in the NAc of mice subjected to CSDS.

(A) Representative western blot of hippocampal BDNF (right) along with GAPDH as the endogenous control. Quantifications are seen on the left. *p<.03, **p<.0021. Interaction: F(1,28) = 15.55, p=0.0005. n= 9 Saline, 8 aKG, 6 Defeat, and 9 Defeat aKG

(B) Representative western blot of hippocampal PGC1a (right) along with GAPDH as the endogenous control. Quantifications are seen on the left. *p<.03. Interaction: F(1,22) = 6.958, p= 0.0150. Treatment: F(1,22) = 9.801, p=0.0049. n= 8 Saline, 8 aKG, 4 Defeat, and 6 Defeat aKG.

(C) Representative western blot of BDNF (right) in the NAc along with GAPDH as the endogenous control. Quantifications are seen on the left. *p<.03. Interaction: F(1,5) = 28.64, p=0.0031. n= 2 for Saline, aKG, and Defeat, 3 for Defeat aKG.

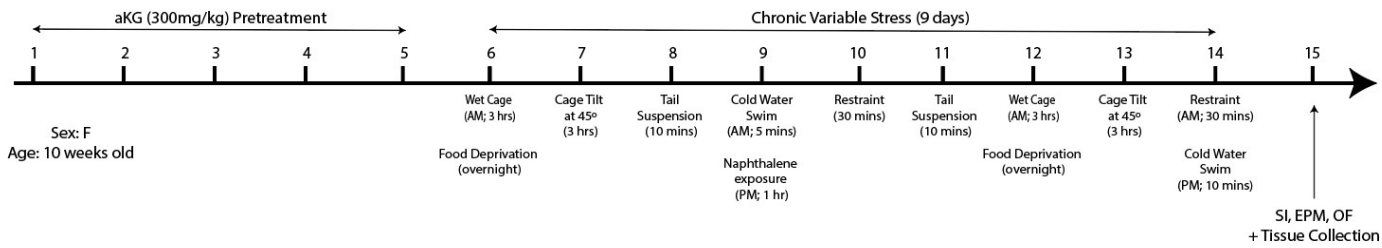
(D) Representative western blot of PGC1a (right) in the NAc along with GAPDH as the endogenous control. Quantifications are seen on the left. No significant changes were seen.

3.3 aKG pretreatment promotes resilience to stress and prevents social avoidance behavior in female C57BL/6J mice

We next assessed whether aKG pretreatment had similar protective effects on females subjected to chronic stress. 10-week-old female C57BL/6J mice were subjected to a CVS paradigm, which is a validated test to mimic symptoms of depression in female mice. Animals were singly housed in separate cages to undergo chronic variable stress (CVS). For 9 days, female mice were exposed to different stressors, followed by behavioral testing on the 10^{th} day (Figure 5A). The mice were divided into four groups: control mice receiving saline, control mice receiving aKG, CVS mice receiving saline, and CVS mice receiving aKG. aKG treatment commenced five days prior to the start of CSDS. First, locomotor activity was analyzed using the

open field test. Both CVS mice and CVS mice receiving aKG travelled significantly higher distances as compared to control mice (Figure 5B). This indicated that CVS does not impair locomotion, but rather significantly enhances locomotor activity and that aKG treatment has no significant effects on its own. The SI test revealed that aKG promotes resilience to CVS: 20% of CVS mice receiving saline were resilient to stress, whereas 90% of CVS mice receiving aKG were resilient to stress (Figure 5C). We next assessed the distribution of the SI ratios of the different groups (Figure 5D). The dotted line represents the threshold above which individual animals are considered to be resilient to stress. As expected, The SI ratio of CVS mice receiving saline was significantly lower than controls. In contrast, the SI ratio of CVS mice receiving aKG was significantly higher than that of CVS mice receiving saline (Figure 5D). Accordingly, CVS mice receiving saline spent significantly less time interacting with the social stimulus as compared to control animals (Figure 5E). This social avoidance phenotype was prevented by aKG pretreatment since the average time spent interacting with the social stimulus was significantly higher in the CVS aKG group as compared to the CVS saline group (Figure 5E). The converse results were observed when we assessed the no interaction time. Together, our results suggest that aKG serves as a protective and prophylactic treatment against the onset of depressive-like symptoms in both male and females.

A



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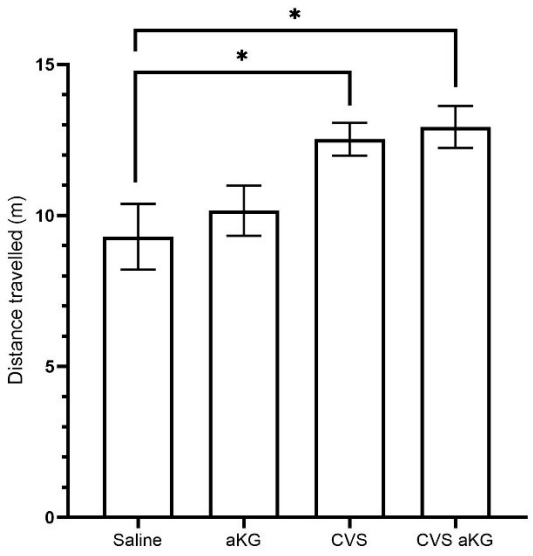
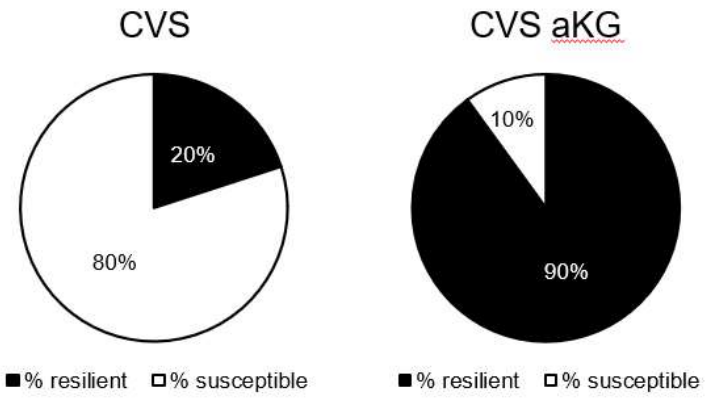
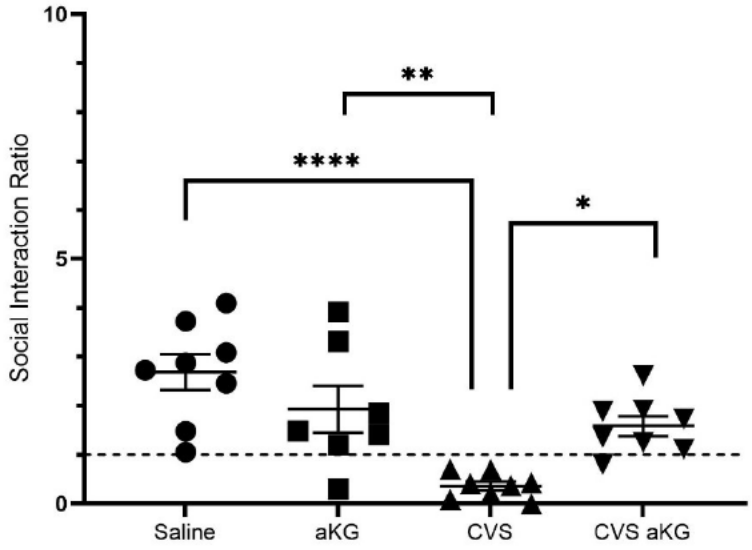

*
*
15
10
5
0
Distance travelled (m)
Saline
aKG
CVS
CVS aKG

C


CVS
20%
80%
% resilient
% susceptible
CVS aKG
10%
90%
% resilient
% susceptible

D



E

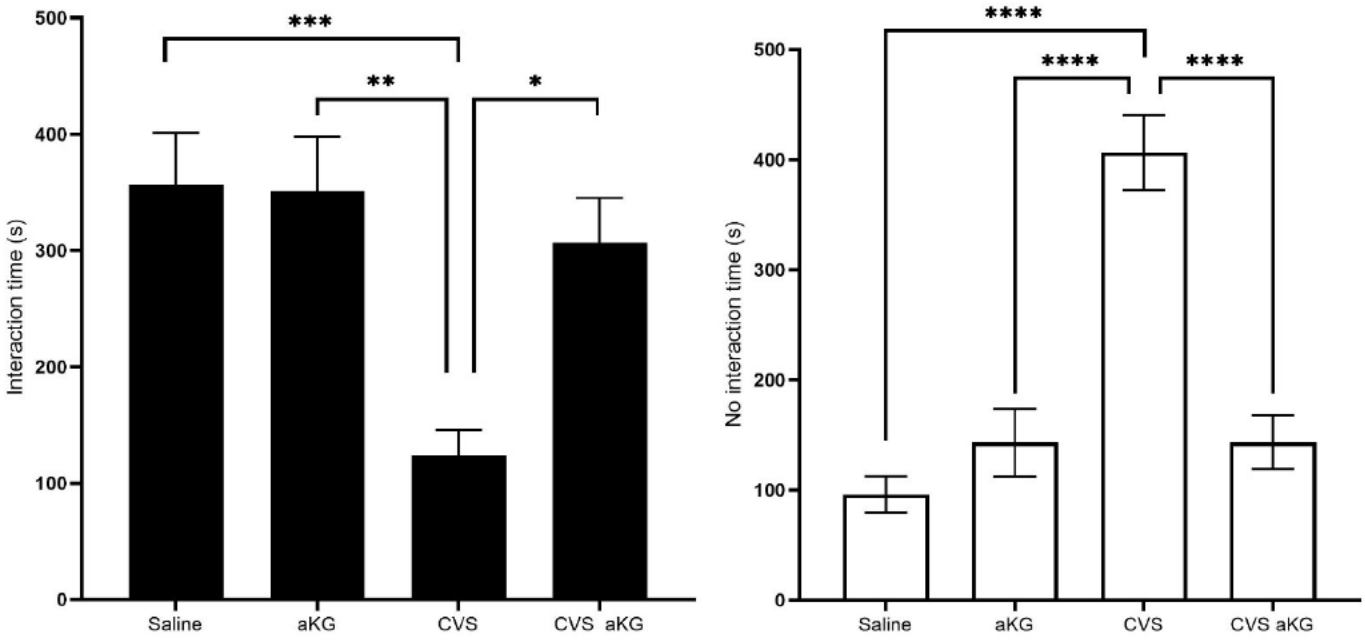


Figure 5: aKG serves as a protective factor that promotes resilience to stress and prevents social interaction deficits in mice that underwent CVS. Statistical significance was measured by 2way ANOVA followed by Tukey's multiple comparison test.

(A) Mice were treated with aKG (300mg/kg) 5 days prior to the start of CVS. The CVS paradigm consists of exposing the experimental mouse to a variety of stressors for 9 consecutive days. On days where more than one stressor is present, a minimum of 2 hours is allowed between each one. On day 10 of the CVS paradigm, behavioral tests and brain tissue collection were conducted.

(B) Locomotor activity was analyzed using the open field test. The average distance travelled is significantly increased in both CVS and CVS aKG groups (Figure 5B). This indicated that CVS does not impair locomotion and that aKG treatment does not enhance locomotion compared to the CVS group treated with saline. (Statistics????)

(C) aKG promotes resilience to stress. In mice treated with saline and then subjected to CVS (n=10), 20% are resilient to stress, while 80% are susceptible. In mice treated with aKG and then subjected to CVS (n=10), 90% are resilient to stress, while 10% are susceptible.

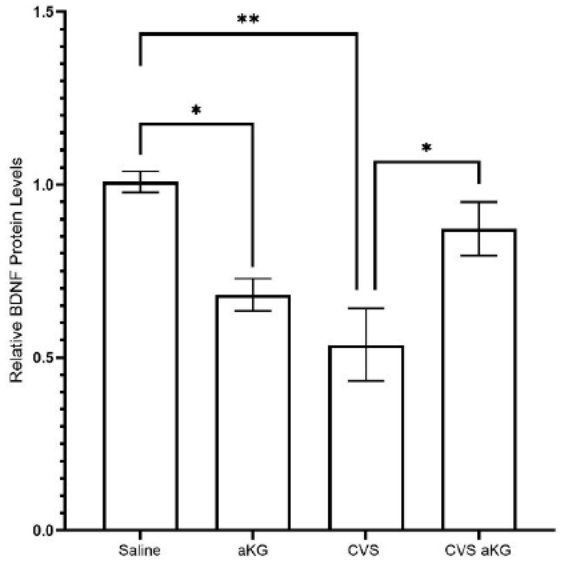
(D) Scatter plot graph that shows the SI ratio distribution across the different mice groups. The dotted line represents the threshold above which animals are classified as resilient to stress. *p<.03, **p<.0021. Interaction: $F(1,27) = 10.41$, $p=0.0033$. CVS: $F(1,27) = 18.81$, $p=0.0002$. n= 8 for Saline, 7 for aKG, 8 for CVS Saline, and 8 for CVS aKG.

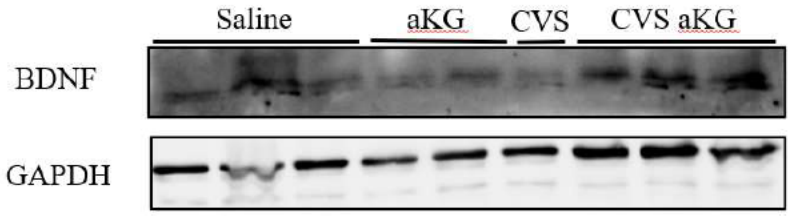
(E) Graphs show the social interaction time (left) and no interaction time (right). i.p. injections of aKG (300mg/kg, 5 days prior to CVS) showed a significant protection against the social avoidance phenotype in CVS mice treated with saline. Interaction time: *p<.03, **p<.0021,***p<.0002. Interaction: $F(1,36) = 5.853$, $p=0.0207$. Defeat: $F(1,36) = 12.70$, $p= 0.0011$. Treatment: $F(1,36) = 5.225$, $p=0.0283$. n= 10 per group. No interaction time: ****p<.0001. Interaction: $F(1,36) = 32.46$, $p<0.0001$. Defeat: $F(1,36) = 32.58$, $p<0.0001$. Treatment: $F(1,36) = 15.74$, $p=0.0003$. n= 10 per group.

3.4 aKG mediates resilience to CVS by modulating BDNF levels

Like before, BDNF protein levels in both the hippocampus and NAc were assessed through western blot analysis. BDNF levels were significantly decreased in the hippocampi of female mice exposed to CVS and receiving saline. These protein levels were restored upon aKG pretreatment (Figure 6A). However, no significant changes were observed in its upstream activators PGC1a (Figure 6B). In contrast, BDNF levels were significantly increased in the NAc of female mice exposed to CVS and receiving saline. These protein levels were restored upon aKG pretreatment (Figure 6C). As seen in the hippocampus, PGC1a levels were not significantly changed in the NAc of all four mice groups (Figure 6D). Taken together, our results suggest that aKG-mediated resilience to CVS is correlated to differential BDNF modulation within specific brain regions.

A





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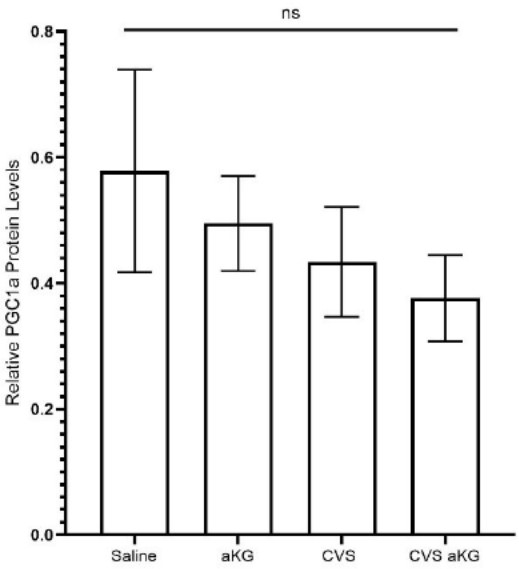
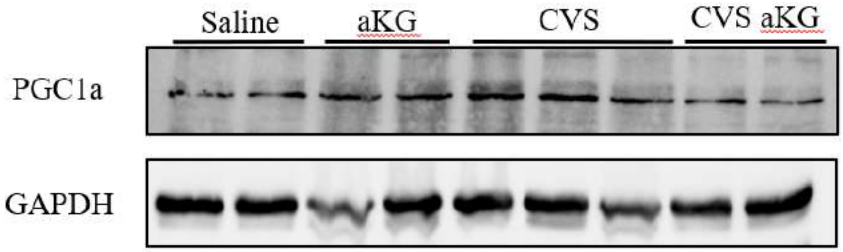
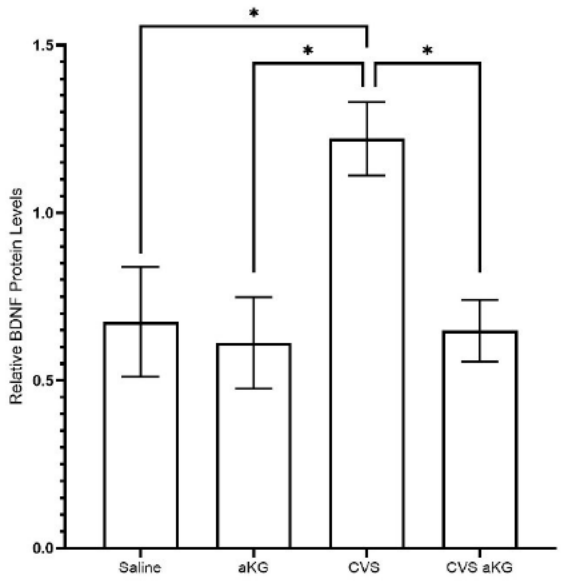
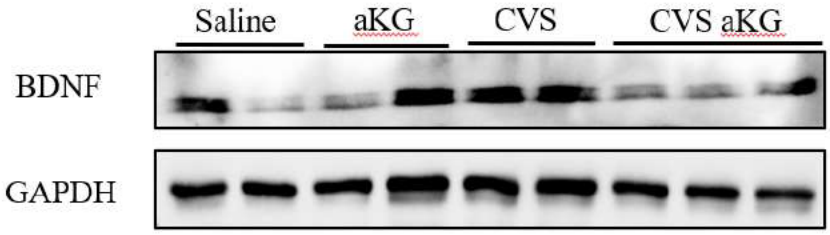

ns
Relative PGC1a Protein Levels
0.8
0.6
0.4
0.2
0.0
Saline
aKG
CVS
CVS aKG


Saline
aKG
CVS
CVS aKG
PGC1a
GAPDH

C


*
*
*
Relative BDNF Protein Levels
1.5
1.0
0.5
0.0
Saline
aKG
CVS
CVS aKG


Saline
aKG
CVS
CVS aKG
BDNF
GAPDH

D

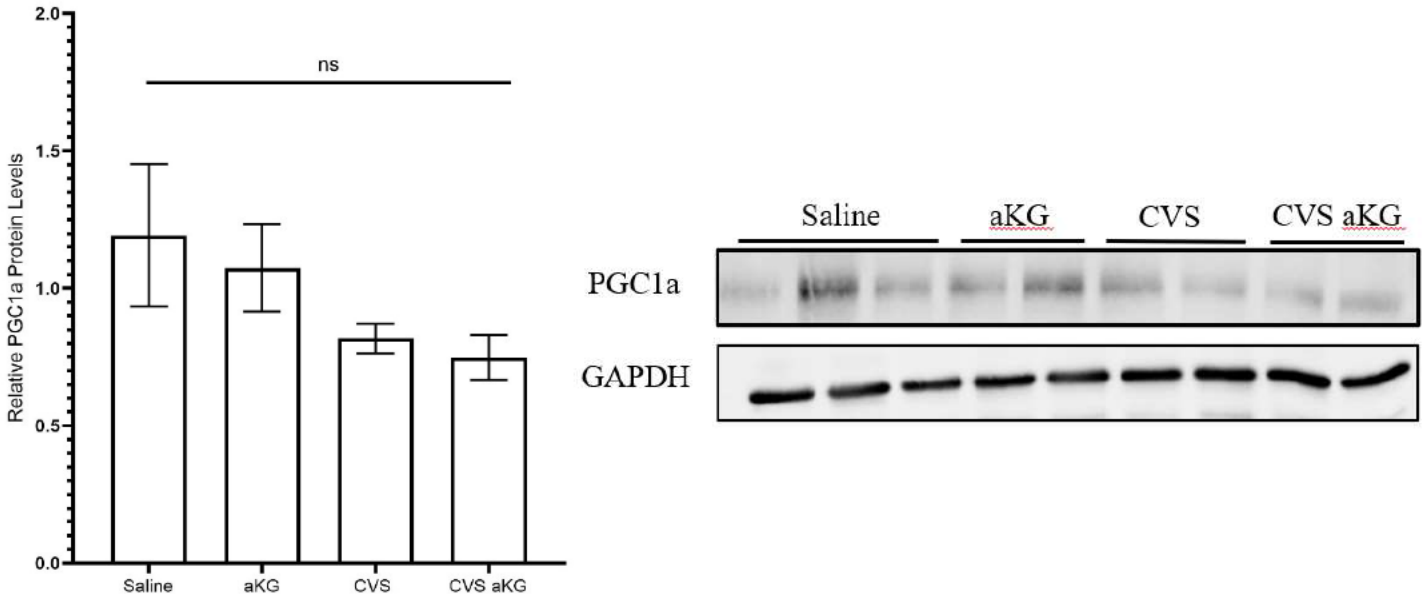


Figure 6: aKG pretreatment restores protein levels of BDNF in the hippocampus and prevents its increase in the NAc of mice subjected to CVS.

(A) Representative western blot of hippocampal BDNF (right) along with GAPDH as the endogenous control. Quantifications are seen on the left. *p<.03, **p<.0021. Interaction: F(1,13) = 20.15, p=0.0006. n= 4 for Saline, 4 for aKG, 3 for CVS, 6 for CVS aKG.

(B) Representative western blot of hippocampal PGC1a (right) along with GAPDH as the endogenous control. Quantifications are seen on the left. No significant changes were seen

(C) Representative western blot of BDNF (right) in the NAc along with GAPDH as the endogenous control. Quantifications are seen on the left. *p<.03. CVS: F(1,23) = 4.680, p=0.0412. Treatment: F(1,23) = 5.558, p=0.0273. n= 8 Saline, 6 aKG, 6 CVS, 7 CVS aKG

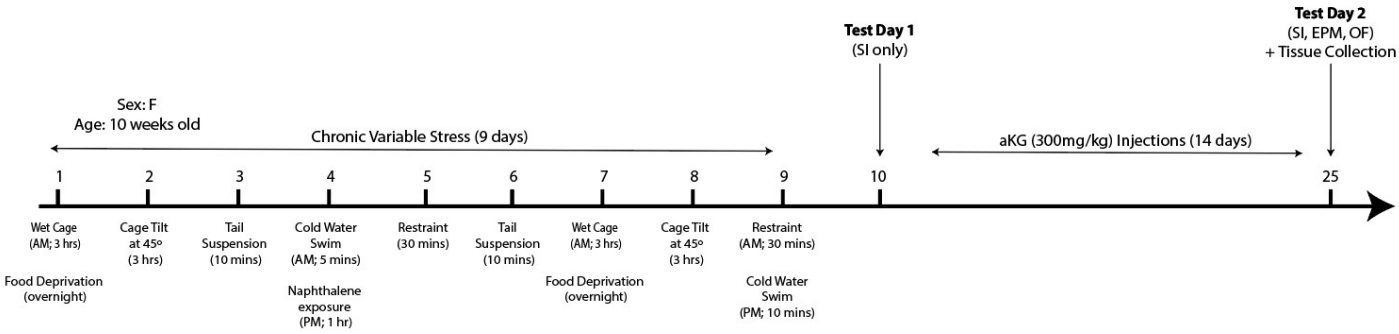
(D) Representative western blot of PGC1a (right) in the NAc along with GAPDH as the endogenous control. Quantifications are seen on the left. No significant changes were observed.

3.5 aKG has antidepressant properties that promotes resilience to stress and rescues social avoidance behavior in female C57BL/6J mice

We next assessed whether aKG has antidepressant effects in addition to its prophylactic effects. To test whether aKG can be used as an antidepressant, we developed a post-treatment paradigm in female mice. 10-week-old female mice were first subjected to CVS. On the 10^{th} day of the CVS paradigm, the SI test was performed (test day 1). Mice were then classified as susceptible or resilient based on their SI ratio on test day 1. Only susceptible mice received either saline or aKG treatment for 14 days. After 14 days, the SI test was performed again (test day 2) (Figure 7A). Mice that were not subjected to CVS and that received either saline or aKG treatment served as controls. No significant difference in the distance travelled in the open field was observed across all groups (Figure 7B). Thus, we can rule out any effects caused by locomotor deficits due to CVS or increased locomotor activity due to aKG treatment. Treatment with aKG significantly and completely reversed social avoidance behavior. Indeed, susceptible mice receiving aKG treatment became completely resilient to stress (12/12) as compared to susceptible mice receiving saline treatment (0/13) (Figure 7C). We next assessed the distribution of the SI ratios in these animals. Susceptible mice treated with saline significantly clustered below the threshold as compared to controls. In contrast, susceptible mice treated with aKG significantly clustered above the threshold indicating a reversal in their social behavior (Figure 7D). Indeed, social avoidance was rescued by aKG. Susceptible mice treated with saline spent significantly less time interacting with the social stimulus as compared to susceptible mice treated with aKG, whereas susceptible mice treated with saline spent significantly more time not interacting with the social stimulus as compared to susceptible mice treated with aKG (Figure

7E). These results confirm that aKG is a potential antidepressant that reverses social avoidance behavior induced by CVS in female mice.

A



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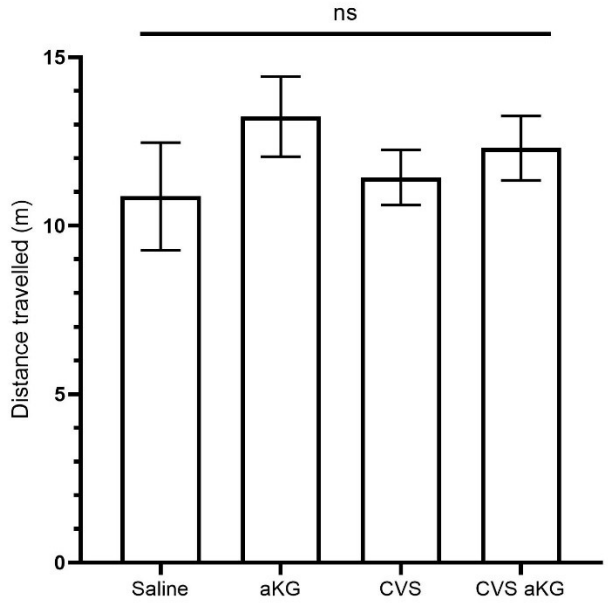

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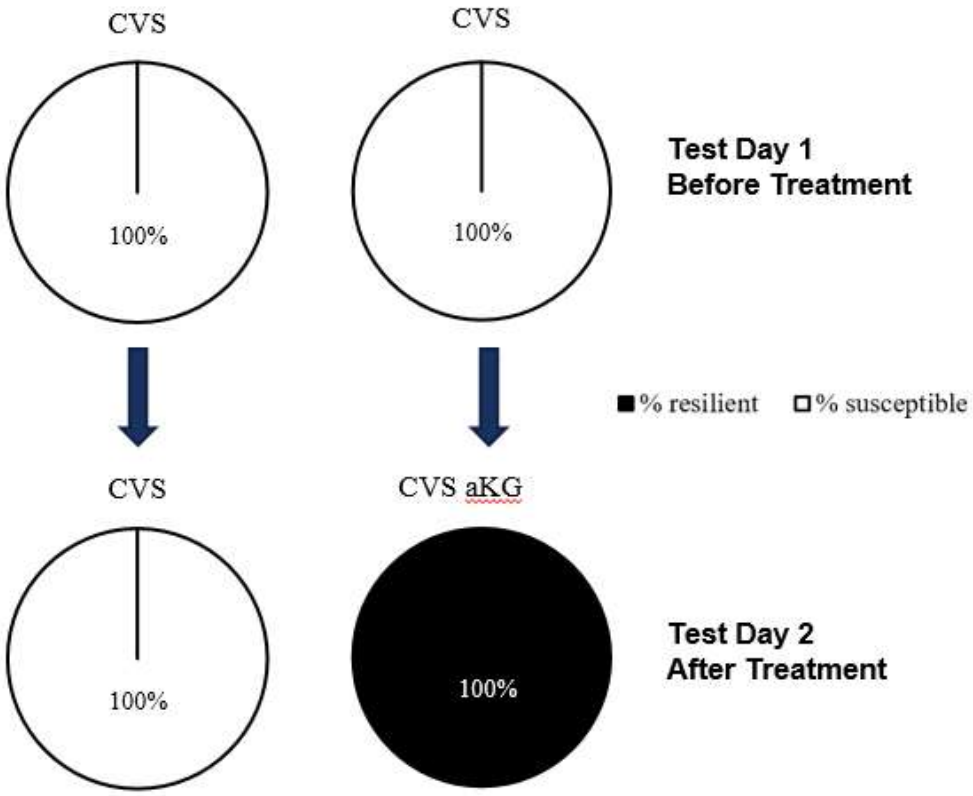
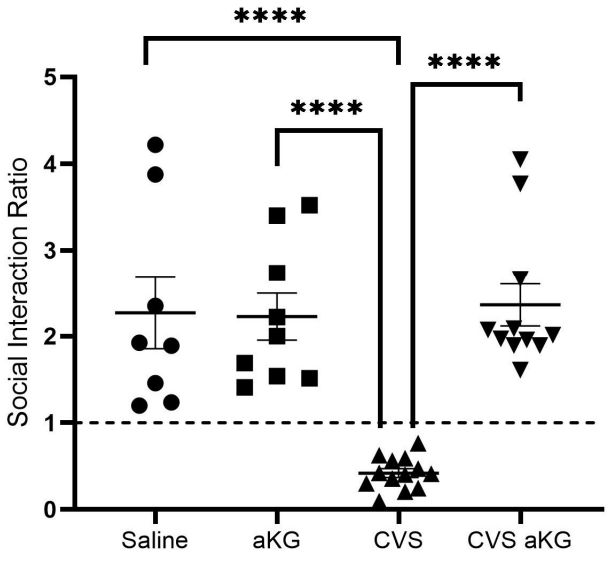

CVS
CVS
100%
100%
Test Day 1
Before Treatment
% resilient
% susceptible
CVS
CVS aKG
100%
100%
Test Day 2
After Treatment

D


****
****
****
Social Interaction Ratio
5
4
3
2
1
0
Saline
aKG
CVS
CVS aKG

E

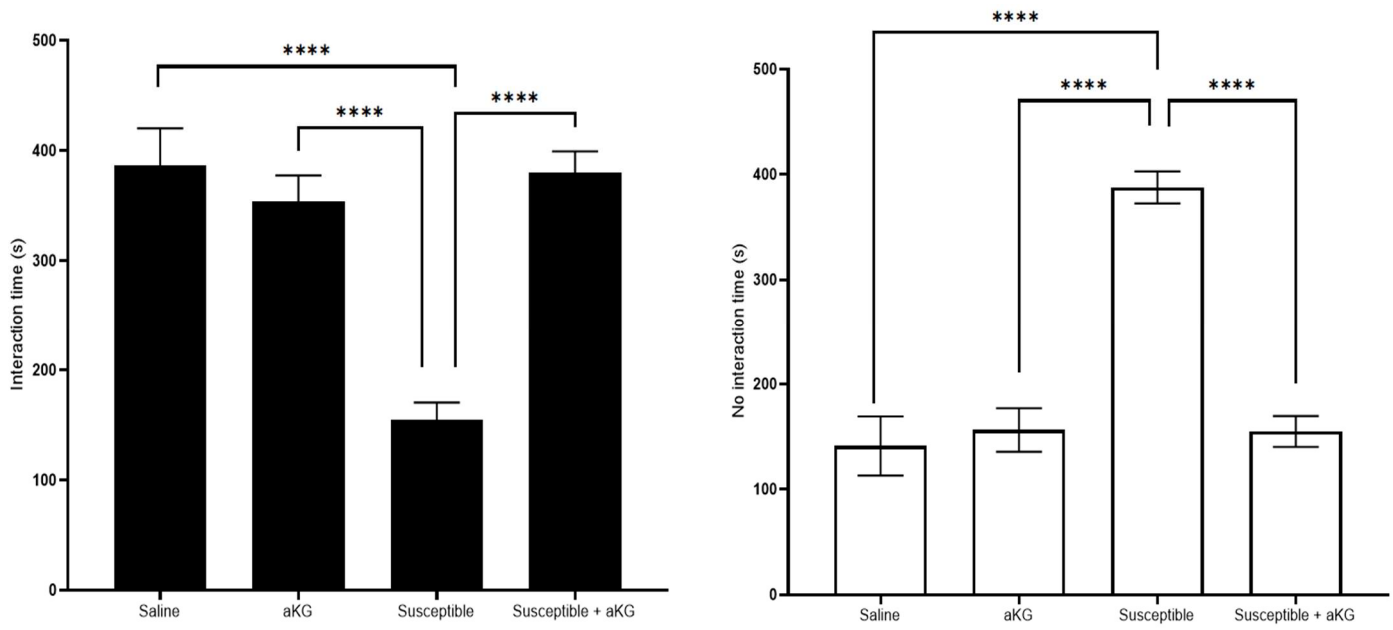


Figure 7: aKG is an antidepressant that promotes resilience to stress and reverses social interaction deficits in mice subjected to CVS. Statistical significance was measured by 2way ANOVA followed by Tukey's multiple comparison test.

(A) The CVS paradigm consists of exposing the experimental mouse to a variety of stressors for 9 consecutive days. On days where more than one stressor is present, a minimum of 2 hours are allowed between stressors. On the 10^{th} day of the CVS paradigm, the SI test was performed (test day 1), and the mice were then divided into susceptible mice and resilient ones. Following this, only susceptible mice received either saline or aKG treatment for 14 days. After 14 days, the SI test was performed again (test day 2).

(B) Locomotor activity was analyzed using the open field test. The average distance travelled was unchanged in all groups. This indicated that both CVS and aKG did not affect locomotor activity

(C) aKG promotes resilience to stress. Susceptible mice receiving saline treatment continued to be susceptible to stress. (n=13, 0% are resilient to stress, while 100% are

susceptible). Susceptible mice treated with aKG all became resilient to stress. In the group of mice treated with aKG (n=12, 100% are resilient to stress, while 0% are susceptible).

(D) Scatter plot graph that shows the SI ratio distribution across the different mice groups. The dotted line represents the threshold above which mice are classified as resilient to stress. ****p<.0001. Interaction: $F(1,37) = 16.60$, $p=0.0002$. CVS: $F(1,37) = 12.36$, $p=0.0012$. Treatment: $F(1,37) = 15.16$, $p=0.0004$. n= 8 Saline, 9 aKG, 13 CVS, 11 CVS aKG.

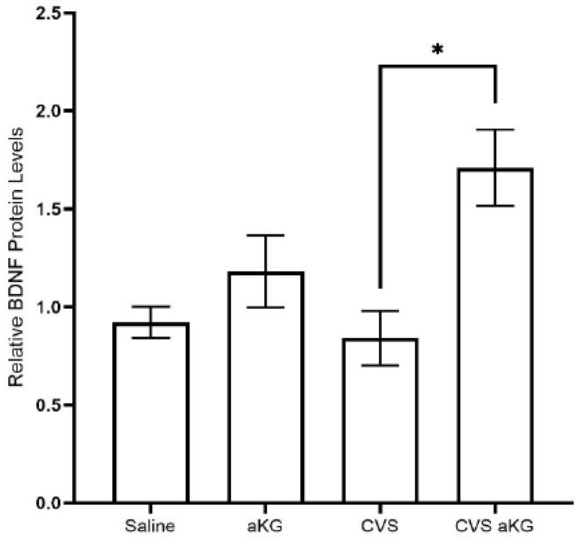
(E) Graphs show the social interaction time (left) and no interaction time (right). IP injections of aKG (300mg/kg, for 14 days) into susceptible mice induced a significant reversal in the social avoidance phenotype. Interaction time: ****p<.0001. Interaction: $F(1,41) = 32.29$, $p<0.0001$. Susceptible: $F(1,41) = 20.30$, $p<0.0001$. Treatment: $F(1,41) = 17.81$, $p=0.0001$. n= 10 Saline & aKG, 13 Susceptible, 12 Susceptible + aKG. No interaction time: ****p<.0001. Interaction: $F(1,41) = 40.29$, $p<0.0001$. Susceptible: $F(1,41) = 39.38$, $p<0.0001$. Treatment: $F(1,41) = 31.01$, $p<0.0001$. n= 10 Saline & aKG, 13 Susceptible, 12 Susceptible + aKG.

3.6 aKG mediates its antidepressant effects in female mice by modulating BDNF levels

Disruptions in BDNF signaling is a hallmark in depression and anxiety disorders (Hasler, 2010). Therefore, we also looked at BDNF levels in the post-treatment paradigm through western blot analysis. In the hippocampus, while we didn't observe significant changes in BDNF expression at this time point as compared to control, we still observed significant increases in BDNF levels in susceptible mice receiving aKG treatment as compared to those treated with saline only (Figure 8A). Our results correlate changes in hippocampal BDNF levels to aKG antidepressant effects. PGC1a, on the other hand, was modulated by both stress and aKG treatment. Indeed, PGC1a levels were decreased in susceptible mice treated with saline, but

restored upon treatment with aKG (Figure 8B). Our results link the hippocampal PGC1a/BDNF axis to the antidepressant effects of aKG. In the NAc, while we also didn't observe significant changes in BDNF expression at this time point as compared to control, we still observed significant decreases in BDNF levels in susceptible mice receiving aKG treatment as compared to those treated with saline only (Figure 8C). In contrast PGC1a levels were not significantly altered (Figure 8D). Interestingly, FNDC5, another upstream modulator of BDNF, was significantly modulated by both stress and aKG (Figure 8E), suggesting that BDNF signaling in the NAc is blocked through the inhibition of FNDC5 expression by aKG.

A



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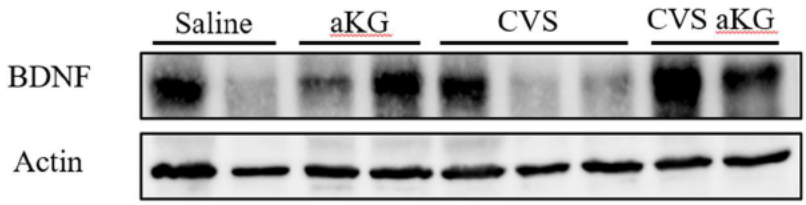
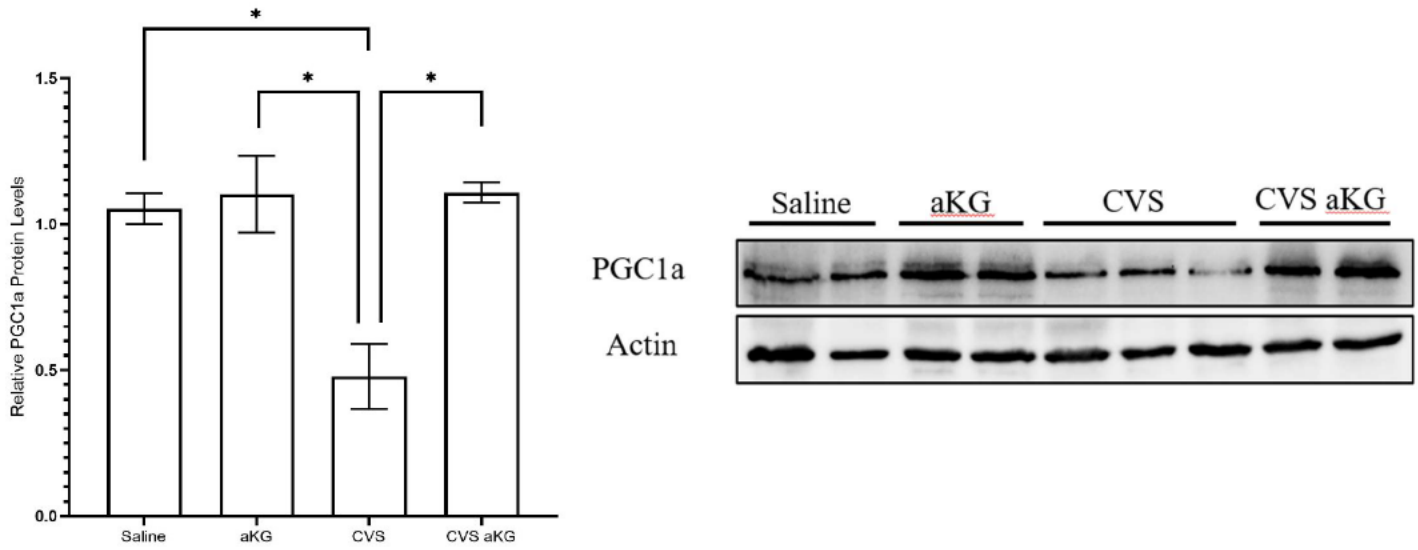
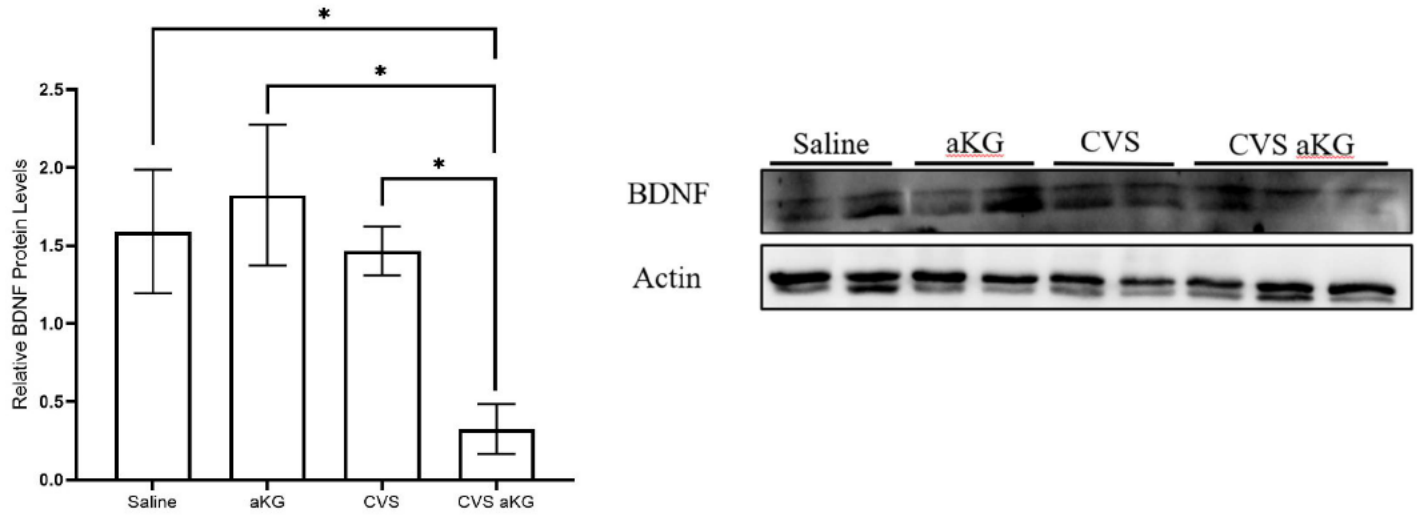
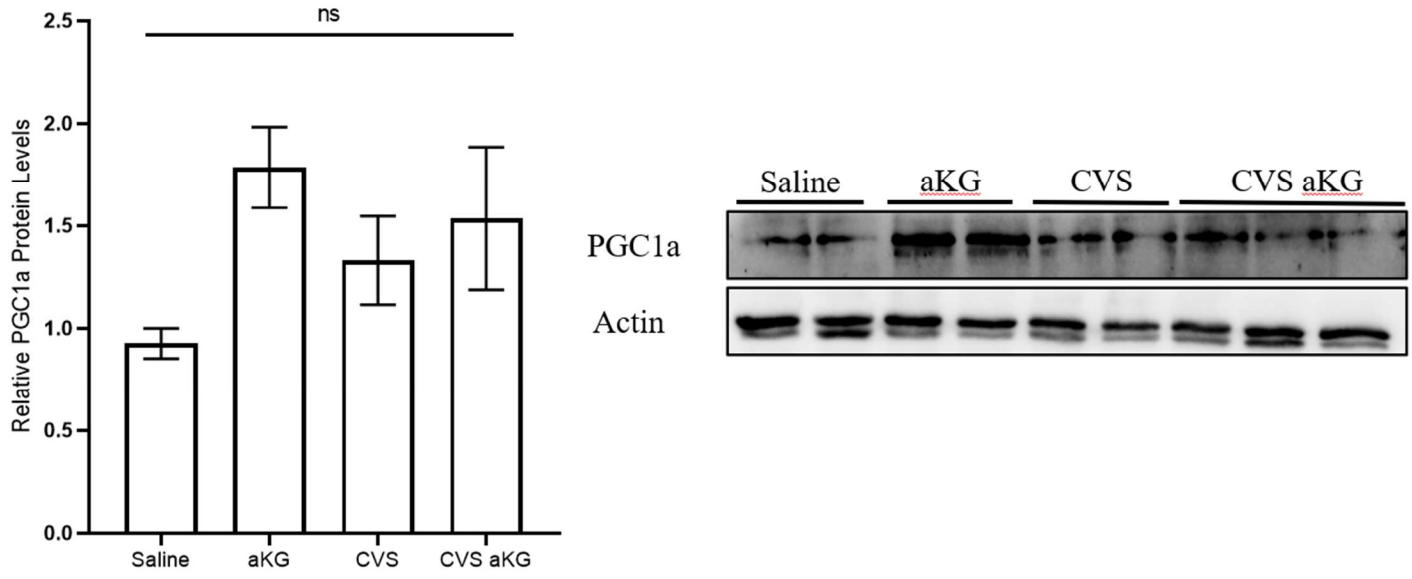



Relative PGC1a Protein Levels
1.5
1.0
0.5
0.0
*
*
*
Saline
aKG
CVS
CVS aKG
Saline
aKG
CVS
CVS aKG
PGC1a
Actin

C


Relative BDNF Protein Levels
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Saline
aKG
CVS
CVS aKG
Saline
aKG
CVS
CVS aKG
BDNF
Actin

D



E

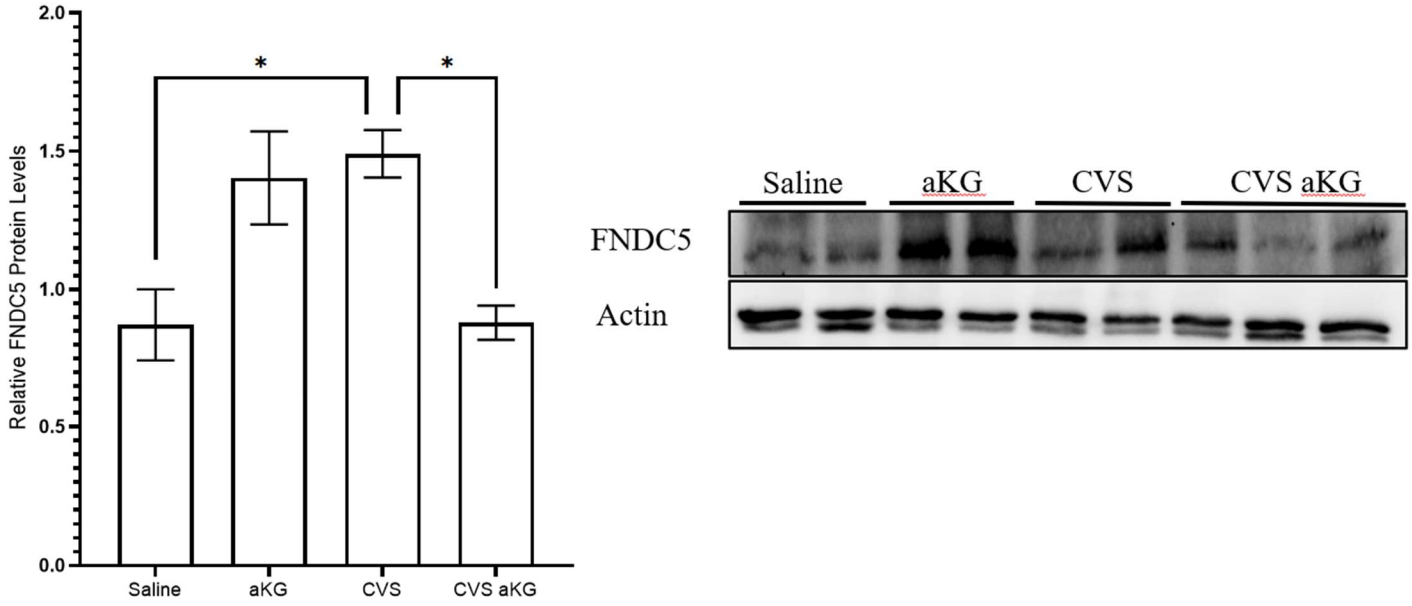


Figure 8:

aKG treatment restores hippocampal BDNF protein levels and prevents their increase in the NAc of susceptible mice.

(A) Representative western blot of hippocampal BDNF (right) along with Actin as the endogenous control. Quantifications are seen on the left. *p<.03. Treatment: $F(1,5) = 12.72$, $p=0.0161$. n= 2 Saline, 2 aKG, 3 CVS, and 2 CVS aKG

(B) Representative western blot of hippocampal PGC1a (right) along with Actin as the endogenous control. Quantifications are seen on the left. *p<.03. Interaction: $F(1,5) = 7.764$, $p=0.0386$. CVS: $F(1,5) = 7.483$, $p=0.0410$. Treatment: $F(1,5) = 10.66$, $p=0.0223$. n= 2 Saline, 2 aKG, 3 CVS, and 2 CVS aKG.

(C) Representative western blot of BDNF (right) in the NAc along with Actin as the endogenous control. Quantifications are seen on the left. *p<.03. Interaction: $F(1,14) = 5.499$, $p=0.0343$. CVS: $F(1,14) = 7.674$, $p=0.0150$. n= 4 Saline, 4 aKG, 5 CVS, and 5 CVS aKG

(D) Representative western blot of PGC1a (right) in the NAc along with Actin as the endogenous control. Quantifications are seen on the left. No significant changes were observed.

(E) Representative western blot of FNDC5 (right) in the NAc along with Actin as the endogenous control. Quantifications are seen on the left. *p<.03. Interaction: $F(1,5) = 27.99$, $p=0.0032$. n= 2 Saline, 2 aKG, 2 CVS, and 3 CVS aKG.

Chapter Four

Discussion

Considering the economic and health care burden that is created by MDD, it is important to identify novel prophylactic and therapeutic strategies. The synergistic effects between environmental and genetic factors are some of the main driving forces for vulnerability towards depression (Henriquez-Alves and Queroz, 2015; Southwick et al, 2005). Therefore, a focus on lifestyle modifications such as regular exercise and a proper diet can allow individuals to become resilient to stress. In this study we showed that: (1) aKG acts as a protective factor against chronic stress, and (2) aKG acts as antidepressant and thus can be developed as part of an exercise pill that can serve as a novel treatment for depression, (3) both the prophylactic and antidepressant effects of aKG are mediated through differential restoration of normal BDNF levels and signaling in different brain regions.

To test our hypotheses, we relied on two validated mouse models of depression that mimic human social stress: the CSDS model and the CVS model. Chronic stress leads mice to develop depressive-like behaviors that are evident through social interaction deficits (Henriquez-Alves and Queroz, 2015). In order to test the prophylactic effects of aKG, we treated the male and female mice with aKG or saline 5 days prior to the start of the CSDS or CVS paradigms respectively. In order to test the antidepressant effects of aKG, we first subjected mice to CSDS or CVS. The mice that exhibited depressive-like behavior were then treated with aKG or saline. Indeed, we observed that aKG is an exercise factor that promotes resilience to stress and acts as antidepressant. Mice that received aKG i.p injections prior to CSDS or CVS did not show social avoidance behavior as observed in the social interaction (SI) test (Figure 3E and 5E) and were

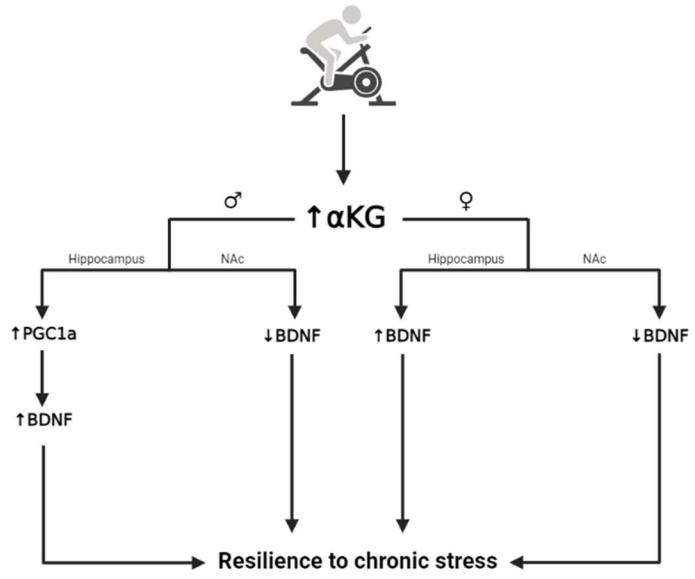
resilient to stress (Figure 3C and 5C). These results were consistent across both sexes suggesting that the pathways regulated by aKG are similar in male and female mice.

We next wanted to identify the signaling pathways that are modulated by aKG. For that reason, we focused on pathways known to be dysregulated in depression and modulated by exercise. Physical exercise rescues depression-like symptoms (Paolucci et al., 2018; Balchin et al., 2016) through regulation of BDNF expression (Cotman et al., 2007). Therefore, we investigated changes in BDNF protein expression in the hippocampus and NAc through western blot analysis. The choice for these brain parts was based on published work that showed that the hippocampus is a main player in the beneficial impacts of exercise (Gray et al., 2013), and that showed that the NAc is a center for reward and a main component for the dopaminergic pathway (Cui et al., 2020). Our results showed that BDNF levels were increased in the hippocampus of defeat male mice that were treated with aKG compared to those treated with saline (Figure 4A), suggesting that aKG modulates BDNF levels in the brain. We next tested whether BDNF upregulation was due to the PGC1a/FNDC5 pathway, which is exercise-regulated (Wrann et al., 2013; El Hayek et al., 2019). Indeed, a significant increase in PGC1a levels were seen in defeated mice that were pretreated with aKG (Figure 4B). Our results are consistent with BDNF upregulation in the hippocampus of male mice being driven by the PGC1a-BDNF pathway. Within the NAc, BDNF levels were significantly decreased in defeat mice receiving aKG compared to those receiving saline (Figure 4C). However, no changes in PGC1a were observed in this brain area, suggesting that another mechanistic pathway may be involved in the regulation of BDNF expression (Figure 4D).

The same pattern in the regulation of BDNF expression was observed as in female mice (Figure 6A and 6C). However, the PGC1a pathway was unaffected in both the hippocampus and NAc (Figure 6B and 6D). Our results suggest that aKG has prophylactic effects in both male and female mice, that it differentially modulates the BDNF signaling pathway in different brain regions, and that the upstream effectors involved in BDNF expression are distinct in the different tissue and possibly the different sexes.

We next tested the antidepressant effects of aKG in female mice. Indeed, we observed that aKG acts as an antidepressant (Figure 7C). aKG rescued social avoidance behavior in susceptible female mice (Figure 7E). This rescued behavior was bolstered by an induction in hippocampal BDNF as compared to the susceptible mice that received saline only (Figure 8A). Unlike the pretreatment paradigm, hippocampal PGC1a was also upregulated in female mice treated with aKG (Figure 8B). This suggests that aKG upregulates hippocampal BDNF in female mice through PGC1a induction. This was only observed in males that were pretreated with aKG previously. Since we are currently testing the antidepressant effects in male mice, we currently can't conclude whether this pathway is sex specific. Finally, western blot analysis in the NAc revealed that aKG blocked BDNF induction in the NAc possibly through the downregulation of FNDC5 (Figure 8C and 8E).

A



B

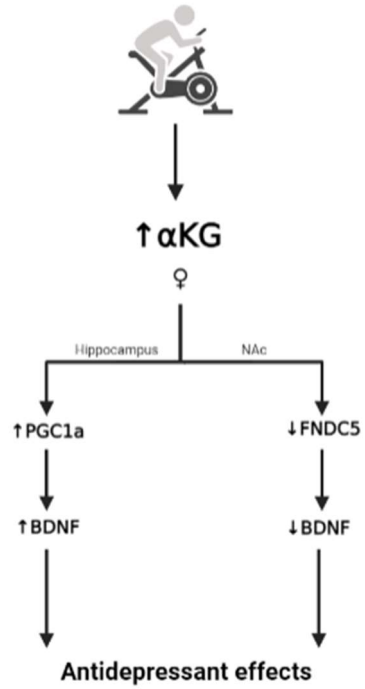


Figure 9: Proposed model of our study.

(A) In the pretreatment paradigm, hippocampal BDNF was restored in depressed mice upon aKG pretreatment. A significant increase in hippocampal PGC1a was observed as well. Our results are consistent with BDNF upregulation in the hippocampus of male mice being driven by a PGC1a-BDNF pathway. Within the NAc, BDNF levels were significantly decreased in defeat mice receiving aKG compared to those receiving saline. However, no changes in PGC1a were observed in this brain area, suggesting that another mechanistic pathway may be involved in the regulation of BDNF expression. The same pattern in the regulation of BDNF expression was observed as in female mice. However, the PGC1a pathway was unaffected in both the hippocampus and NAc.

(B) In the post-treatment paradigm, induction of hippocampal BDNF was seen in female mice who were treated with aKG. Unlike the pretreatment paradigm, hippocampal PGC1a was also upregulated in female mice treated with aKG. This suggests that aKG upregulates hippocampal BDNF in female mice through PGC1a induction. This was only observed in males within the pretreatment paradigm. Finally, aKG blocked BDNF induction in the NAc possibly through the downregulation of FNDC5.

It is crucial to further dissect the underlying mechanisms by which aKG promotes resilience to chronic stress and reverses social avoidance phenotypes. More studies are needed to understand whether exercise mediates resilience to stress via aKG production. This can be achieved by blocking aKG production in exercising mice and testing whether exercise fails to mediate resilience to stress. If indeed exercise mediates its effects through aKG, then aKG may be a pivotal component of an exercise pill along with lactate and DBHB that can serve as a both a prophylactic and antidepressant treatment for depression.

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