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Ketogenic diet changes microglial morphology and the hippocampal lipidomic profile differently in stress susceptible *versus* resistant male mice upon repeated social defeat

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Highlights

- Ketogenic diet tends to promote resistance to psychological stress
- Hippocampal microglia show morphological adaptations to stress and diet
- Microglia of stress-susceptible mice make less synaptic contacts
- Microglia of ketogenic diet-fed mice show less signs of cellular stress
- Lipids are differentially regulated in the hippocampi of susceptible mice

Abstract

Psychological stress confers an increased risk for several diseases including psychiatric conditions. The susceptibility to psychological stress is modulated by various factors, many of them being modifiable lifestyle choices. The ketogenic diet (KD) has emerged as a dietary regime that offers positive outcomes on mood and health status. Psychological stress and elevated inflammation are common features of neuropsychiatric disorders such as certain types of major depressive disorder. KD has been attributed anti-inflammatory properties that could underlie its beneficial consequences on the brain and behavior. Microglia are the main drivers of inflammation in the central nervous system. They are known to respond to both dietary changes and psychological stress, notably by modifying their production of cytokines and relationships among the brain parenchyma. To assess the interactions between KD and the stress response, including effects on microglia, we examined adult male mice on control diet (CD) *versus* KD that underwent 10 days of repeated social defeat (RSD) or remained non-stressed (controls; CTRLs). Through a social interaction test, stressed mice were classified as susceptible (SUS) or resistant (RES) to RSD. The mouse population fed a KD tended to have a higher

proportion of individuals classified as RES following RSD. Microglial morphology and ultrastructure were then analyzed in the ventral hippocampus CA1, a brain region known to present structural alterations as a response to psychological stress. Distinct changes in microglial soma and arborization linked to the KD, SUS and RES phenotypes were revealed. Ultrastructural analysis by electron microscopy showed a clear reduction of cellular stress markers in microglia from KD fed animals. Furthermore, ultrastructural analysis showed that microglial contacts with synaptic elements were reduced in the SUS compared to the RES and CTRL groups. Hippocampal lipidomic analyses lastly identified a distinct lipid profile in SUS animals compared to CTRLs. These key differences, combined with the distinct microglial responses to diet and stress, indicate that unique metabolic changes may underlie the stress susceptibility phenotypes. Altogether, our results reveal novel mechanisms by which a KD might improve the resistance to psychological stress.

Key words

Ketogenic diet, repeated social defeat, psychological stress, hippocampus, microglia, electron microscopy, lipidomics

1. Introduction

In recent years, dietary interventions have garnered increased scientific and clinical interest for the treatment of psychiatric disorders, including major depressive disorder (MDD) (Brietzke et al. 2018; Opie et al. 2018; Igwe et al. 2021; Huang et al. 2018; Ricci et al. 2020; Martin-McGill et al. 2018; Guan et al. 2020; Włodarczyk, Cubała, and Stawicki

2021; Guan et al. 2020; Murphy et al. 2004). In particular, the ketogenic diet (KD), already established as a treatment for epilepsy (Brietzke et al. 2018; Nei et al. 2014), has gained attention for psychiatric disorders, as well as for promoting optimal cognition in healthy individuals (Ashton et al. 2021; Carneiro and Pellerin 2022; Jiwani et al. 2022; Ricci et al. 2020; Włodarczyk, Cubała, and Stawicki 2021). A standard KD consists of up to 80% of lipids, with adequate protein and minimal carbohydrate contents (Martin-McGill et al. 2018; Włodarczyk, Cubała, and Stawicki 2021; Ricci et al. 2020). Contrary to a typical carbohydrate-rich diet, in which glucose is the primary energy source for most tissues, including the brain (Brietzke et al. 2018; Mergenthaler et al. 2013), a KD regimen forces the body into ketosis, a metabolic state in which lipids are converted to ketone bodies, such as β-hydroxybutyrate (BHB) and acetoacetate, in the absence or reduced presence of glucose (Brietzke et al. 2018; Włodarczyk, Cubała, and Stawicki 2021). Energy is derived from the catabolism of fatty acids and ketone bodies, with the brain relying heavily on ketone bodies (Boison 2017; Brietzke et al. 2018; Włodarczyk, Cubała, and Stawicki 2021). A strong anti-oxidative effect has been proposed to underlie the beneficial effects of KD in epilepsy (de Lima, de Brito Sampaio, and Damasceno 2014). Furthermore, the metabolic changes induced by a KD regimen may also exert positive physiological and cognitive outcomes under normal homeostatic conditions (Carneiro and Pellerin 2022). Thus, a KD may represent a potential approach to prevent the emergence of psychiatric disorders in healthy individuals, notably considering the preclinical evidence suggesting that a KD may promote resilience to stress-induced depression (Guan et al. 2020).

Recent preclinical and clinical reports have revealed the potential of a KD to exert strong antidepressant effects (Huang et al. 2018; Guan et al. 2020). In a model of repeated social

defeat (RSD), a KD ameliorated depressive-like behavior in stress-susceptible mice, as well as improved the social interaction ratios, sucrose preference and performance in the tail suspension and forced swim tests (Guan et al. 2020). A prior study on adult male mice demonstrated significant reductions in anxious and depressive-like behaviors following two weeks of KD (Huang et al. 2018). Evidence from humans is still limited, but improvements in mood, cognitive function, and anxious behavior were noted in young patients undergoing KD therapy for epilepsy (IJff et al. 2016). Compromised hippocampal function is thought to underlie the classical symptoms of depression such as impaired concentration and declarative memory, and affective changes accompanied by anxiety (Clark et al. 2009; Brown et al. 2014). Significant reductions in the volume of the ventral hippocampus, involved in anxiety (Parfitt et al. 2017), were reported in rodent models of environmental challenges including mice subjected to psychological stress (Vyas et al. 2002; Schoenfeld et al. 2017). Similarly, reduction in hippocampal volume has been reported in human patients with MDD (Campbell and MacQueen 2004; Videbech and Ravnkilde 2004). Synaptic loss was identified as a driver of hippocampal volume reduction in mouse models of chronic stress (Woodburn, Bollinger, and Wohleb 2021; Magarinos and McEwen 1995). This is supported by observations in patients with MDD where volume reduction (Frodl et al. 2002) and synaptic loss (Holmes et al. 2019) in the hippocampus were associated with MDD severity.

Acute and chronic stress, but also cumulative stress exposure, are major risk factors for MDD (Gold 2015; Saveanu and Nemeroff 2012). Recent work has demonstrated that psychological stress and depression are strongly linked to inflammation, which is thought to play a causal role at least in a subset of depression cases (Beurel, Toups, and

Nemeroff 2020; Casaril, Dantzer, and Bas-Orth 2021; Krishnan et al. 2007; Miller and Raison 2016; Kitaoka 2022; Koo and Wohleb 2021). Notably, RSD stress in mice triggers central and peripheral inflammatory processes, inducing similar depressive-like behaviors (Guan et al. 2020), which further supports a causal link between inflammation and depression. Elevated basal levels of inflammatory markers were also linked to depressive symptoms in humans (Bonaccorso et al. 2002; Capuron and Castanon 2017; Walker et al. 2014; Eisenberger et al. 2010; Harrison et al. 2009). Conversely, anti-inflammatory interventions, such as dietary regimens, may benefit patients with MDD who display chronic inflammation (Allison, Sharma, and Timmons 2019; Dantzer et al. 2018; Tolkien, Bradburn, and Murgatroyd 2019). Among the underlying mechanisms, microglia, the resident innate immune cells of the central nervous system, are key mediators of brain inflammation (Bohlen et al. 2019; Carrier et al. 2021; Nimmerjahn, Kirchhoff, and Helmchen 2005; Kettenmann et al. 2011; Walker et al. 2014). Emerging evidence suggests that microglia play an important role in the pathophysiology of stress and depression, particularly as mediators of pathological inflammation, but also vascular, neuronal, and synaptic remodeling (Enache, Pariante, and Mondelli 2019; Morris et al. 2020; L. Zhang, Zhang, and You 2018). Morphological and functional alterations of microglia were described notably among the hippocampus in mouse models of RSD (Guan et al. 2020; Wohleb et al. 2015). Similar observations were made in patients with MDD (Setiawan et al. 2015). The microglial alterations observed in rodent models of psychological stress include accelerated cellular aging, as well as senescence and metabolic dysregulation, associated with energetic deficits (Carrier et al. 2021; Franklin et al. 2018). Stress compromises the physiological role of microglia in maintaining

homeostasis, resulting in increased basal levels of inflammatory cytokines in the brain, considered to be detrimental to microglia-neuron interactions and cognitive function (Carrier et al. 2021). However, microglial release of anti-inflammatory cytokines and neurotrophic factors, which promote adult hippocampal neurogenesis (Stratoulias et al. 2019; Morris et al. 2020; L. Zhang, Zhang, and You 2018), among other beneficial functions, may contribute to counteracting some of these changes.

The evidence linking inflammation with chronic psychological stress and depression has prompted an interest in developing therapeutic interventions acting on inflammation, to treat depressive disorders and favor optimal cognitive health (Troubat et al. 2021; Toenders et al. 2022; Morris et al. 2020). Among the proposed strategies, a KD regimen was shown to exert anti-inflammatory effects, by reducing the peripheral and central levels of pro-inflammatory cytokines notably in response to RSD in adult male rats and mice (Dupuis et al. 2015; Guan et al. 2020). Furthermore, the major ketone body BHB is a known inhibitor of the nucleotide-binding and oligomerization domain-like receptors pyrin domain-containing protein 3 (NLRP3) inflammasome, which acts as an important mediator of inflammation in innate immune cells that include microglia (Youm et al. 2015; Iwata et al. 2016). Microglia are emerging as a critical mediator of several beneficial effects of a KD, such as reducing brain inflammation and improving depressive behaviors in humans (Morris et al. 2020), while changes in microglia immune-metabolic pathways have been highlighted as a central mechanism underlying MDD (Rahimian et al. 2022). A KD is thus hypothesized to attenuate stress-related cellular aging and inflammation through the normalization of microglial metabolism and functions.

To provide further insight into the outcomes of KD (*versus* a control diet; CD) on stress resilience, inflammation, and microglia, we utilized a RSD paradigm in adult male mice. The study was performed on the ventral hippocampus CA1 *stratum radiatum* considering its key role in the plasticity impairment observed upon chronic social stress and in MDD (Milior et al. 2016; Laine et al. 2017; Parfitt et al. 2017; Bannerman et al. 2003; Schoenfeld et al. 2017; Campbell and MacQueen 2004; Videbech and Ravnkilde 2004). We first assessed the prevalence of susceptible *versus* resistant mice under CD and KD following the RSD. We then compared blood levels of anti- and pro-inflammatory mediators with the diets at steady-state and after psychosocial stress. Furthermore, we characterized changes in hippocampal microglial density, morphology, and ultrastructure (including organelles and relationships with parenchymal elements such as synapses), as well as analyzed the hippocampal lipidomic profile under KD *versus* CD at steady-state and in the context of psychosocial stress.

2. Methods

2.1 Animals

All animal experiments were performed under approval of the institutional animal ethics committees, in conformity with the Canadian Council on Animal Care guidelines. Male mice were used considering that RSD relies on inter-male interactions (Golden et al. 2011; Henry et al. 2018). C57BL/6J mice (7–8 weeks old) were acquired from The Jackson Laboratories and CD1 retired breeder mice (4–6 months old) from Charles River (St. Constant, QC, Canada). A total of 82 animals were used in this study. The animals

were housed under a 12 h light-dark cycle at 22–25 °C with *ad libitum access* to food and water.

2.2 Ketogenic diet

Starting 4 weeks prior to the RSD paradigm, the experimental C57BL/6J mice gradually transitioned to KD or remained on CD over 1 week (Fig. 1A). The KD (high fat and low carbohydrate content) had a composition of 8.6% protein, 75.1% fat, 4.8% fiber, 3.2% carbohydrate, caloric profile: protein 0.34 kcal/g, fat 6.76 kcal/g and carbohydrate 0.13 kcal/g [Ketogenic Diet AIN-76A-Modified, High Fat, F3666 Bio-Serve]. The CD was composed of 24% protein, 18% fat, 58% carbohydrate, caloric profile of protein 0.744 kcal/g, fat 0.558 kcal/g and 1.798 kcal/g [Teklad Global 18% protein, 2018S, ENVIGO]. The mice were weighted daily.

2.3 Blood samples

Blood samples were collected from all the experimental C57BL/6J mice through the mandibular vein, without anesthesia, on Day 0, Day 6 of the social defeat and on Day 12 of the social interaction (SI) test (Fig. 1A). Blood was collected in heparinized tubes and centrifuged at 3600 revolutions per min for 10' at 4°C to collect plasma.

2.3.1 Corticosterone measurement

Plasma corticosterone (CORT) levels were determined using blood samples collected on Day 12 using a commercial CORT ELISA kit (item No. 501320, Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Plates were read at 405

nm with a microplate reader (iMarkTM, Biorad, Hercules, CA, USA). Sample concentrations were determined using a standard curve (logarithmic scale), followed by a four-parameter logistic fit analysis (Henry et al. 2018).

2.3.2 β-hydroxybutyrate measurement

Plasma BHB levels were determined to confirm the increase of circulating ketone bodies in the KD fed animals, using a β-hydroxybutyrate or 3-hydroxybutyric acid Colorimetric Kit Essay (item No. 700190Cayman, Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Absorbance was read at 445–455 nm using a plate reader.

2.3.3 Cytokine measurement

Plasma levels of 31 cytokines were measured with the Discovery Assay® by Eve technologies (Mouse Cytokine/Chemokine 31-Plex Discovery Assay® Array (MD31); Calgary, Canada). These cytokines were as follows: eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN)gamma, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, C-X-C motif chemokine ligand 10 (IP-10), keratinocytes-derived chemokine (KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemoattractant protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF), monokine induced by interferon-γ (MIG), macrophage inflammatory protein 1-alpha (MIP-1α), macrophage inflammatory protein 1β (MIP-1beta), macrophage inflammatory protein 2 (MIP-2), C–C chemokine ligand 5 (RANTES), TNF-α, and vascular endothelial growth factor (VEGF).

2.4 Social defeat

2.4.1 Social defeat paradigm

CD1 retired breeder mice were selected for their level of aggressiveness in presence of naïve C57BL/6J mice as previously published by Golden *et al*., 2015 and Henry *et al.,* 2018. Experimental C57BL/6J mice were randomly assigned to the RSD or non-stressed control (CTRL) group. Experimental mice (intruder) were subjected to 10 days of consecutive RSD (Day 1 is the first day of RSD). The social defeat arena is a cage with two equal sections divided by a physical barrier with holes that allow for visual, olfactory, and auditory interactions between the sections. An aggressor CD1 mouse (resident mouse) is first placed on one side of the barrier, then an intruder mouse is placed for 5 min with the resident mouse. After 5 min, the intruder mouse is moved to the other side of the barrier for the next 24 h until the intruder mouse is exposed to a new resident mouse (and the resident mouse to a new intruder) (Golden et al. 2011; Henry et al. 2018). Each intruder was randomly exposed to the same group of aggressor mice in a different order. The health status of the intruder mice was monitored closely. Injuries are not required for this protocol. In order to control for unnecessary physical lesions, the social defeat was interrupted after 10 physical attacks within a session or when attacks lasted more than 3 seconds. After every social defeat, the mice were examined by animal care technicians, and their wounds were treated. In our study, only one mouse was heavily wounded, then euthanized and excluded from the experiment according to the veterinarian guidelines.

For non-stressed controls, the mice were paired with partners, and housed in the same type of cage changed daily in the same room as RSD animals. All groups, stressed and

non-stressed on both diets, underwent the SI test. SI test was performed on Day 11 and animal euthanasia on Day 12. The animal movement was tracked in the arena, before and after the introduction of a novel CD1 aggressor. Using the time mice spent in each zone, a SI ratio was determined as described below.

2.4.2 Social interaction test

One day after the final defeat (Day 11), experimental animals were placed alone inside an open field arena (42 cm x 42 cm x 42 cm) for 150 s. The social interaction arena was divided into corner zones (CZ), an interaction and peri-interaction zones (Fig. 1A). After 150 s, an aggressor mouse not previously used in the RSD paradigm was placed inside a wire mesh in the arena, contained in the interaction zone (IZ). The aggressor mouse remained there for 150 s. Tracking videos were captured and analyzed using ANY-maze (Stoelting Co, Wood Dale, IL, USA). The test was performed by an observer blind to the experimental conditions. The SI ratio was calculated by dividing the time spent in the interaction zone and peri-interaction zone in the presence *versus* absence of the CD1 mouse. Susceptible mice (SUS) tended to freeze, avoiding interaction with the aggressor mouse, and spending more time in the CZs, thus showing social avoidance. Resistant mice (RES) still interacted with the aggressor mice, staying in the interaction and peri-interaction zones, adapted from (Golden et al. 2011; Henry et al. 2018) (Fig. 1A). To classify mice either as RES or SUS, a theoretical cut-off of 1 was used for the SI ratio. Mice with a SI ≥1 were considered as RES and mice with SI ratio < 1 were considered as SUS (Golden et al. 2011; Henry et al. 2018). CTRL mice showing a SI <1 were excluded,

following (Berton et al. 2006; Krishnan et al. 2007; Menard et al. 2017; Henry et al. 2018). Excluded CTRL mice with a SI < 1: CD = 2 mice; KD = 9 mice.

2.4.2 Euthanasia and tissue preparation

On Day 12 (i.e., 1 day after the SI test to exclude acute effects of the social interaction), mice were anesthetized with ketamine and xylazine (80 and 10 mg/kg, respectively, by intraperitoneal injection), and euthanized (Fig. 1A). For imaging techniques, mice were perfused with phosphate-buffered saline (PBS; 50 mM pH 7.4) followed by a mixture of 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde in phosphate buffer (PB; 100 mM, pH 7.4). Using a vibratome (Leica VT1000S), 50 µm thick coronal sections were obtained and stored in cryoprotectant at -20 °C until use. For lipidomics, the mice were perfused with PBS, the hippocampus was collected and flash-frozen on dry ice and stored at -80 °C. To allocate mice to different experiments (e.g., microscopy *versus* omics), this was planned beforehand given that different experiments required different perfusion methods. The animals were randomly assigned to an experiment. The only experiment where mice were selected was for lipidomics, samples from the 3 mice presenting the strongest phenotype, based on their SI ratio, were sent for analysis. There was no calculation of the average, all SUS and RES animals were treated equally, except for lipidomics, as mentioned previously.

2.5 Density and morphology

2.5.1 Fluorescence staining

Fluorescence staining was performed as described in Gonzalez-Ibanez *et al*., 2019. Utilizing 3–5 animals per experiment group, 2–5 sections containing the ventral hippocampus corresponding to Bregma levels -2.88 to -3.38 mm were elected based on the stereotaxic atlas of Paxinos and Franklin (4^{th} edition). Sections were washed with PBS five times for 5 min and incubated with 10 mM citrate buffer at 70 °C. After reaching room temperature (RT), they were washed with PBS five times for 5 min and incubated in 0.1% $NaBH_4$ for 30 min followed by 5 times for 5 min washes with PBS. Sections were then incubated in a blocking buffer (BB, 0.5% gelatin, 5% normal goat serum, 5% normal donkey serum and 0.01% Triton X-100) for 1 h. Subsequently, sections were incubated overnight at 4 °C with BB containing a primary antibody cocktail (1:150 mouse anti-ionized calcium-binding adapter molecule 1 (Iba1; EMD-Millipore cat# MABN92+), 1:300 rabbit anti-transmembrane protein 119 (TMEM119; Abcam cat# ab209064)). The next day, the sections were washed with PBS containing Triton X-100 (PBST, 0.01% Triton X-100 in PBS) five times for 5 min, followed by an incubation in BB with a secondary antibody cocktail. For the morphology analysis, we used: 1:300 donkey anti-mouse Alexa555 (Invitrogen-ThermoFisher cat# A-31570) and 1:300 goat anti-rabbit Alexa647 (Invitrogen-ThermoFisher cat# A-21245). For the density and distribution analysis: 1:300 donkey anti-mouse Alexa555 (Invitrogen-ThermoFisher cat# A-31570), 1:300 goat anti-rabbit Alexa647 (Invitrogen-ThermoFisher cat# A-21245) or 1:300 donkey anti-mouse Alexa488 (Invitrogen-ThermoFisher cat# A-21202) and 1:300 goat anti-rabbit Alexa568 (Invitrogen-ThermoFisher cat# A-11011) for 90 min. Sections were then washed five times for 5 min with PBST, incubated with DAPI 1:20,000 for 5 min and washed with PB three times for

5 min. The sections were mounted, dried overnight and coverslipped with mounting medium Fluoromount G (cat# 0100-01, Southern-Biotech, Birmingham, AB, USA).

2.5.2 Microglial density and distribution

Density and distribution analyses were performed in the ventral hippocampus CA1 *stratum radiatum* where stress-driven microglia-synapse interactions were previously studied (Peng et al. 2019). In each of 3 animals per experimental group, 2–3 sections containing the ventral hippocampus CA1 *stratum radiatum* were used to build a mosaic at 20x. Images were acquired with an Axio Imager M2 epifluorescence microscope equipped with an AxioCam MRm camera using the Zen Pro 2012 software (Zeiss, Oberkochen, Germany). With ImageJ, the freehand tool was used to delimit the region of interest (ROI) which was then measured in mm^2. Using DAPI as a confirmation of cellular identity, all Iba1+/TMEM119+ cells were considered as microglia and all Iba1+/TMEM119- cells were considered as peripheral infiltrating macrophages (Bennett et al. 2016; Ibanez et al. 2019). Using the paintbrush tool, all cell bodies were registered and quantified. A total of 200–300 cells per animal was included in the analysis. To assess density, the total number of microglia was divided by the measured area. Using ImageJ's nearest neighbor distance (NND) plug in, the distance (µm) of each microglia to its closest neighbor was obtained. The average NND of all microglia per ROI was calculated. Spacing index was calculated as the square average NND multiplied by the density (arbitrary units). This value was averaged across mosaics to determine the value per animal as in Tremblay *et al.* 2012 and Ibáñez *et al.* 2019.

2.5.3 Microglial morphology

For morphology analysis, 3–5 animals per experimental group were utilized. In each animal, 14–19 microglia (Iba1+/Tmem119+ cells) from the ventral hippocampus CA1 *stratum radiatum* were randomly selected and analyzed, resulting in 51–88 cells/experimental group, a sample size which was considered sufficient to obtain statistical power based on the G*Power software V3.1 (effect size of 0.231 and power of 0.95 estimated to a total of 378 individual cells). Cells were imaged at 40x with a Z-interval of 0.33 µm using a Quorum WaveFX spinning disc confocal microscope (Quorum Technologies, Guelph, ON, Canada) equipped with an ORCA-R2 camera (512 x 512 pixels; Hamamatsu Photonics, Hamamatsu, Japan). A Z-project projection maximum intensity image was generated using the ImageJ Z-stack tool. Morphological analysis was done using the Iba1 channel in ImageJ. Using the ImageJ freehand tool, the soma of each cell was traced and measured in μm^2. With the ImageJ polygon tool, the arborization area was traced by selecting the tips of microglial processes and then measured in μm^2. The morphological index was performed by dividing the soma area by the arborization area, as performed in Tremblay *et al.* 2012 and González Ibáñez *et al.* 2019.

2.6 Ultrastructural analysis

2.6.1 Microglial ultrastructural analysis

In each of 3 animals per group, 3 sections containing the ventral hippocampus CA1 *stratum radiatum* were selected. Sections were washed with PBS five times for 5 min. Samples were post-fixed with 3.5% acrolein in PB for 2 h. Sections were washed with PBS five times for 5 min and incubated in 0.3% $NaBH_4$ for 30 min followed by five times

for 5 min washes with PBS. Sections were then incubated in BB (10% fetal bovine serum, 3% bovine serum albumin, 0.01% Triton-X) for 1 h. Subsequently, the sections were incubated overnight at 4 °C in BB with primary antibody cocktail ([1:1000] rabbit anti-Iba1 polyclonal primary antibody (FUJIFILM, Wako Chemical, Osaka, Japan, cat#019-19741). After reaching RT, the sections were washed with Tris-buffered saline (TBS, 50 mM, pH 7.4) five times for 5 min and incubated in BB containing biotinylated goat anti-rabbit polyclonal secondary antibody ([1:200] Jackson ImmunoResearch, West Grove, PA, USA, cat# 111-066-046) in TBS for 1.5 h. Sections were next incubated with avidin-biotin complex solution (Vector Laboratories, Burlingame, CA, USA, cat# PK-6100,) [1:100] in TBS; for 1 h at RT. The staining was revealed in 0.05% diaminobenzidine (DAB; Millipore Sigma cat# D5905-50TAB,) with 0.015% H_2O_2 in Tris-buffer (TB, pH 8.0) for 4.5 min at RT. Samples were post-fixed in osmium-thiocarbohydrazide-osmium to enhance contrast for scanning electron microscopy (SEM). Sections were incubated in a 1:1 solution of 4% aqueous osmium tetroxide (Electron Microscopy Sciences (EMS), Hatfield, PA, USA cat#19170) and 3% potassium ferrocyanide (Bio-Shop, Burlington, ON, Canada, cat# PFC232.250) in double distilled (dd)H_2O for 1 h. Sections were washed with ddH_2O three times 5 min and incubated in 1% thiocarbohydrazide (EMS, cat# 2231-57-4) diluted in ddH_2O for 20 min. After washing the sections three times for 5 min, they were incubated for 30 min in 2% osmium tetroxide diluted in ddH_2O and then dehydrated in ascending concentrations of ethanol (two times in 35%, one time in 50%, 70%, 80%, 90%, three times 100%) followed by three incubations of 5 min in propylene oxide. After dehydration, the sections were flat-embedded in Durcupan ACM resin (Millipore Sigma, cat# 44611-44614). In brief, the sections infiltrated the resin at RT overnight. They were carefully

placed on a fine layer of resin between 2 sheets of ACLAR® embedding films (EMS, cat# 50425-25) for polymerization at 55 °C for 72 h. After polymerization, a section containing the region of interest was excised and glued to a Durcupan resin block for ultrathin sectioning (Ultracut UC7 ultramicrotome, Leica Biosystems). Ultrathin sections, of ~75 nm thickness, were collected on a silicon nitride chip and placed on specimen mounts for SEM. In each animal, 10–14 randomly selected microglial cell bodies located in the CA1 *stratum radiatum* were imaged, resulting in a total of 33–38 cells per condition, a sample size which was considered sufficient to obtain statistical power based on the G*Power software V3.1 (effect size of 0.313 and power of 0.95 estimated to 210 individual cells). The cells were imaged at 5 nm of resolution using a Crossbeam 540 field emission SEM with a Gemini column (Zeiss). The quantitative analysis was performed blind to the experimental conditions using QuPath Software.

2.6.2 Ultrastructural identification

Microglial cell bodies were identified by their dark irregular cytoplasm, heterogeneous chromatin pattern, distinctive long stretches of endoplasmic reticulum (ER) and lipidic inclusions (i.e., lipofuscin, lipid bodies or droplets, lysosomes), as well as frequent contacts with axon terminals (Alan Peters, Sanford Palay, and Webster Henry 1990; Nahirney and Tremblay 2021). Contacts with blood vessels (BV), astrocytic cell bodies and neuronal cell bodies were quantified. Neurons were identified by their pale nuclei, pale cytoplasm, common presence of a nucleolus and their round shape with a frequent apical dendrite or axon projecting from the cell body. Astrocytes were identified by their pale nuclei, a fine rim of heterochromatin lining the nuclear membrane, acute angles, and

frequent intermediate filaments, among other features. BV were identified by their lumen, endothelial cells, and surrounding basal membrane. Contacts to BV were considered when microglial cell body was directly touching the basal membrane of the BV or in proximity to the basement membrane (Alan Peters, Sanford Palay, and Webster Henry 1990; Nahirney and Tremblay 2021). Microglial contacts with other neuronal structures, particularly pre-synaptic axon terminals and post-synaptic dendritic spines, were quantified. Pre-synaptic axon terminals were identified by their synaptic vesicles, with a minimum of 5 vesicles required for recognition. Post-synaptic dendritic spines were identified by their post synaptic density and apposition with a pre-synaptic axon terminal (Alan Peters, Sanford Palay, and Webster Henry 1990; Nahirney and Tremblay 2021). Contacts to synaptic clefts were considered when microglial cell bodies directly juxtaposed both excitatory synapse-forming elements (Tremblay, Lowery, and Majewska 2010; St-Pierre et al. 2022). Microglial-synaptic contacts were classified as axon terminals, dendritic spines, or synaptic clefts.

Microglial mitochondria, ER, Golgi apparatus, lysosomes, lipofuscin granules, nuclear membrane alterations, nuclear pores and autophagosomes were quantified and their health status was assessed (Nahirney and Tremblay 2021). Mitochondria longer than 1 µm were considered elongated (St-Pierre et al. 2022). Mitochondria containing electron-lucent circular hollow membrane rings were categorized as "holy" mitochondria (St-Pierre et al. 2022). Swollen mitochondria with abnormal cristae structure were considered dystrophic mitochondria. Mitochondria with clear appearance and small fractured cristae were defined as white mitochondria (Nahirney and Tremblay 2021). Total dystrophic mitochondria count was obtained by adding dystrophic mitochondria, white mitochondria

and holy mitochondria. Mitochondria without any of these alterations were considered as standard mitochondria. A percentage of dystrophic mitochondria was calculated based on total mitochondria count. Dilation of the ER was noted when the cisternae had an electron-lucent appearance and the intracisternal distance was 100 nm or higher (El Hajj et al. 2019; Chavez-Valdez et al. 2016). The presence of inclusions refers to electron-dense material within the intracisternal space. ER without signs of dilation or inclusions was considered standard ER. The total dystrophic ER number was calculated by the sum of total ER with dilation and inclusions. A total ER count was calculated by the sum of standard ER and total dystrophic ER. Percentage of dystrophic ER was calculated based on total ER count. Dilation of the Golgi apparatus was noted when the cisternae had an electron-lucent appearance and the intracisternal distance was 100 nm or more (El Hajj et al. 2019; Chavez-Valdez et al. 2016). Inclusions refer to the accumulation of electron-dense material within the intracisternal space. Golgi apparatus without signs of dilation or inclusions was considered standard Golgi apparatus. Total dystrophic Golgi apparatus was calculated by the sum of total Golgi apparatus with dilation and inclusions. A total Golgi apparatus count was calculated by the sum of standard Golgi apparatus and total dystrophic Golgi apparatus. Percentage of dystrophic Golgi apparatus was calculated based on the total Golgi apparatus count. Lipid inclusions were identified by their round shape and electron-dense color and smooth texture. Lipofuscin granules were identified by their round or oval shape, and granular appearance with thread-like structures resembling a fingerprint-like pattern (Savage et al. 2020). Phagosomes were quantified, discriminating between empty phagosomes and phagosomes with content (El Hajj et al. 2019). The presence of content was defined as electron-dense material contained in the

phagosome. Total phagosomes were calculated by adding empty phagosomes and phagosomes with content. A percentage of phagosomes with content was calculated based on the total number of phagosomes. Primary lysosomes were recognized by their dense homogeneous salt and pepper texture, round shape, and single membrane enclosure (St-Pierre et al. 2022; Lecours et al. 2020). Secondary lysosomes were identified by their association with endosomes, small lipid droplets and inhomogeneous texture. Tertiary lysosomes were identified by their association to lipofuscin granules and lipid droplets (St-Pierre et al. 2022; Lecours et al. 2020; Savage et al. 2020). A total lysosomal count was calculated by adding primary, secondary and tertiary lysosomes. Nuclear pores were identified as an interruption of the nuclear membrane, when the outer and inner nuclear membranes were joined. Nuclear indentations were defined as an invagination of the nuclear membrane. Nuclear alterations consisted in an alteration of the nuclear integrity or presence of inclusions within the nuclear membrane (Nahirney and Tremblay 2021). All data was registered per cell and averaged by experimental condition.

2.7 Lipidomic analysis

2.7.1 Liquid chromatography/mass spectrometry (LC/MS)

Whole hippocampi were collected from 4 mice per treatment group and stored frozen at -80°C prior to performing untargeted lipidomic analyses. For lipidomic analyses, each frozen hippocampus was weighed in a 1.5-mL safe-lock Eppendorf tube. The tube was added with two metal beads and 2 µL of water per mg of raw tissue. The samples were homogenized at a shaking frequency of 30 Hz on a MM 400 mill mixer for 1 min twice.

Methanol-chloroform (3:1, v/v) at 18 μL per mg of raw tissue was then added. The samples were homogenized again for 1 min twice, followed by sonication in an ice-water bath for 3 min before centrifugal clarification at 21 000 g and 5ºC for 10 min. The clear supernatants were quantitatively transferred to another set of Eppendorf tubes, where 240 μL of the clear supernatant was mixed with 120 μL of water-methanol (2:1 v/v) and 100 μL of chloroform. The mixture was vortex-mixed for 1 min at 3 000 rpm and then centrifuged to split the whole phase into an upper aqueous phase and a lower organic phase. The organic phase of each sample was carefully collected and dried under a nitrogen gas flow. The dried residue was dissolved in 120 μL of HPLC-grade ethanol. Aliquots of 6 μL from each solution were injected into a Waters BEH C4 LC column (2.1 I.D. * 50 mm, 1.7 μm for UPLC-high resolution mass spectrometry (HRMS) on a Thermo Ultimate 3000 UHPLC system coupled to a Thermo LTQ-Orbitrap Velos Pro mass spectrometer through an atmospheric pressure electrospray ionization (ESI) interface. The mobile phase was (A) 0.01% formic acid in water and (B) 0.01% formic acid in acetonitrile-isopropanol (1:1 v/v). The LC elution gradient was 5–50% B in 6 min; 50-100% B in 14 min and 100% B for 4 min, before the column was equilibrated at the initial solvent composition for 4 min between injections. The column flow rate was 400 μL/min and the column temperature was maintained at 40°C. Two UPLC-HRMS runs per sample were conducted in two rounds of LC injections with positive-ion and negative-ion detection, respectively. For lipid detection and relative quantitation, the MS instrument was operated with full-mass-Fourier transform MS detection, and at a mass resolution of 60 000 full width at half maximum (FWHM) at a mass-to-charge (*m/z*) ratio of 400. The mass range of HRMS detection was *m/z* ratios of 80 to 1800. A solution pooled from 20

μL aliquots of 12 randomly selected sample solutions was used as the quality control (QC) sample. This QC solution was injected at the beginning, in the middle and at the end of the UPLC-HRMS batch runs for each round of LC injections. During data acquisition, all the sample solutions were injected in a random order and two UPLC-HRMS datasets were acquired. Along with the UPLC-HRMS data acquisitions, LC-MS/MS of the QC sample solution was carried out using collision-induced dissociation (CID), with the 6 most abundant ions of each survey scan chosen for subsequent CID at normalized collision energies of 28–35%.

2.7.2 Lipidomic data processing and analysis

The raw data files of UPLC-HRMS datasets with positive-ion and negative-ion detection were respectively processed using the XCMS module (Benton, Want, and Ebbels 2010; Smith et al. 2006; Tautenhahn, Böttcher, and Neumann 2008) in R with a custom-written script for peak detection, retention time shift correction, peak grouping and peak alignment in two rounds for each step. Mass deisotoping and removal of chemical background noise peaks were performed according to the seven golden rules described by (Kind and Fiehn 2007). The output files of XCMS processing were in the format of *m/z*, retention time and peak area pair for each detected lipid, which was amenable for subsequent statistical analyses using MetaboAnalyst v5.0 (Pang et al. 2022). Prior to statistical analysis, the XCMS output data were quantile-normalized and log-transformed using the interquartile range (IQR) filtration and the detected lipid features detected in the QC samples, which showed relative standard deviations (RSDs) of >30% were removed. Group means were compared using two-sample t-test and fold-change (FC) analysis.

Differentially-regulated lipids (DRLs) for each comparison were defined as having a false discovery rate (FDR)-adjusted p-value ≤0.05 and a fold change >1.5. DRLs were assigned by mass-matching against the Human Metabolome Database (HMDB) and LIPID MAPS databases within a maximum of mass errors of 5 ppm, in combination with spectral elucidation of the acquired lipid MS/MS spectra, with the aid of the MS/MS libraries of HMDB, METLIN, MASSBANK and LIPID MAPS and an in-house library of authentic compounds of different classes of >100 lipids. For lipid ontology enrichment analyses, LION software (v.2020.07.14) (Molenaar et al. 2019) was utilized. DRLs identified in the positive ion and negative ion detection modes were combined, and comparisons were made in 'ranking mode', using 2-log [fold change] analyses with a two-tailed alternative hypothesis in the K-S settings. Lipids that could not be matched to a LION identifier were retained in datasets during analysis.

2.8 Statistics

Data are reported as means ± standard error of the mean. Statistical analyses were conducted using Prism 9 (v.9.2, GraphPad Software, San Diego, CA, USA). Normality was verified using a Shapiro-Wilk test and assessed by QQ plot. All metabolic and immunological parameters were analyzed using a two-way analysis of variance (ANOVA) to compare diet (CD *versus* KD) and stress phenotypes (CTRL, SUS, or RES) as between-subject factors. Significant ANOVA tests with a main effect of diet or stress or a diet × stress interaction were reported. The different stress phenotypes (CTRL, SUS, or RES) were examined as different levels of the stress phenotype factor. Significant ANOVA tests with a main effect of either diet or stress or a diet × stress interaction were

followed by Tukey *post-hoc* tests to identify significant differences between the relevant groups (CD CTRL *versus* KD CTRL, CD CTRL *versus* CD SUS, CD CTRL *versus* CD RES, CD SUS *versus* CD RES, KD CTRL *versus* KD SUS, KD CTRL *versus* KD RES, KD SUS *versus* KD RES, CD SUS *versus* KD SUS, CD RES *versus* KD RES). The differences were considered statistically significant with a p value < 0.05. Asterisks (*) were used to represent diet x stress interactions, hashtags (#) were used to represent a main effect of stress, ampersands (&) were used to represent a main effect of diet. Sample size (n) refers to individual animals for behavioral, metabolic, and molecular analyses. For morphology and ultrastructural analysis, n refers to individual microglia considering microglia as a biological unit, while N refers to the population size (number of animals). Analysis of microglia as a biological unit, instead of the animal, takes into account the high heterogeneity of the microglia population, allowing for the assessment of individual cellular contributions to the population response (Stratoulias et al. 2019; Hui et al. 2018; Q. Li et al. 2019). No statistical outliers were removed.

3. Results

3.1 KD increases the proportion of stress-resistant mice following social stress

To study the mechanisms underlying the protective effects of a KD, we compared the outcomes of a KD *versus* CD in adult male mice exposed to RSD *versus* non-stressed CTRLs. Two-month-old C57BL/6J mice were introduced to KD or kept on CD starting 4 weeks prior to starting the paradigm until the end of the experiment (Fig. 1A). Ketosis was confirmed by measuring the levels of BHB from blood samples collected at Day 0 and Day 12 (Fig. 1B).

After 4 weeks, mice on both diets were exposed to 10 days of RSD (Days 1 to 10) while non-exposed mice served as CTRLs. The animals remained on their respective diet throughout the RSD protocol. To assess the susceptibility or resistance to stress, a SI test measuring social avoidance to a new CD1 mouse which is associated with stress susceptibility (Golden et al. 2011) was performed on Day 11. As described in details in the Methods, the social interaction arena was divided into CZ, an interaction and peri-interaction zones (Fig. 1A). A SI ratio was determined by dividing the time spent in the interaction zone and peri-interaction zone in the presence *versus* absence of a novel CD1 mouse. Mice with a SI ratio < 1 were classified as SUS and those with a ratio ≥ 1 as RES. The results were compared to paired-housed, non-stressed CTRL mice which received either CD or KD without exposure to social stress paradigm.

Blood levels of CORT were elevated in stressed animals thus confirming the effectiveness of RSD. Interestingly, when analyzing the SUS and RES separately, we also found an effect of stress ($F(2, 40) = 7.933$, $p= 0.0013$), showing increased levels of CORT in the SUS group compared to the CTRL and RES groups (Fig. 1C). SUS animals on both diets further displayed increased social avoidance compared to respective CTRLs as shown by their SI ratio ($F(5, 65) = 18.75$, $p< 0.0001$; CD CTRL 1.252 ± 0.2192 *versus* CD SUS 0.7184 ± 0.2187 *versus* CD RES 1.150 ± 0.1198; KD CTRL 1.169 ± 0.1805 *versus* KD SUS 0.6895 ± 0.2809 *versus* KD RES 1.096 ± 0.0848) (Fig. 1D). Quantification of the CZ time before *versus* after the introduction of a CD1 aggressor showed that SUS animals on both diets increased their presence in the CZ (Fig. 1E). SI ratio also revealed that animals following KD *versus* CD were more likely to be classified as RES (CD: 63.63% SUS: 36.36% RES; KD: 42.85% SUS: 57.14% RES) (Fig. 1F). We then performed a chi-

square test, which showed a tendency for an increase in the proportion of mice classified as RES under the KD [chi-square, df: 2.131,1; z: 1.460; $p = 0.0722$] (Fig. 1F). The shift in the SUS:RES ratio observed in KD mice indicates a potential increase in stress resistance, compared to the expected proportion of 30% to 40% of RES using the same paradigm (Golden et al. 2011; Henry et al. 2018). Overall, these results support the idea that KD as a dietary intervention could confer stress protection and result in behavioral improvements. Our findings thus reveal that KD also tended to increase the proportion of RES mice after RSD.

The KD diet exerts anti-inflammatory properties, which could mediate its stress-resistance capacities (Ashton et al. 2021; Carneiro and Pellerin 2022; Jiwani et al. 2022; Ricci et al. 2020; Włodarczyk, Cubała, and Stawicki 2021). To better understand the mechanisms underlying the tendency for KD to have a protective effect, we performed multiplex ELISA and measured blood levels of 31 pro- and anti-inflammatory cytokines in mice exposed to RSD. To look at the effects of stress over time, our analyses were conducted on Day 6 and Day 12 of the RSD. On Day 6, there was a significant increase in 4 pro-inflammatory cytokines in the SUS animals *versus* CTRLs related to stress:, namely G-CSF, IL-6, IL-13 and IP-10 (G-CSF ($F(1,14) = 4.253$, $p = 0.0374$), IL-6 ($F(2,16) = 3.719$, $p = 0.0472$), IL-13 ($F(2,16 = 8.230)$ $p = 0.0035$), IP-10 ($F(2,16) = 4.181$, $p = 0.0346$) (Fig. 2A-E). On Day 12, only G-CSF presented an effect of stress, being significantly higher in the SUS *versus* CTRL and RES groups ($F(2,16) = 7.723$, $p = 0.0045$) (Fig. 2A-E'). A main effect of diet was observed in IFN-ɣ on day 6 ($F(1, 13 = 5.165)$ $p = 0.0407$) (Fig. 2B) and on G-CSF on day 12 ($F(1, 16) = 9.754$ $p = 0.0066$) (Fig. 2A').

Overall, our results indicate that 4 weeks of KD *versus* CD elevated the circulating levels of ketone bodies in mice. Ten days of RSD also caused behavioral changes in mice, resulting in distinct SUS and RES phenotypes. Ten days of RSD induced inflammation in the stressed mice, which differed in their circulating levels of inflammatory cytokines. Furthermore, the population following a KD showed a tendency toward an increased proportion of RES animals upon RSD

3.2 KD results in different microglial morphological adaptations to social stress

Microglia have emerged as key mediators of chronic stress outcomes by coordinating the peripheral and central immune responses to environmental challenges (Cunningham et al. 2005; Picard et al. 2021; Tay et al. 2017). To study whether changes in microglia might underlie the effects of KD at steady-state and upon psychosocial stress, we first analyzed possible changes in their density and distribution among the ventral hippocampus CA1 *stratum radiatum,* comparing the two diets, CD and KD, and three stress phenotypes, CTRL, SUS, and RES, on Day 12 of the paradigm. A double immunostaining for the microglia/macrophage marker Iba1 and largely microglia-specific TMEM119 was performed (Ibanez et al. 2019). Microglia were identified as Iba1-positive (+)/TMEM119+ cells (Fig. 3A). Microglial density (cells/area), nearest neighbor distance (NND; average distance of each microglia to its nearest neighbor) and spacing index (square average NND multiplied by the density) were assessed. This analysis revealed that the different parameters analyzed remained unchanged across all groups (Fig. 3B-D) (Table 1).

We then assessed in the same animals and hippocampal region, also on Day 12, possible changes in microglial morphology under the two diets (Table 1). We found in the KD-fed

mouse population that microglia had morphological differences at steady-state, among the non-stressed CTRL group (Table 1). Two-way ANOVA showed changes in soma ($F(2,374) = 5.628$ $p = 0.0039$) and *post-hoc* analysis indicated that microglia had a bigger soma in KD CTRL compared to CD CTRL mice (CD CTRL 42.41 $\mu m^2 \pm 1.336$ *versus* KD CTRL 48.86 $\mu m^2 \pm 1.545$ $p = 0.0169$). Two-way ANOVA revealed a difference in arborization area ($F(2,374) = 5.42$ $p = 0.0048$) and *post-hoc* analysis indicated that microglia of KD CTRL *versus* CD CTRL animals had a larger arborization area (CD CTRL 1339 $\mu m^2 \pm 58.28$ *versus* KD CTRL 1573 $\mu m^2 \pm 47.88$ $p = 0.0391$). These differences in microglial soma and arborization area observed in unstressed CTRL animals following a KD indicate that this diet modifies microglial properties at the basal level, under steady-state conditions.

We further examined possible changes in microglial morphology induced by stress under the two diets (Table 1).Two-way ANOVA revealed an effect of stress ($F(2, 374) = 7.108$ $p = 0.0009$) and *post-hoc* analysis indicated that microglia in the CD SUS group had increased soma area compared to the CD CTRL group (CD CTRL 42.41 $\mu m^2 \pm 1.336$ *versus* CD SUS 52.19 $\mu m^2 \pm 1.678$ $p = 0.0002$) (Fig. 3E). Microglia in CD RES mice had an increased arborization area compared to those in CD CTRL animals (CD CTRL 1339 $\mu m^2 \pm 58.28$ *versus* CD RES 1668 $\mu m^2 \pm 65.01$ $p = 0.0017$) (Fig. 3E). These morphological changes were not observed upon stress in KD animals, for soma area (KD CTRL 48.86 $\mu m^2 \pm 1.545$ *versus* KD SUS 49.81 $\mu m^2 \pm 1.555$) and arborization area (KD CTRL 1573 $\mu m^2 \pm 47.88$ *versus* KD RES 1561 $\mu m^2 \pm 43.46$) (Fig. 3E-F). A main effect of stress ($F(2,374) = 9.632$, $p < 0.0001$) was, however, observed for the morphology index. *Post-hoc* analysis revealed that microglia in KD RES *versus* KD SUS mice had a smaller

morphology index (KD SUS 0.03852 a.u. ± 0.0023 *versus* KD RES 0.3094 a.u. ± 0.0010 $p = 0.0066$) (Fig. 3G), meaning that microglia have a smaller soma in relationship to their arborization, a ratio that describes more ramified microglia. While changes in microglial morphology can provide relevant insights into their physiological functions (Savage et al. 2019; Paolicelli et al. 2022), these results indicate that microglia display diverse morphological adaptations to diet and stress.

3.3 KD changes microglia-synapse interactions differently in stress susceptibility and resistance

To characterize how KD and RSD affect microglial interactions within the hippocampus, a detailed ultrastructural analysis was performed with SEM in the CA1 *stratum radiatum* of the same animals, also on Day 12. Microglial contacts with neuronal and astrocytic cell bodies as well as BV (basement membrane) were first quantified. There were no changes in these microglial interactions between CTRL, SUS, and RES mice under both diets (Table 2).

As microglia-synapse interactions are relevant to the regulation of neuronal activity and plasticity processes, notably upon stress and in MDD (Gonçalves de Andrade, González Ibáñez, and Tremblay 2022), microglial direct contacts with excitatory synapses were next quantified. They were classified as contacts with presynaptic axon terminals (identified by their synaptic vesicles), dendritic spines (recognized by their post-synaptic density) and synaptic clefts (contact with both pre- and post-synaptic elements) (Fig. 4A-D). Diet had no effect on microglial contacts with synaptic elements between the groups (Fig. 4E-H) (Table 2). However, we observed a main effect of stress on the number of direct microglial

contacts with axon terminals ($F(2, 206) = 7.542$, $p = 0.0007$), dendritic spines ($F(2, 206) = 5.036$, $p = 0.0073$) and synaptic clefts ($F(2, 206) = 4.951$, $p = 0.0079$) (Fig. 4E-G). *Post-hoc* analysis further revealed that the CD SUS mice had a reduced number of microglial contacts with dendritic spines than CD CTRL and CD RES (CD CTRL 0.5882 ± 0.1586 *versus* CD SUS 0.0606 ± 0.0421 $p < 0.0174$ *versus* CD RES 0.526 ± 0.118 $p = 0.0426$) (Fig. 4F). KD SUS microglia also showed fewer contacts than KD CTRL with axon terminals (KD CTRL 8.229 ± 0.6586 *versus* KD SUS 5.361 ± 0.7073 $p = 0.0486$) and synaptic clefts (KD CTRL 1.286 ± 0.2267 *versus* KD SUS 0.4167 ± 0.1344 $p = 0.007$) (Fig. 4E-G). To assess the overall impact of stress on microglial interventions at neuronal circuits, when synaptic element types were pooled, 2-way ANOVA analysis showed a main effect of stress ($F(2, 206) = 9.745$, $p < 0.0001$) on the total number of microglial contacts with synaptic elements (Fig. 4H). Following *post-hoc* analysis a decrease in the number of these microglial contacts with synaptic elements between KD SUS and KD CTRL groups (KD CTRL 9.886 ± 0.8031 *versus* KD SUS 6.0 ± 0.7807 $p = 0.0113$) was observed (Fig. 4H).

Together, these results reveal that SUS mice display reduced microglial contacts with synaptic elements. Increasing evidence has revealed the importance of membrane-to-membrane contacts between microglia and their neighboring cells in shaping neuronal networks, for instance via neuronal synchronization, synaptic plasticity, and structural remodeling (Cserép, Pósfai, and Dénes 2021; Umpierre et al. 2020).

3.4 KD and stress differently modify microglial phagolysosomal inclusions and other organelles

To provide further insights into possible changes in microglial function with KD and RSD, we next examined their accumulation of cellular stress markers, indicative of altered or compromised activities (Nahirney and Tremblay 2021). We performed an ultrastructural characterization of microglial organelles as well as classified and quantified their markers of cellular stress in samples from the same animals and region imaged by SEM on Day 12. We examined microglial mitochondria (healthy, long, altered), lysosomal inclusions (primary, secondary, tertiary lysosomes), autophagosomes, phagosomes (with content or empty), lipofuscin granules, lipid droplets, ER and Golgi apparatus (standard or with alterations), and nucleus (pores, indentations, alterations).

We detected an interaction of diet x stress for the number of tertiary lysosomes ($F(2,206) = 4.715$, $p < 0.01$) (Fig. 5A-C). *Post-hoc* analysis further showed that microglia in CD CTRL *versus* KD CTRL mice had more tertiary lysosomes (CD CTRL 0.294 ± 0.099 *versus* KD CTRL 0 $p = 0.0032$) (Fig. 5C). We observed a main effect of stress on a series of ultrastructural characteristics associated with phagocytic activity as well as cellular stress and aging (Table 2). In particular, we found a main effect of stress on the number of healthy ER ($F(2, 206) = 3.840$ $p = 0.0230$) (Fig. 5G). *Post-hoc* analysis revealed that CD SUS animals compared to CD CTRL had a reduced number of healthy ER (CD CTRL 7.088 ± 0.8748 *versus* CD SUS 3.303 ± 0.4656 $p = 0.0247$) (Fig. 5G). We further observed diet-related ultrastructural changes associated to cellular stress in ER and Golgi apparatus (Fig. 5D-F). Two-way ANOVA analysis revealed a main effect of the diet, notably for the number of microglial ER with dilation ($F(1,206) = 7.020$ $p = 0.0087$) (Fig. 5H). Two-way ANOVA analysis revealed a main effect of the diet, notably for the total number of microglial ER with dystrophy ($F(1,206) = 7.020$ $p = 0.0087$) (Fig. 5I). *Post-hoc*

analysis indicated that microglia in KD CTRL *versus* CD CTRL mice had a reduced number of ER showing dystrophy (CD CTRL 1.912 ± 0.5575 *versus* KD CTRL 0.600 ± 0.1650 &p = 0.0431) (Fig. 5I). Other features presenting a significant effect of the diet included the number of dilated Golgi apparatus ($F(1,206) = 6.766$ $p = 0.0337$) (Fig. 5J), the proportion of dilated Golgi ($F(1,206) = 7.724$ $p = 0.006$) (Fig. 5K), the number of elongated mitochondria ($F(1,206) = 3.913$ $p = 0.0493$) (Fig. 5L), and the number of empty phagosomes ($F(1,206) = 10.45$ $p = 0.0014$) (Fig. 5M).

We further observed a main effect of stress on mitochondria and the nuclear envelope. Total number of healthy mitochondria showed a main effect of stress (Fig. 5K) ($F(2,206) = 5.391$ $p = 0.0052$) (Fig. 5N). *Post-hoc* analysis revealed that CD SUS compared to CD CTRL had a reduced number of healthy mitochondria (CD CTRL 3.206 ± 0.4464 *versus* CD SUS 1.515 ± 0.2923 $p < 0.0205$) (Fig. 5N). Additionally, we observed a main effect of stress on the number of nuclear alterations, which include indentations and alterations to the nuclear envelope ($F(2,206) = 3.102$ $p = 0.0471$) (Fig. 5O).

These changes in ultrastructural features reveal that a KD diet and stress differently influence microglial properties, including phagolysosomal activity as well as cellular stress and aging.

3.5 KD alters the hippocampal lipidomic profile at steady-state and upon social stress

To deepen our understanding of the effects of KD on the hippocampal metabolism and function, we next performed lipidomic analyses of diet-related differences between mice with a shared stress phenotype (CTR, SUS or RES). Datasets from positive and negative

ion data acquisition modes were analyzed separately for initial statistical investigations. Principal component analysis (PCA) demonstrated differential clustering of KD and CD samples (Fig. 6A). In CTRL mice, the major principal component (PC1) explained 22.5% and 26.2% of the variation among groups in the negative ion and positive ion datasets, respectively. Similarly, PC1 explained 26.4% and 28.3% of the variation in the SUS group, and 21.3% and 22.6% in the RES group, in negative ion and positive ion datasets, respectively. Two-sample t-tests and fold change analyses further revealed substantial numbers of differentially-regulated lipids (DRLs) between the KD and CD groups (Fig. 6B, Supplementary Figure 1A, Supplementary Table 1). The DRLs were subsequently annotated by matching to the Human Metabolome Database (HMDB), combining the positive and negative ion mode datasets, which led to successful annotation and identification of 32 DRLs between CTRL KD and CD mice, 21 DRLs between RES KD and CD mice, and 52 DRLs between SUS KD and CD mice. In response to the KD regime, hippocampal lipids belonging to several classes were highly upregulated (Fig. 6B, Supplementary Figure 1A, Supplementary Table 1). This included multiple phospholipids, such as phosphatidylglycerols, sphingomyelins, lysophosphatidylethanolamines (LPEs) and phosphatidylserines (PSs), with DRL profiles differing distinctly at the lipid species level among CTRL, SUS and RES mice in response to a KD. Several phospholipid classes were also downregulated in response to a KD in CTRL and SUS mice, whereas certain conjugated N-acyl taurines were uniquely downregulated in RES mice (Supplementary Figure 1A, Supplementary Table 1).

Lipid ontology analyses using LION software (v.2020.07.14) (Molenaar et al. 2019) were performed to investigate the functional relevance of the DRLs identified (Fig. 6C,

Supplementary Figure 1B, Supplementary Table 2). Among the lipids assigned with HMDB, 29 of 32 (91%) DRLs identified between diet groups in CTRL mice, 17 of 21 (81%) DRLs in RES mice, and 48 of 52 (92%) in SUS mice, were matched to the LION database. In CTRL mice, lipids with positively charged head groups (effect size (ES) = 0.615, q = 0.047) and ceramide phosphocholines (ES = 0.923, q = 0.047) were significantly enriched in the KD group, while lipids with negatively charged head groups (ES = -0.800, q = 0.002), monoacyl glycerophosphoinositols (ES = -1.00, q = 0.011) and glycerophosphoserines (ES = -0.840, q = 0.047) were significantly underrepresented. In SUS mice, lysoglycerophospholipids (ES = -0.615, q = 0.0422) and lipids with a positive intrinsic curvature (ES = -0.571, q = 0.048) were significantly underrepresented in the KD group. No significant ontological differences between the KD and CD groups were identified in RES mice.

4. Discussion

Our study investigated the effects of KD, in which ketone bodies instead of glucose are used as a source of energy, on stress resistance as well as microglial properties and the lipidomic profile in the ventral hippocampus. Mice following a KD did not show reduced weight compared to mice on a CD, an observation in line with those of another study in which 8–10-week-old male mice on a KD were exposed to chronic unpredictable stress (Huang et al. 2018). After 4 weeks, mice on a KD had elevated circulating levels of the ketone body BHB, confirming that these mice were deriving energy from the oxidation of ketone bodies instead of glucose.

Characterization of our model revealed that after 10 days of RSD, KD tended to increase the proportion of adult male mice classified as RES, defined as presenting social behavior similar to unstressed CTRLs during the SI test, compared to mice following CD. We observed an effect of psychosocial stress on blood CORT, with SUS mice showing increased levels compared to CTRL and RES animals. RSD also caused a transient elevation at Day 6 in blood levels of the inflammatory cytokines G-CSF, IL-6, IL-13 and IP-10 in SUS animals compared to CTRL. G-CSF was previously shown to have anti-inflammatory and neuroprotective properties in the brain (Rahi, Jamwal, and Kumar 2021). IL-6 is responsible for stimulating the production of C-reactive protein, a protein serving as a general marker of inflammation that correlates with depressive symptoms (Kappelmann et al. 2021). IL-13 is produced by peripheral immune cells and microglia (Ellis et al. 2010; Miao et al. 2020) and can exacerbate or resolve central nervous system inflammation, depending on the context (Miao et al. 2020). By contrast, IFN-γ, a key mediator of microglial inflammatory response that was shown to drive depressive-like behavior in mice (J. Zhang et al. 2021; Kann, Almouhanna, and Chausse 2022), presented no significant variation with the diet or stress paradigm. Nevertheless, we observed changes in IFN-γ-related cytokines such as IP-10, which is increased in blood samples of patients with Alzheimer's disease (AD) and in cerebrospinal fluid of patients with frontotemporal dementia encephalitis (Curtin et al. 2009; Galimberti et al. 2015). A study showed that 10 days of RSD caused an increased number of peripheral neutrophils and monocytes in adult male mice (Ishikawa et al. 2021). In our study, IFN-γ levels were also reduced in the blood of mice following a KD.

The 10 days of RSD did not induce changes in microglial density and distribution in the CA1 *stratum radiatum*. Unchanged microglial density was previously reported in the same hippocampal region and layer in male mice following other protocols of stress such as chronic unpredictable mild stress (Milior et al. 2016; Alboni et al. 2016). A limitation of the density analysis in our and previous studies might arise from the fact that different microglial states downregulating classical microglial markers might not be captured by Iba1 staining (combined with TMEM119 in this case) (Paolicelli et al. 2022). However, our study revealed that microglia from KD-fed mice showed morphological changes at baseline, with larger soma and increased arborization area, within the CA1 *stratum radiatum*. It was previously observed that a KD on its own, at steady-state, can modify microglial morphology. In particular, microglia from the dorsal hippocampus of KD-fed adult male rats tended to have increased branching (Gzielo et al. 2019). In our study, microglia displayed increased soma and increased arborization without affecting the size ratio between soma and arborization with a KD, as seen with the morphological index in CTRL mice on both diets. Together, microglia of mice on a KD presented morphological adaptations that can be related to the increased prevalence of RES phenotype under this diet. We also observed microglial morphological changes linked to the stress phenotypes, hinting to specific stress-induced microglial adaptations that could underlie these phenotypes. We observed an increase of microglial soma size in KD CTRL and CD SUS animals, compared to CD CTRL animals. In the same region, CA1 *stratum radiatum*, microglia with enlarged soma were previously described in an adult male mouse model of chronic unpredictable mild stress (Milior et al. 2016). Increased microglial soma area has been linked to elevated production of trophic factors and pro- or anti-inflammatory

mediators (Alboni et al. 2016; Savage et al. 2019), which could explain the observed soma increase associated to KD in the KD CTRL group and associated to stress in the CD SUS group. We further observed increased microglial arborization area at baseline in KD CTRL and in CD RES (CD CTRL *versus* CD RES) animals. This aligns with a surveillant function, considering that hyper-ramified microglia were associated with a stress resistant phenotype in the context of chronic unpredictable stress (Huang et al. 2018). A consequence of hyper-ramification is the higher number of functional contacts between microglia and the brain parenchyma (Cserép et al. 2019). A recent study examining microglia in the CA1 region linked hyper-ramified microglia to stress resilience in a male mouse model of RSD (Fujikawa and Jinno 2022). Similar observations were made in the habenula, a region involved in aversive negative behavior (Proulx, Hikosaka, and Malinow 2014). When studying the effect of RSD, microglia in the habenula of SUS male mice showed increased cell body and decreased arborization volumes (Guan et al. 2020). In our study, no differences in microglial cell body and arborization area were linked to KD at steady-state, when comparing CD CTRL and CD RES mice. However, microglia from KD-fed *versus* CD-fed SUS mice were previously shown to display reduced cell body volume and increased arborization volume, highlighting the capacity of a KD to restore microglial morphology in a context of RSD (Guan et al. 2020).

Ultrastructural characterization of microglia by SEM allowed us to investigate at the nanoscale level possible changes in microglial intracellular organelles and contacts with specific elements of the parenchyma. SEM analysis showed that microglia of KD *versus* CD mice had a reduction of both empty phagosomes and tertiary lysosomes, hinting at possibly decreased contents that require degradation with the dietary change (Majumdar

et al. 2011; El Hajj et al. 2019). Microglial cell bodies can establish functional membrane-to-membrane contacts with various types of elements although their specific functions remain to be more deeply understood (Wogram et al. 2016; Cserép, Pósfai, and Dénes 2021). In our study, we observed microglial contacts with pre-synaptic axon terminals, dendritic spines and synaptic clefts, and with all categories combined. Ultrastructural analysis showed that microglia of SUS mice make fewer contacts with pre-synaptic axon terminals, post-synaptic dendritic spines and synaptic clefts compared to CTRL microglia. Additionally, we observed that microglia of SUS mice made overall less direct contacts with excitatory synapses. Given that further ultrastructural characterization of SUS mice did not reveal changes in microglial organelles involved in phagolysosomal activity, these reduced contacts were probably not associated to an active synaptic elimination at the time examined. Additional electrophysiological studies are warranted to determine the outcomes of these microglial contacts on synaptic density over the course of RSD.

We also found that microglia of mice following a KD had a reduced prevalence of cellular stress markers, seen as a reduced number of dilated Golgi apparatus and ER cisternae, as well as ER cisternae with dystrophy, and microglia with a lower percentage of Golgi apparatus displaying dilation. Elevations in reactive oxygen species (ROS), which are an important source of cellular stress, were shown to compromise the function of several cytoplasmic organelles (Pizzino et al. 2017; Chavez-Valdez et al. 2016). This could lead to impaired microglial function and a shift towards detrimental microglial states (Nahirney and Tremblay 2021). The ketone body BHB can protect neurons from calcium-induced excitotoxicity and was shown to reduce reactive oxygen species (ROS) levels in neurons isolated from the rat brain (Maalouf et al. 2007). Increased ROS production in the brain

was also linked to psychiatric diseases including mood disorders and major depression (Salim 2014). Interestingly, one study showed that the behavioral changes associated with RSD require microglia and are driven by oxidative stress in adult male mice (Lehmann et al. 2019).

Microglial function is tightly regulated by mitochondrial dynamics (Katoh et al. 2017). The structure of the mitochondrial network tightly influences microglial metabolism and function, notably in response to pathological stimuli (Katoh et al. 2017; Peruzzotti-Jametti et al. 2021). Our analysis showed a reduction in the number of mitochondria in microglia from SUS mice on a CD diet, hinting that metabolic alterations in the CA1 region may contribute to the SUS phenotype under CD. We did not observe a different prevalence of dystrophic mitochondria in microglia from mice undergoing RSD, but an increased frequency of elongated mitochondria was found, suggesting mitochondrial adaptations. Mitochondria of different lengths were proposed to have different functions and energetic properties, including energy production efficiency (Skulachev 2001; Karbowski and Youle 2003; Collins et al. 2002). Further 3D analysis of the mitochondrial network would provide better understanding of the complex relationships between mitochondrial structure and function in microglia (Lounas et al. 2022). Further analyses of functional mitochondrial output would help linking the ultrastructural modifications of mitochondria to their energetic proficiency among the different behavioral phenotypes.

Although glucose is the primary source of energy for the brain, microglial lipid metabolism is emerging as a key player in neuroimmune homeostasis (Loving and Bruce 2020). Microglia exist in a variety of homeostatic and inflammatory states, while recent evidence indicates that microglial uptake of lipids drives oxidative metabolism and an anti-

inflammatory phenotype (Chausse et al. 2019; Morris et al. 2020), and FAO-related genes are repressed in response to proinflammatory stimuli (Mauerer, Walczak, and Langmann 2010). Microglia take up free FAs through the scavenger receptor CD36 and import the cholesterol-containing apolipoproteins ApoE and ApoA1 through lipoprotein lipase (LPL) and the low-density lipoprotein receptor (LDL-R) (Loving and Bruce 2020). The uptake and subsequent degradation of microglial lipids is critical for the clearance of cellular debris, while deficiency in the triggering receptor expressed on myeloid cells 2 (TREM2), a key microglial sensor of apoptotic cells and extracellular debris, impairs microglial function in pathological states (Nugent et al. 2020). As discussed below, our lipidomic data suggest an altered lipid profile in response to a KD that may reduce brain inflammation, promoting a homeostatic microglial state and supporting the possibility that microglia may underlie the proposed benefits of KD treatment for MDD.

Here, we identified profound changes in hippocampal lipid profiles in response to a KD, which differed among CTRL, SUS and RES mice. In general, a far greater number of lipids were differentially regulated by a KD in SUS mice than in CTRL or RES mice, potentially indicating that lipid metabolism in SUS mice varies in response to psychological stress, which may facilitate beneficial changes introduced by interventions such as a KD. In CTRL and RES mice, which may represent a more homeostatic state, a KD resulted in fewer changes to hippocampal lipid profile. It tended to broadly upregulate individual lipids, which suggests a general enrichment consistent with a lipid-rich diet. Perhaps surprisingly, a KD intervention did not significantly enrich any lipid ontology terms in RES mice, likely due to the low number of DRLs identified in these groups.

Lipid ontology analyses identified several terms significantly enriched by a KD across both CTRL and SUS mice. A KD treatment upregulated sphingomyelins, with SM(d18:1) species also trending upwards. These results are strikingly similar to the known effects of the antidepressants amitriptyline and fluoxetine, which were shown to induce sphingomyelin accumulation in an autophagy-dependent manner in cultured hippocampal neurons, thus promoting neurogenesis (Gulbins et al. 2018). As impaired neurogenesis is a major pathological mechanism in mood disorders, the ability of a KD to induce sphingomyelin accumulation may be highly desirable in a clinical context. Similarly chronic unpredictable stress leads to decreased hippocampal sphingomyelins in rats, which correlated negatively with blood CORT levels (Oliveira et al. 2016), supporting the idea that KD treatment could normalize stress-related alterations in lipid profiles. However, we did not observe consistent upregulation of sphingomyelins in SUS mice under KD, suggesting that a longer treatment period or combining KD treatment with conventional antidepressants may be necessary to increase sphingomyelin levels.

In both CTRL and SUS mice, KD treatment significantly altered lipid ontology terms related to glycerophospholipids, including downregulation of monoacylglycerophosphoinositols and glycerophosphoserines in CTRL mice and monoacylglycerophosphoglycerols in the SUS group. Glycerophospholipids are highly prevalent components of cell membranes, dictating membrane structure and function, while some less abundant lipids of this class, such as glycerophosphoinositols and glycerophosphoserines, are involved in cell-cell communication and intracellular signaling pathways (Miranda and Oliveira 2015). Moreover, the charged headgroups of phospholipids play a key role in determining membrane structure, which can in turn

modify cellular function (K. W. Li, Ganz, and Smit 2019; Ma et al. 2017). For example, lipid headgroups with various charges differentially influence the activity of transmembrane receptors, such as the stress-related beta-adrenergic receptor, by modulating protein stability (Bruzzese et al. 2018). Our lipid ontology results in CTRL mice showed significant upregulation of lipids with positively charged headgroups, which include phosphatidylethanolamines and phosphatidylcholines, and downregulation of those with negatively charged headgroups, such as PSs, in response to a KD. Thus, KD produces important changes in cell membrane structure, and by extension, may modify cellular function.

Specific lipids can also act as potent mediators of pro- or anti-inflammatory effects. Lysoglycerophospholipids are generally considered proinflammatory, promoting MIP-2 production and chemotaxis to apoptotic cells in macrophages (Olofsson et al. 2008; Yang et al. 2005). Lipidomic analyses have identified lysophosphatidylcholines (LPCs) and LPEs as significantly upregulated in rodent models of chronic restraint stress and serotonin deficiency (Chen et al. 2014; Weng et al. 2016), consistent with a role for inflammation and immune reactivity in depressive disorders. Meanwhile, KD treatment is widely reported to exert anti-inflammatory effects in rodent models of depression, potentially through its actions on microglia (Guan et al. 2020; Morris et al. 2020). Importantly, our results identified lysoglycerophospholipids as significantly downregulated by a KD in SUS mice, implying that a KD may attenuate inflammation in part through reducing proinflammatory lipid species such as lysoglycerophospholipids. It is worth noting that another study demonstrated increases in LPC upon treatment with conventional antidepressants (Lee et al. 2009). However, this study investigated lipidomic

changes in healthy, rather than stressed mice. Indeed, our analyses of CTRL mice generally showed an upregulation of individual LPC species in response to KD, highlighting potentially similar mechanisms of KD and conventional antidepressant action that may differ between healthy and stressed subjects.

Notably, our results indicate that PSs were generally downregulated by KD treatment, with lipid ontology analyses showing a significant decrease in glycerophosphoserines in CTRL mice and a decreasing trend in monoacylglycerophosphoserines in SUS mice. PSs, normally localized to the internal leaflet of the plasma membrane, are externalized in apoptotic cells, marking the cell for phagocytosis by macrophages and microglia (Neher et al. 2011). The microglial receptor TREM2 is critical for this homeostatic process, as a loss of TREM2 impairs PS-mediated synaptic pruning of new hippocampal neurons during development and adult neurogenesis (Kurematsu et al. 2022; Scott-Hewitt et al. 2020). Thus, increased brain PS content could indicate either increased neuronal cell death or defective microglial clearance of apoptotic neurons, both of which may contribute to pathology (Loving and Bruce 2020). The decrease in PSs observed in our study suggests either decreased neuronal death, notably in the context of adult neurogenesis, or improved microglial homeostatic function, consistent with the established effects of the KD in promoting anti-inflammatory microglial states (Morris et al. 2020).

As a limitation, this study was done only in male mice given that the RSD paradigm used relies on male-to-male aggressivity. Nevertheless, recent modifications of this paradigm can circumvent these behavioral limitations and have allowed other groups to characterize stress related changes in the female mouse brain. Future work using these models would be key for the study of sex differences in the outcomes of KD on stress

related brain changes (Yin et al. 2019; van Doeselaar et al. 2021). While we focused on whole hippocampus for lipidomic analysis, RSD further increased the activity of the prefrontal cortex, bed nucleus of stria terminalis, and periaqueductal gray in mice (Laine et al. 2017). As a consequence, the results from this study should not be extrapolated to other brain regions which may differ in their susceptibilities to stress. It will be important in the future to ensure their further characterization, as behavior is the result of concerted changes among brain regions in constant communication. Furthermore, inflammatory responses are exacerbated with aging. A study showed that RSD caused a stronger inflammatory reaction in older *versus* younger mice. When analyzing the supernatants harvested from cultured splenocytes, the samples belonging to older mice contained higher IL-6 and TNF-α compared to samples from younger male mice (Kinsey et al. 2008). It would be interesting to study whether RSD in older mice would result in similar SUS to RES ratios and whether KD would still be able to promote a RES phenotype.

A KD has shown promising results as a potential beneficial dietary regime for many health complications including epilepsy and mood improvement (Ashton et al. 2021; Carneiro and Pellerin 2022; Ricci et al. 2020; Jiwani et al. 2022; Włodarczyk, Cubała, and Stawicki 2021). Nevertheless, low adherence rates are a limitation with this diet (Kumar et al. 2022). Therefore, research that sheds light onto the mechanisms underlying the described beneficial effects could identify and isolate the responsible chemical compounds which could eliminate the requirement to adhere to this dietary regime. Exogenous supplementation of diets with ketone-derived products have shown promising results in preclinical and clinical studies. Supplementation with a ketone body-containing drink improved cognition in patients with mild cognitive impairment (Fortier et al. 2021),

while another study revealed that supplementation with ketogenic medium chain triglycerides increased energy metabolism in AD (Croteau et al. 2018).

5. Conclusion

In conclusion, KD promoted resistance to psychological stress, by tending to increase the number of mice with preserved sociability after 10 days of RSD. Our model allowed us to study specific changes linked to KD as well as study differences between the SUS and RES phenotypes under both diets. We further identified a transient elevation of circulating inflammatory cytokines, changes in microglial morphology, ultrastructural evidence of altered microglial direct contacts with synapses, cytoplasmic organelles, and cellular stress markers, as well as hippocampal lipidic expression due to stress and diet. Together, our results shed light onto the complex relationships between diet, immune system and stress resistance, contributing to a deeper understanding of the effects of stress and diet on the brain and behavior.

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7. Author contributions

K.S., N.V., F.G.I. and M.E.T. conceived the project and designed the experiments. Animal handling and diet control were done by K.S. and N.V. with the help of K.B. Behavioral experiments were done by K.S. N.V. and K.B. Tissue processing was performed by K.S., N.V., K.B., and F.G.I. The immunofluorescence staining, epifluorescence and confocal imaging, as well as analysis were done by C.M., K.P., and F.G.I. Electron microscopy image acquisition and analysis were done by F.G.I. In addition, T.H., M.C. and M.L. were in charge of the lipidomic sample processing and bioinformatic analyses. F.G.I. and T.H. prepared the manuscript, as well as figures and tables, under the supervision of M.E.T.

Proof reading and approval of the final manuscript was done by F.G.I., T.H., J.D., M.L. and M.E.T.

8. Declaration of Competing Interest

The authors declare no competing interest.

9. Data Availability

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

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11. Figure Legends

Figure 1: Ketogenic diet tends to increase stress resistance in a mouse model of repeated social defeat. Experimental timeline, mice were fed 4 weeks of ketogenic diet (KD) in KD group, or control diet (CD) in CD group. Mice were exposed to ten days of repeated social defeat (RSD), first day of social defeat is considered Day 1. Social interaction (SI) test was performed on Day 11. Blood draws were performed on Days 0, 6 and 12. Perfusion and brain collection were performed on Day 12 **(A)**. Four weeks of KD increased blood levels of β-hydroxybutyrate (BHB) throughout RSD until brain collection **(B)**. Susceptible (SUS) mice on CD and KD had increased blood levels of CORT corticosterone compared to control (CTRL) and resistant (RES) mice **(C)**. SUS mice on CD and KD showed increased levels of social avoidance in a SI test **(D)**. SUS mice on CD and KD spent more time in corner zone (CZ) once the CD1 mouse entered the arena **(E)**. RES mice on KD spent less time in CZ once the CD1 entered the arena. Mice on KD *versus* CD showed an increased proportion of mice classified as RES **(F)**. n = 5–10 mice/group for the BHB and corticosterone tests; n = 8–20 mice/group for the behavioral tests. Data are expressed as mean ± standard error of the mean. Statistical significance was assessed by 2-way ANOVA followed by Tukey *post-hoc* analysis, where **p < 0.01 and ****p < 0.0001. For corticosterone analysis, statistical significance was assessed by 1-way ANOVA, where **p < 0.01. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible; RES: resistant. Created with the help of BioRender.

Figure 2: Ketogenic diet and repeated social defeat stress modify blood levels of inflammatory cytokines. On Day 6, granulocyte colony stimulating factor (G-CSF) **(A)**,

interleukin 6 (IL-6) **(C)**, interleukin 13 (IL-13) **(D)** and C-X-C motif chemokine ligand 10 (IP-10) **(E)** presented an effect of stress. Interferon gamma (IFN-ɣ) showed an effect of diet **(B)**. By Day 12, only G-CSF maintained changes related to stress **(A')**. On Day 12, G-CSF showed an effect related to diet. n = 3–4 mice/group. Data are expressed as mean ± standard error of the mean. Statistical significance was assessed by 2-way ANOVA where #p < 0.05; ##p < 0.01 represent a main effect of stress; &p < 0.05; &&p < 0.01 represent a main effect of diet. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible; RES: resistant; G-CSF: granulocyte colony stimulating factor; IFN-γ: interferon-gamma; IL: interleukin; IP-10: C-X-C motif chemokine ligand 10.

Figure 3: Ketogenic diet results in different microglial morphological adaptations to social stress. Representative confocal images at 40x magnification showing Iba1 (red) and TMEM119 (green) stained microglia in the ventral hippocampus CA1 *stratum radiatum* of the 6 experimental groups **(A)**. Scale bar is equivalent to 25 μm. Microglial density and distribution remained unchanged by stress or diet as observed by density **(B)**, nearest neighbor distance (NND) **(C)** and spacing index **(D)** of Iba1+/TMEM119+ cells. Microglia of KD fed mice showed an increase in soma **(E)** and arborization **(F)** area in non-stressed controls (CTRL). Microglia of stressed animals showed morphological adaptations to stress: an increase of soma area **(E)** of control diet (CD) susceptible (SUS) microglia compared to CD CTRL, and an increase in arborization **(F)** area of CD resistant (RES) microglia compared to CD CTRL. Stress modified the microglial morphology index **(G)**. Microglia of SUS animals had a bigger soma to arborization ratio compared to CTRL and RES animals of KD group. n = 51–88 microglia/group; N = 3–5 mice/group. Data are

expressed as mean ± standard error of the mean. Statistical significance was assessed by 2-way ANOVA followed by Tukey *post-hoc* analysis, where *p < 0.05, **p < 0.01, ***p < 0.001. ##p < 0.01, ####p < 0.0001 represent a main effect of stress. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible; RES: resistant; NND: nearest neighbor distance; a.u.: arbitrary units.

Figure 4: Microglia-synapse interactions change differently in stress susceptibility and resistance. Representative 5 nm resolution scanning electron microscopy images showing microglia captured in the ventral hippocampus CA1 *stratum radiatum* of the 6 experimental groups **(A)**. There is a reduction in the number of direct contacts between microglial cell bodies and pre-synaptic axon terminals **(B,F)**, post-synaptic dendritic spines **(C,G)** and simultaneous contact to two synapse-forming elements classified as synaptic clefts **(D,H)**. Stress affects plasticity as reflected by total number of contacts between microglial cell bodies and all three categories of synaptic elements **(I)**. n = 34–38 cells/group; N = 3 mice/group. Data are expressed as mean ± standard error of the mean. Statistical significance was assessed by 2-way ANOVA followed by Tukey *post-hoc* analysis, where #p < 0.05, ##p < 0.01, ###*p < 0.001,* ####p < 0.0001 represent a main effect of stress. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible; RES: resistant; red outline: microglial plasma membrane; yellow outline: nuclear membrane; green pseudo-coloring: pre-synaptic axon terminals; magenta pseudo-coloring: post-synaptic dendritic spines.

Figure 5: Ketogenic diet modifies microglial organelle number and ultrastructure. Representative 5 nm resolution scanning electron microscopy images showing microglia captured in the ventral hippocampus CA1 *stratum radiatum* of unstressed control animals **(A)**. Ultrastructural example of a tertiary lysosome **(B)**. There is a reduction in the number of tertiary lysosomes in microglial cell bodies from KD fed control animals compared to control diet (CD) fed controls **(C)**. Representative 5 nm resolution scanning electron microscopy images showing microglia captured in the ventral hippocampus CA1 *stratum radiatum* of non-stressed controls (CTRL). Ultrastructural examples of healthy and dystrophic endoplasmic reticulum (ER) and Golgi apparatus **(E, F)**. KD diet modifies in microglial cell bodies the number and ultrastructural characteristics of ER as seen by the total number of healthy ER **(G)**, ER showing signs of dilation **(H)** and total number of ER with signs of dystrophy (endoplasmic reticulum with inclusions and dilation) **(I)**. KD diet affects in microglial cell bodies the number and ultrastructural features of Golgi apparatus, as seen by changes in the number of dilated Golgi apparatus **(J)** and total number of Golgi apparatus with signs of dystrophy (Golgi apparatus with inclusions and dilation) **(K)**. Diet affects in microglial cell bodies the number of elongated mitochondria **(L)** and empty phagosomes **(M)**. Stress affects in microglial cell bodies the number of total healthy mitochondria (standard and long mitochondria with absence of dystrophy), **(N)** elongated mitochondria and total nuclear alterations **(O)**. n = 34–38 cells/group; N = 3 mice/group. Data are expressed as mean ± standard error of the mean. Statistical significance was assessed by 2-way ANOVA followed by Tukey *post-hoc* analysis, where **p < 0.01. #p < 0.05, ##p < 0.01 represent a main effect of stress. &p < 0.05, &&p < 0.01 represent a main effect of diet. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible;

RES: resistant; ER: endoplasmic reticulum. Red outline: microglial plasma membrane; yellow outline: nuclear membrane; yellow pseudo-coloring: tertiary lysosome; green pseudo-coloring: Golgi apparatus; blue pseudo-coloring: endoplasmic reticulum; white star dilated Golgi apparatus; red star: dilated ER.

Figure 6: Ketogenic diet differentially alters hippocampal lipidomic profiles in non-stressed mice and mice exposed to repeated social defeat stress. PCA plots showing the separation in CTRL, SUS and RES groups, in positive and negative ion modes, respectively **(A)**. Volcano plots showing hippocampal differentially regulated lipids by a ketogenic diet (KD) *versus* control diet (CD) in the CTRL or SUS mice. Lipids indicated in red (up) or blue (down) are differentially regulated lipids, identified based on a fold change, FC >1.5 and a two-sample t-test p-value ≤0.05 that was considered significant **(B)**. Ontological analysis of differentially regulated lipids between KD and CD diets in CTRL and SUS mice. Lipids identified through two-sample t-test analyses as differentially regulated were annotated by matching to the human metabolome database (HMDB), followed by analysis in LION software in ranking mode. Lipid ontology terms that displayed differential regulation were considered to be significantly enriched (up) or underrepresented (down) are indicated by an asterisk (*) **(C)**. n = 4 mice/group. CD: control diet; KD: ketogenic diet; CTRL: control; SUS: susceptible; RES: resistant; GlcCer, glucosylceramide; PA, phosphatidic acid; PE, phosphatidylethanolamine; PGF1α, prostaglandin F1α; PI, phosphatidylinositol; PIP, phosphatidylinositol-monophosphate; PS, phosphatidylserine; SM, sphingomyelin.

Table 1 Microglial number, distribution and morphological properties in CD *versus* KD fed mice exposed to RSD. a.u.: arbitrary units; CD: control diet; KD: ketogenic diet; RSD: repeated social defeat.

	Density and distribution									
Parameters	mean ± standard error of the mean								F	P
	Control		Susceptible		Resistant					
	CD	KD	CD	KD	CD	KD				
Density	262.5	272.6	275.1	264.2	299.6	257.1	F (2, 15)	Stress x diet	1.59	0.2365
	± 4.017	± 11.22	± 20.73	± 16.65	± 20.12	± 14.37	F (2, 15)	Stress	0.2948	0.7489
							F (1, 15)	Diet	1.34	0.2651
Nearest neighbor distance (µm)	42.21	40.98	40.59	42.95	39.31	42.12	F (2, 15)	Stress x diet	1.035	0.3791
	± 0.5627	± 1.714	± 1.630	± 2.399	± 1.250	± 0.748	F (2, 15)	Stress	0.2539	0.7791
							F (1, 15)	Diet	1.023	0.3279
Spacing index (a.u.)	0.4598	0.4453	0.448	0.457	0.455	0.4456	F (2, 15)	Stress x diet	0.2322	0.7965
	± 0.0037	± 0.0228	± 0.0180	± 0.0077	± 0.0050	± 0.0158	F (2, 15)	Stress	0.0105	0.9895
							F (1, 15)	Diet	0.1144	0.7399
	Morphology									
	mean ± standard error of the mean									
	Control		Susceptible		Resistant					
	CD	KD	CD	KD	CD	KD				
Soma area (μm^2)	42.41	48.86	52.19	49.81	47.2	45.76	F (2, 374)	Stress x diet	5.628	**0.0039 ****
	± 1.336	± 1.545	± 1.678	± 1.555	± 1.432	± 1.110	F (2, 374)	Stress	7.108	**0.0009 *****
							F (1, 374)	Diet	0.5411	0.4624

Arborization area (µm)	1339 ± 58.28	1573 ± 47.88	1515 ± 62.59	1453 ± 76.82	1668 ± 65.01	1561 ± 43.46	F (2, 374)	Stress x diet	5.42	**0.0048 ****
							F (2, 374)	Stress	4.614	**0.0105 ***
							F (1, 374)	Diet	0.2088	0.648
Morpho-logical index (a.u.)	0.0346 ± 0.0016	0.0327 ± 0.0013	0.0374 ± 0.002	0.0385 ± 0.0023	0.0309 ± 0.0015	0.0309 ± 0.001	F (2, 374)	Stress x diet	0.4414	0.6435
							F (2, 374)	Stress	9.632	**<0.0001 ******
							F (1, 374)	Diet	0.355	0.8507

Table 2 Microglial ultrastructural features in CD *versus* KD fed mice exposed to RSD. CD: control diet; KD: ketogenic diet; RSD: repeated social defeat.

		Ultrastructural analysis mean ± standard error of the mean								F	P
		Control		Susceptible		Resistant					
		CD	KD	CD	KD	CD	KD				
Contacts with other cells	Blood vessels	0.264 ± 0.077	0.171 ± 0.065	0.182 ± 0.068	0.222 ± 0.070	0.079 ± 0.044	0.243 ± 0.072	F(2, 206)	Stress x diet	1.992	0.139
								F(2, 206)	Stress	0.3478	0.7066
								F(1, 206)	Diet	0.5246	0.4697
	Astrocytes	0.088 ± 0.049	0.057 ± 0.040	0.091 ± 0.051	0.194 ± 0.067	0	0.194 ± 0.067	F(2, 206)	Stress x diet	1.393	0.2506
								F(2, 206)	Stress	1.864	0.1576
								F(1, 206)	Diet	2.466	0.1179
	Neurons	0.177 ± 0.099	0.086 ± 0.048	0.121 ± 0.072	0.028 ± 0.028	0.121 ± 0.072	0.108 ± 0.052	F (2, 206)	Stress x diet	0.4356	0.6475
								F (2, 206)	Stress	0.4217	0.6565
								F (1, 206)	Diet	1.41	0.2364
Mitochondria	Standard	3 ± 0.431	2.571 ± 0.383	1.424 ± 0.275	1.972 ± 0.254	2.421 ± 0.319	2.243 ± 0.348	F (2, 206)	Stress x diet	1.076	0.3429
								F (2, 206)	Stress	5.003	**0.007****
								F (1, 206)	Diet	0.003	0.95
	Long	0.205 ± 0.082	0.114 ± 0.068	0.091 ± 0.667	0	0.263 ± 0.134	0.054 ± 0.054	F (2, 206)	Stress x diet	0.3563	0.7007
								F (2, 206)	Stress	1.327	0.2675
								F (1, 206)	Diet	3.913	**0.0493***

	Total healthy	3.206 ± 0.446	2.686 ± 0.399	1.515 ± 0.292	1.972 ± 0.254	2.684 ± 0.392	2.297 ± 0.359	F (2, 206)	Stress x diet	1.025	0.3606
								F (2, 206)	Stress	5.391	**0.0052****
								F (1, 206)	Diet	0.243	0.6226
	Dystrophic	0.677 ± 0.201	0.6 ± 0.170	0.485 ± 0.139	0.417 ± 0.122	0.29 ± 0.130	0.351 ± 0.111	F (2, 206)	Stress x diet	0.162	0.8506
								F (2, 206)	Stress	2.285	0.1044
								F (1, 206)	Diet	0.0406	0.8405
	White	0.823 ± 0.251	0.8 ± 0.158	0.485 ± 0.180	0.389 ± 0.115	0.29 ± 0.092	0.838 ± 0.184	F (2, 206)	Stress x diet	2.097	0.1254
								F (2, 206)	Stress	2.754	0.066
								F (1, 207)	Diet	1.222	0.2703
	Long and dystrophic	0.088 ± 0.049	0.057 ± 0.040	0.061 ± 0.061	0	0.053 ± 0.037	0	F (2, 206)	Stress x diet	0.0799	0.9233
								F (2, 206)	Stress	0.9164	0.4016
								F (1, 206)	Diet	2.426	0.1209
	Holy	0.294 ± 0.115	0.114 ± 0.054	0.09 ± 0.050	0	0.105 ± 0.062	0.27 ± 0.107	F (2, 206)	Stress x diet	2.503	0.0843
								F (2, 206)	Stress	2.478	0.0864
								F (1, 206)	Diet	0.4614	0.4977
	Total dystrophic	2 ± 0.347	1.571 ± 0.276	1.545 ± 0.237	0.944 ± 0.173	0.842 ± 0.215	1.514 ± 0.281	F (2, 206)	Stress x diet	2.464	0.0876
								F (2, 206)	Stress	2.012	0.1363
								F (1, 206)	Diet	0.1194	0.73
	% of dystrophic mitochondria	30.55 ± 5.288	38.04 ± 6.159	37.27 ± 6.783	27.54 ± 4.797	19.94 ± 5.166	31.7 ± 5.146	F (2, 206)	Stress x diet	1.998	0.1383
								F (2, 206)	Stress	1.384	0.2529
								F (1, 206)	Diet	0.4339	0.5108

Contacts with synaptic elements	Axon terminal contacts	7.353 ± 0.973	8.229 ± 0.659	4.727 ± 0.627	5.361 ± 0.707	5.816 ± 0.542	6.378 ± 0.657	F (2, 206)	Stress x diet	0.0199	0.9803
								F (2, 206)	Stress	7.542	**0.0007** ***
								F (1, 206)	Diet	1.535	0.2168
	Dendritic spine contacts	0.588 ± 0.159	0.371 ± 0.101	0.061 ± 0.042	0.222 ± 0.106	0.526 ± 0.118	0.297 ± 0.109	F (2, 206)	Stress x diet	1.891	0.1535
								F (2, 206)	Stress	5.036	**0.0073** **
								F (1, 206)	Diet	1.006	0.317
	Synaptic cleft contacts	0.912 ± 0.186	1.286 ± 0.227	0.667 ± 0.178	0.417 ± 0.134	0.684 ± 0.161	0.946 ± 0.155	F (2, 206)	Stress x diet	1.711	0.1833
								F (2, 206)	Stress	4.951	**0.0079** **
								F (1, 206)	Diet	0.6898	0.4072
	Total synaptic contacts	8.853 ± 1.145	9.886 ± 0.803	5.455 ± 0.702	6 ± 0.781	7.026 ± 0.631	7.622 ± 0.761	F (2, 206)	Stress x diet	0.049	0.9521
								F (2, 206)	Stress	9.745	**<0.0001** ****
								F (1, 206)	Diet	1.228	0.2691
Other organelles	Primary lysosomes	0.118 ± 0.070	0.343 ± 0.136	0.212 ± 0.113	0.139 ± 0.071	0.158 ± 0.110	0.135 ± 0.069	F (2, 206)	Stress x diet	1.4	0.249
								F (2, 206)	Stress	0.4844	0.6168
								F (1, 206)	Diet	0.1918	0.6619
	Secondary lysosomes	0.118 ± 0.056	0.114 ± 0.068	0	0.028 ± 0.028	0	0.108 ± 0.085	F (2, 206)	Stress x diet	0.6626	0.5166
								F (2, 206)	Stress	0.91	0.1508
								F (1, 206)	Diet	1.135	0.2881
	Tertiary lysosomes	0.294 ± 0.099	0	0.091 ± 0.051	0.111 ± 0.053	0.079 ± 0.044	0.054 ± 0.038	F (2, 206)	Stress x diet	4.715	**0.0100** **
								F (2, 206)	Stress	1.069	0.3451
								F (1, 206)	Diet	4.866	**0.0285** *

Total lysosomes	0.529 ± 0.159	0.457 ± 0.166	0.303 ± 0.119	0.278 ± 0.094	0.237 ± 0.116	0.297 ± 0.109	F (2, 206)	Stress x diet	0.0983	0.9064
							F (2, 206)	Stress	1.943	0.1459
							F (1, 206)	Diet	0.0319	0.8584
Phagosomes with content	0.265 ± 0.114	0.343 ± 0.100	0.121 ± 0.072	0.5 ± 0.180	0.447 ± 0.167	0.378 ± 0.098	F (2, 206)	Stress x diet	1.627	0.1991
							F (2, 206)	Stress	0.3757	0.6873
							F (1, 206)	Diet	1.334	0.2495
Phagosomes with myelin	0.324 ± 0.173	0.286 ± 0.145	0.061 ± 0.042	0.167 ± 0.075	0.079 ± 0.044	0.027 ± 0.027	F (2, 206)	Stress x diet	0.3845	0.6813
							F (2, 206)	Stress	3.51	**0.0317 ***
							F (1, 206)	Diet	0.0049	0.9439
Empty phagosomes	1.265 ± 0.305	0.486 ± 0.126	1.273 ± 0.299	0.75 ± 0.230	1 ± 0.226	0.487 ± 0.184	F (2, 206)	Stress x diet	0.1741	0.8403
							F (2, 206)	Stress	0.7707	0.464
							F (1, 206)	Diet	10.45	**0.0014 ****
Total phagosomes	1.853 ± 0.489	1.114 ± 0.245	1.455 ± 0.296	1.417 ± 0.366	1.526 ± 0.326	0.833 ± 0.247	F (2, 206)	Stress x diet	0.6657	0.515
							F (2, 206)	Stress	0.4791	0.62
							F (1, 206)	Diet	3.16	0.0769
Lipofuscin granules	0.471 ± 0.121	0.171 ± 0.087	0.121 ± 0.058	0.306 ± 0.118	0.395 ± 0.153	0.351 ± 0.111	F (2, 206)	Stress x diet	2.237	0.1094
							F (2, 206)	Stress	0.8058	0.4481
							F (1, 206)	Diet	0.5399	0.4633
Lipid droplets	0.765 ± 0.164	0.629 ± 0.229	0.727 ± 0.236	0.361 ± 0.183	0.447 ± 0.222	0.297 ± 0.109	F (2, 206)	Stress x diet	0.222	0.8011
							F (2, 206)	Stress	1.358	0.2595
							F (1, 206)	Diet	1.806	0.1805

Standard Golgi apparatus	0.265 ± 0.097	0.514 ± 0.155	0.091 ± 0.067	0.417 ± 0.175	0.263 ± 0.082	0.297 ± 0.128	F (2, 206)	Stress x diet	0.8598	0.4248
							F (2, 206)	Stress	0.6911	0.5022
							F (1, 206)	Diet	3.715	0.0553
Dystrophic Golgi apparatus	0.353 ± 0.102	0.286 ± 0.112	0.697 ± 2282	0.278 ± 0.116	0.342 ± 0.120	0.139 ± 0.070	F (2, 206)	Stress x diet	0.8884	0.4129
							F (2, 206)	Stress	1.839	0.1616
							F (1, 206)	Diet	4.571	**0.0337 ***
Proportion dystrophic Golgi apparatus	22.550 x ± 0.070	11.86 ± 5.005	30.3 ± 7.861	15.05 ± 5.768	22.37 ± 6.450	8.333 ± 4.033	F (2, 206)	Stress x diet	0.0927	0.9115
							F (2, 206)	Stress	0.899	0.4086
							F (1, 206)	Diet	7.724	**0.0060 ****
Nuclear pores	0.76 ± 0.270	1.2 ± 0.306	1.485 ± 0.547	1.667 ± 0.384	1.132 ± 0.267	0.865 ± 0.187	F (2, 206)	Stress x diet	0.4772	0.6212
							F (2, 206)	Stress	1.862	0.158
							F (1, 206)	Diet	0.1713	0.6794
Nuclear indentations	0.177 ± 0.066	0.2 ± 0.090	0.03 ± 0.030	0.167 ± 0.075	0.079 ± 0.044	0.054 ± 0.038	F (2, 206)	Stress x diet	0.9027	0.4071
							F (2, 206)	Stress	2.111	0.1237
							F (1, 206)	Diet	0.8304	0.3632
Nuclear alterations	0.2941 ± 0.0992	0.3429 ± 0.0999	0.2424 ± 0.1308	0.2222 ± 0.0807	0.1316 ± 0.0555	0.1389 ± 0.0707	F (2, 206)	Stress x diet	0.0714	0.9311
							F (2, 206)	Stress	2.068	0.1291
							F (1, 206)	Diet	0.0259	0.8721
Total nuclear alterations	0.677 ± 0.304	0.543 ± 0.150	0.273 ± 0.140	0.389 ± 0.134	0.211 ± 0.077	0.189 ± 0.094	F (2, 206)	Stress x diet	0.1523	0.8589
							F (2, 206)	Stress	3.102	**0.0471 ***
							F (1, 206)	Diet	0.3263	0.5685

Standard endoplasmic reticulum	7.088 ± 0.875	6.029 ± 0.885	3.303 ± 0.466	5.139 ± 0.930	6.158 ± 0.931	4.838 ± 0.745	F (2, 206)	Stress x diet	2.161	0.1178
							F (2, 206)	Stress	3.84	**0.0230** *
							F (1, 206)	Diet	0.0647	0.7994
Dilated endoplasmic reticulum	1.559 ± 0.401	0.486 ± 0.144	0.901 ± 0.315	0.389 ± 0.115	0.711 ± 0.346	0.541 ± 0.158	F (2, 206)	Stress x diet	1.483	0.2293
							F (2, 206)	Stress	1.325	0.2681
							F (1, 206)	Diet	7.02	**0.0087** **
Endoplasmic reticulum with content	0.353 ± 0.242	0.114 ± 0.055	0.091 ± 0.051	0	0	0.081 ± 0.060	F (2, 206)	Stress x diet	1.246	0.2897
							F (2, 206)	Stress	2.256	0.1073
							F (1, 206)	Diet	0.9559	0.3294
Total dystrophic endoplasmic reticulum	1.912 ± 0.558	0.6 ± 0.165	1 ± 0.323	0.389 ± 0.115	0.711 ± 0.346	1.135 ± 0.575	F (2, 206)	Stress x diet	1.991	0.1391
							F (2, 206)	Stress	2.202	0.1132
							F (1, 206)	Diet	6.766	**0.0100** **
Proportion dystrophic endoplasmic reticulum	15.72 ± 3.743	10.23 ± 3.369	16.88 ± 4.066	10.68 ± 4.066	7.983 ± 3.386	9.152 ± 3.034	F (2, 206)	Stress x diet	0.6525	0.5218
							F (2, 206)	Stress	1.1	0.3348
							F (1, 206)	Diet	1.264	0.2622
Autophagosomes	0.206 ± 0.102	0.143 ± 0.073	0.091 ± 0.067	0.083 ± 0.047	0.053 ± 0.037	0.054 ± 0.038	F (2, 206)	Stress x diet	0.156	0.8556
							F (2, 206)	Stress	1.93	0.1477
							F (1, 206)	Diet	0.1905	0.6629

Supplementary Figure 1: Effect of KD treatment on hippocampal lipidomic profiles in stress-resistant mice. A) Volcano plots illustrating differentially upregulated (red) and downregulated (blue) lipids in whole hippocampus of stress-resistant (RES) mice fed a ketogenic diet (KD), relative to a control diet (CD) (n = 4 mice per group). Positive and negative ion mode datasets were analyzed separately using two-sample t-tests, with a fold change of >1.5 and an FDR-adjusted p-value (q) ≤ 0.05 that were considered significant. Cer, ceramide; NAT, N-acyltaurine; PC, phosphatidylcholine; PE, phosphatdiylethanolamine; PS, phosphatidylserine; SM, sphingomyelin. B) Ontological analysis of differentially regulated lipids between KD and CD diets in RES mice. Differentially regulated lipids were annotated by matching to the human metabolome database (HMDB), followed by analysis in LION software in ranking mode. Lipid ontology terms with a fold change of >1.3 in either direction q ≤ 0.05 were considered significantly enriched (top) or underrepresented (bottom) in response to a ketogenic diet. No significantly enriched or underrepresented terms were identified in RES mice.

Supplementary Table 1: Hippocampal lipids differentially regulated by a ketogenic diet in non-stressed and stress-susceptible mice. Differentially regulated hippocampal lipids between non-stressed control (CTRL), stress-susceptible (SUS) and stress-resistant (RES) mice fed either a ketogenic (KD) or control diet (CD) prior to repeated social defeat stress exposure (n = 4 mice per treatment). Positive and negative-ion mode lipidomic datasets were analyzed separately using two-sample t-tests, with features considered significant with a fold change >1.5 in either direction and a false-discovery rate (FDR)-adjusted p-value ≤0.05. Features identified as significant were annotated by matching m/z ratios and retention times to the human metabolome database (HMDB). Displayed here are lipids successfully matched to an identifier. Cer, ceramide; CL, cardiolipin; HexCer, hexosylceramide; FA, fatty acid; GlcCer, glucosylceramide; NAT, N-acyltaurine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol-monophosphate; PG, phosphatidylglycerol; PS, phosphatidylserine; SHexCer, sulfated hexosylceramide; SM, sphingomyelin; SQDG, sulfoquinovosyl-diacylglycerol.

Supplementary Table 2: Lipid ontology term enrichment analyses. Ontological analyses of differentially regulated lipids between (CTRL), stress-susceptible (SUS) and stress-resistant (RES) mice fed a ketogenic (KD) compared to control diet (CD), and exposed to repeated social defeat stress (n = 4 mice per treatment). Lipids identified as differentially regulated by two-sample t-test analyses were annotated by matching to the human metabolome database (HMDB), and annotated lipids were analyzed using LION

software (v.2020.07.14) in ranking mode. All matched lipid ontology terms are displayed below. Terms with a fold change of >1.3 in either direction with a false-discovery rate (FDR)-adjusted p-value (q) of ≤0.05 were considered significantly enriched or underrepresented. No significantly enriched or underrepresented groups of lipids were identified in stress-resilient mice.

12. List of abbreviations

+: positive

AD: Alzheimer's disease

ANOVA: analysis of variance

BB: blocking buffer

BHB: β-hydroxybutyrate

BV: blood vessel

CA1: *cornu ammonis*

CD: control diet

CID: collision induced dissociation

CORT: corticosterone

CTRL: control

CZ: corner zone

DAB: 3,3' diaminobenzidine

DRLs: differentially-regulated lipids

ER: endoplasmic reticulum

ES: effect size

FC: fold change

FDS: false discovery range

FWHM: full width at half maximum

G-CSF: granulocyte colony stimulating factor

GlcCer: glucosylceramide

GM-CSF: granulocyte macrophage colony stimulating factor

HMDB: human metabolome data base

Iba1: ionized calcium-binding adapter molecule 1

IFN-ɣ: interferon gamma

IL: interleukin

iNOS: inducible nitric oxide synthase

IP-10: C-X-C motif chemokine ligand 10

IQR: interquartile range

IZ: interaction zone

KC: keratinocytes-derived chemokine

KD; ketogenic diet

LC/MS: Liquid Chromatography/Mass Spectrometry

LDL-R: low-density lipoprotein receptor

LIF: leukemia inhibitory factor

LIX: lipopolysaccharide-induced CXC chemokine

LPCs: lysophosphatidylcholines

LPEs: lysophosphatidylethanolamines

LPL: lipoprotein lipase

LPS: lipopolysaccharide

M-CSF: macrophage colony stimulating factor

m/z: mass to charge ratio

MCP-1: monocyte chemoattractant protein-1

MDD: major depressive disorder

MIG: monokine induced by interferon-gamma

MIP-1α: macrophage inflammatory protein 1-alpha

MIP-1β: macrophage inflammatory protein 1-beta

MIP-2: macrophage inflammatory protein 2

n: sample size

NF-kb: nuclear factor kappa b

NLPR3: nucleotide-binding and oligomerization domain-like receptors pyrin domain-containing protein 3

NND: nearest neighbor distance

PA: phosphatidic acid

PB: phosphate buffer

PBS: phosphate-buffered saline

PBST: PBS containing Triton X-100

PC: principal component

PE: phosphatidylethanolamine

PFA: paraformaldehyde

PGF1α: prostaglandin F1 alpha

PI: phosphatidylinositol

PIP: phosphatidylinositol-monophosphate

PSs: phosphatidylserines

QC: quality control

RANTES: C–C chemokine ligand 5

RES: resistant

ROS: reactive oxygen species

RSD: repeated social defeat

RSDs: relative standard deviations

RT: room temperature

SEM: scanning electron microscope

SI: social interaction

SM: sphingomyelin

SUS: susceptible

TB: Tris-buffer

TBS: Tris-buffered saline

TMEM119: transmembrane protein 119

TNF-α: tumor necrosis factor alpha

TREM2: triggering receptor expressed on myeloid cells 2

VGEF: vascular endothelial growth factor