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## ARTICLE

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**Intermittent and Limited Exposure to a High-Fat Diet Prevents Social Defeat-Induced Increase in Ethanol Intake and Neuroinflammation**M.Carmen Arenas<sup>a,d,†\*</sup>, Irene Pérez-Esteban<sup>a,†</sup>, Héctor Cañeque-Rufo<sup>b,c</sup>, Esther Gramage<sup>b,d,e</sup>, Gonzalo Herradón<sup>b,d,e</sup>, Marta Rodríguez-Arias<sup>a,d</sup>

Social stress is widely recognized for increasing ethanol consumption, an effect mediated by an enhanced neuroinflammatory response. High-fat diets (HFDs) alleviate stress and have been shown to reduce cocaine-seeking behavior, likely by serving as an alternative reinforcer. This study examines whether intermittent HFD administration following social defeat (SD) mitigates long-term increases in ethanol (EtOH) consumption and neuroinflammatory responses in adult male mice. Two intermittent HFD protocols were tested: HFD1x2 (one-hour access twice a week), and HFD2x3 (two-hour access three times a week), initiated immediately after the last SD episode and maintained through the drinking-in-the-dark (DID) protocol. Defeated mice were categorized as resilient or susceptible using the social interaction test (SIT). At the end of the procedure, gene expression analysis of neuroinflammatory markers was conducted in the hippocampus and striatum. Intermittent HFD did not increase body weight, despite promoting binge-eating behavior. As expected, only susceptible SD mice on a standard diet (STD) showed increased ethanol consumption, whereas those on either HFD protocol consumed ethanol at levels comparable to non-stressed controls. Elevated IL-6 and TNF $\alpha$  levels, along with mitochondrial dysfunction, were observed in the hippocampus and striatum of STD-fed defeated mice but were entirely absent in HFD-fed mice. These findings suggest that intermittent HFD access effectively prevents stress-induced ethanol consumption and neuroinflammatory alterations. Limited HFD access may represent a promising nutritional strategy to counteract stress-related alcohol use disorders.

**1. Introduction**

Stress is a major risk factor for the initiation, escalation, and maintenance of substance use disorders (SUD)<sup>1,2</sup>. Among humans, social stress—stemming from interpersonal relationships and the developmental environment—is particularly important<sup>3</sup>. The Social Defeat (SD) paradigm is a well-established animal model designed to explore the neurobiological mechanisms underlying social stress and to identify potential therapeutic targets<sup>4</sup>. Extensive preclinical research using this model has shown that social stress leads to long-term effects such as heightened anxiety, depression-like behaviours, and increased ethanol (EtOH) consumption<sup>5-10</sup>. Employing oral self-administration (SA) or drinking-in-the-dark (DID) protocols, studies have consistently demonstrated that SD results in both short- and long-term increases in EtOH intake and motivation for alcohol in adult and adolescent rodents<sup>11-14</sup>.

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Supplementary Information available: correlations between the gene expression levels of neuroinflammation markers



However, not all rodents exhibit the same response when exposed to SD. Resilience refers to the process of adaptation that allows individuals to confront and recover from complex or unpleasant life experiences, thereby developing more adaptive coping strategies<sup>15,16</sup>. Numerous reports from our laboratory and others have shown that defeated resilient mice do not develop anxiety- or depression-like behaviours, nor do they exhibit increased ethanol intake or cocaine reward<sup>8,17-20</sup>. In contrast, only adult mice with a susceptible phenotype to SD-induced depressive-like behaviours show increased ethanol intake<sup>8,10</sup>.

Among the several mechanisms that could underlie these stress effects, numerous studies point to the immune system. Social stress experiences amplify the neuroimmune response, activating neuroinflammatory events such as microglial activation and increased cytokine production<sup>21,22-27</sup>. Notably, susceptible mice exposed to social stress exhibit elevated levels of pro-inflammatory cytokines, such as *IL-6*, weeks after stress exposure in reward-related key brain areas, such as the striatum and prefrontal cortex<sup>8,17</sup>. In contrast, stress resilience is associated with reduced pro-inflammatory signalling, suggesting therapeutic targets to promote resilience<sup>28</sup>.

Importantly, stress also influences nutritional habits. Clinical research has revealed that the intake of palatable foods increases when individuals are exposed to stressful situations<sup>29-31</sup>. This increase is attributed to the alleviating effects of palatable food on psychological distress, serving as a form of comfort food<sup>32</sup>. For instance, in humans, the consumption of palatable food reduces plasma cortisol levels and perceived stress<sup>33</sup>. In preclinical studies, rodents exposed to chronic stress exhibit an increased preference for high-fat diets (HFD) compared to standard diets (STD)<sup>34</sup>. Along these lines, HFD reduces the response to acute stressors, such as hyperactivity of the hypothalamic-pituitary-adrenal axis<sup>31,35,36</sup>. Like drugs of abuse, hypercaloric foods high in fat and/or sugar induce a pleasurable response by elevating dopamine (DA) release in the nucleus accumbens (NAcc) and activating the ventral tegmental area, the prefrontal cortex, and the amygdala—all critical structures of the reward system<sup>37,38</sup>. Preclinical studies show that HFD increases the reinforcing effects of psychostimulants<sup>39-41</sup> and reduces cocaine-seeking behaviour during the extinction of memories associated with conditioned reinforcement<sup>42</sup>. Hence, exposure to this palatable diet during the extinction phase of conditioned place preference (CPP) could function as an alternative reinforce by reducing compulsive drug-seeking behaviour. Consumption of this diet triggers DA release in the NAcc, attenuates reinforcing properties, and alters the rewarding aspects of psychostimulants<sup>43</sup>. Continuous exposure to fats and sugars causes metabolic effects, such as significant weight gain and increased adipose tissue, along with elevated leptin levels. It may even induce withdrawal syndrome upon cessation of diet administration. However, these effects are not observed with intermittent HFD administration<sup>42-45</sup>. In a recent study, our laboratory demonstrated that limited and intermittent exposure to HFD was capable of blocking reinstatement processes of cocaine-induced CPP<sup>46</sup>. However, no studies have

evaluated the effect of HFD on SD-induced increases in ethanol intake.

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The aim of the present study was to evaluate whether limited and intermittent administration of a HFD after exposure to SD could potentiate a resilient response in adult male mice. Defeated mice were categorized into resilient or susceptible phenotypes using the social interaction test (SIT), and two types of restricted access to HFD were employed (1-hour access per day, 2 days a week; and 2-hour access, 3 days a week) before evaluating the animals' EtOH consumption using the DID protocol. At the end of the experimental procedure, the expression of genes related to the neuroinflammatory response, mitochondrial biogenesis, dynamics and the mitochondrial function, triggered by social stress and ethanol intake, was also analysed.

## 2. Materials and methods

### 2.1. Animals

A total of 89 OF1 adult male mice, obtained from Charles Rivers (France) were used in this study. Mice were accommodated in plastic cages (28 x 28 x 14.5 cm) in groups of 3-4 during the experimental procedures. Non-experimental adult males (N=20) were selected as aggressive opponents and individually housed in plastic cages (21 x 32 x 20 cm) for a minimum of one month before starting the experiments to enhance their aggressive behaviour. All mice were maintained under controlled laboratory conditions, which included a consistent temperature and humidity level, as well as a reversed light schedule (white lights on from 19:30 to 7:30). Food and water were made available ad libitum to all mice, except during the behavioural tests. All experimental procedures adhered to the regulations outlined in the European Council Directive 2010/63/UE governing animal research and received approval from the Local Ethics Committees of the University of Valencia for the use of animal subjects (Comité d'Ètica d'Experimentació i Benestar Animal, reference number 2022 VSC PEA 0008).

### 2.2. Feeding conditions

The study involved three different feeding conditions. The control group received a Standard Diet (STD), consisting of 13% of its caloric content from fat, 67% from carbohydrates, and 20% from protein, with an energy density of 2.9 Kcal per gram. Alternatively, the High-Fat Diet (HFD) (TD.06415), with 45% of its caloric content from fat, 36% from carbohydrates, and 19% from protein, and an energy density of 4.6 Kcal per gram, was provided in a restricted manner to the HFD binge groups. This limited-access approach was based on the model developed by Corwin et al.<sup>47</sup>, in which non-food-deprived animals with sporadic and limited access to an HFD exhibit binge-type behaviour. Both diets were provided by Harlan Laboratories Models, SL (Barcelona, Spain). The individual components of the HFD (TD.06415) included casein (245 g/kg), L-cystine (3.5 g/kg), corn starch (85 g/kg), maltodextrin (115 g/kg), sucrose (200 g/kg), lard (195 g/kg), soybean oil (30 g/kg), cellulose (58 g/kg), mineral mix (43 g/kg), calcium phosphate dibasic (3.4 g/kg), vitamin mix (19 g/kg),



choline bitartrate (3 g/kg), and red food colour (0.1 g/kg). The fatty acid profile (% of total fat) consisted of 36% saturated, 47% monounsaturated, and 17% polyunsaturated fats.

Upon arrival at the laboratory, the mice were randomly divided into groups with a similar average body weight (30–40 g) and assigned to either the STD group or one of the intermittent HFD groups. The HFD groups had either 1-hour access on Tuesdays and Thursdays (HFD1x2) or 2-hour access on Mondays, Wednesdays, and Fridays (HFD2x3), beginning 3–4 hours after the start of the dark phase. All groups were provided ad libitum access to the STD (except during the HFD administration) and water in their cages. Behavioural tests were conducted before the binge sessions to maintain consistent daily conditions throughout the experiment and avoid interference with behavioural outcomes.

### 2.3. Experimental Design

All mice arrived at the laboratory on PND 42 and were acclimated to the vivarium for five days before the experiments began. They were randomly divided into six groups based on stress (exploration-EXP; stressed-SD) and feeding conditions: EXP-STD (n=13), SD-STD (n=14), EXP-HFD1x2 (n=15), SD-HFD1x2 (n=17), EXP-HFD2x3 (n=15), and SD-HFD2x3 (n=16).

First, mice were subjected to stress using the SD paradigm, which consisted of four episodes spaced 72 hours apart (PND47 to PND56). Following the final SD encounter (PND 56), intermittent sessions of HFD intake were initiated for a total duration of five weeks (HFD1x2 or HFD2x3). On PND 57, animals performed the social interaction test (SIT) to evaluate depressive-like behaviours and were categorized as

resilient or susceptible based on their SIT ratio, resulting in a total of nine experimental groups (see Fig. 1). DOI: 10.1039/D5FO00584A

Three weeks after the final SD episode (PND75), the animals underwent the drinking in the dark (DID) test, which lasted for 8 days. Two weeks after completing the DID test, the mice were sacrificed, and brain samples were collected (PND 85). For the DID protocol, ethanol (Merck, Madrid, Spain) was diluted in water to create a 20% (v/v) ethanol solution.

A more detailed description of the experimental design is presented in Fig. 1.

### 2.4. Apparatus and procedures

**2.4.1. Body weight, feeding and binge-eating measures.** Throughout the study, animals were weighed weekly, and the percentage of weight gain each week following the initiation of HFD consumption was assessed. Four measurements were obtained: Week 1—the percentage of weight gain after the first session of HFD consumption (PND 60) relative to the last recorded weight prior to the diet initiation (PND 53); Week 2—the percentage of weight gain at the start of the second week of HFD consumption (PND 67) relative to the weight recorded in the previous week (PND 60); Week 3—the percentage of weight gain prior to the DID test (PND 74) compared to the weight recorded in the preceding week (PND 67); and Week 4—the percentage of weight gain during the DID test (PND 81) compared to the weight recorded in the prior week (PND 74). Weekly standard diet consumption in the home cage was also monitored. During each HFD session, the high-fat food pellet was

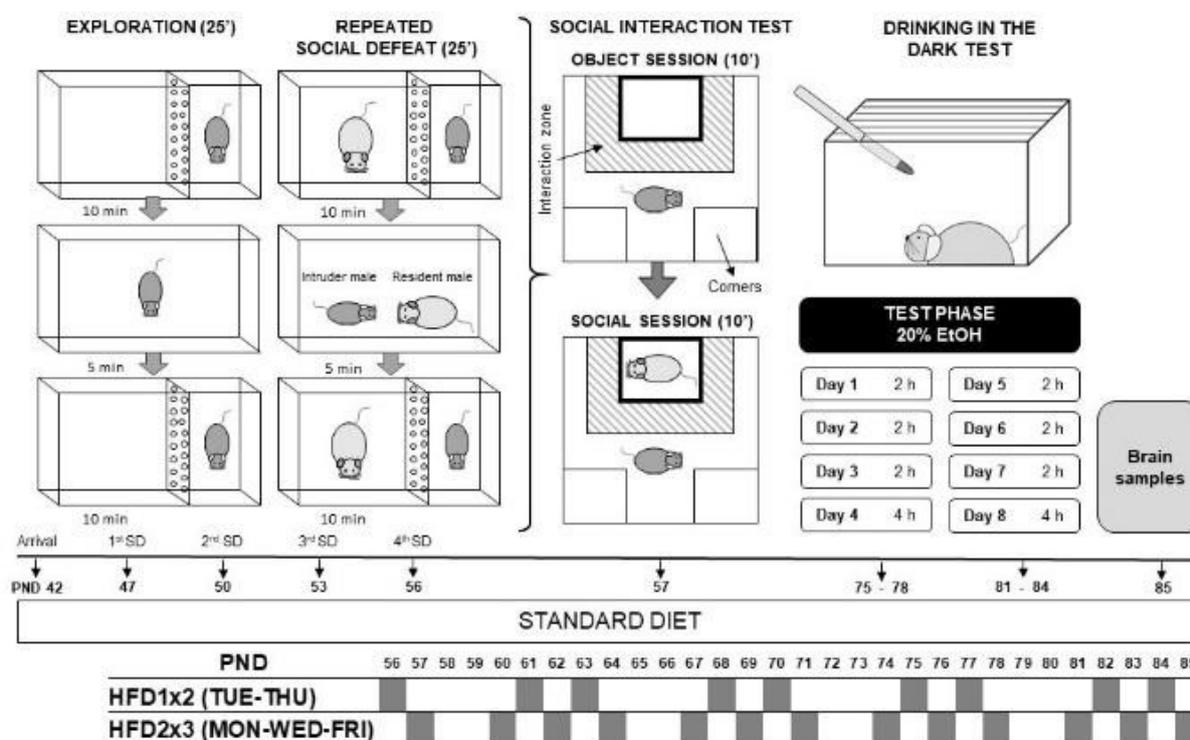


Fig. 1. Experimental design. The animals were divided into the following 9 groups: EXP-STD, R-STD, S-STD, EXP-HFD1x2, R-HFD1x2, S-HFD1x2, EXP-HFD2x3, R-HFD2x3, and S-HFD2x3



weighed before and after the exposure period to calculate the total kilocalories consumed.

**2.4.2. Procedure of repeated Social Defeat (SD).** Stressed/defeated mice were exposed to four 25-minute episodes of SD, each with three phases. The first stage commenced with the introduction of the "intruder" (the experimental mice) into the home cage of the "resident" (the aggressive opponent) for a duration of 10 minutes<sup>48</sup>. In this initial phase, the intruder was protected from direct attacks, while the wire mesh walls of the cage permitted social interactions and species-typical threats from the male aggressive resident. This setup facilitated instigation and provocation<sup>49</sup>. During the second phase, the wire mesh was taken off the cage to enable a 5-minute confrontation between the two mice. Then, the wire mesh was reinstated in the cage, separating the two mice for an additional 10 minutes to permit social threats by the resident. The exploration groups not subjected to stress followed the identical protocol, although without a "resident" mouse present in the cage. During the SD episode, the intruder mice encountered each time a distinct aggressor mouse. The criterion for identifying a mouse as defeated involved the adoption of a specific posture indicating defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears<sup>50</sup>.

**2.4.3. Social Interaction Test (SIT).** The social withdrawal ratio utilized in this study was derived from the social approach-avoidance test outlined by Berton et al.<sup>51</sup>. Conducted during the dark cycle, 24 hours after the last social defeat, the test was conducted in a different environment than the confrontation sessions. Mice were acclimated to a quiet, dimly lit room an hour before the test. After habituation, mice were placed in the centre of an arena with four different squares (black Plexiglas open field, 30 cm per side, and 35 cm high), and each mouse's behaviour was recorded via video (EthoVision XT 11, 50 fps; camera placed above the arena). Mice explored the arena twice, spending 600 seconds in each session. In the object session, an empty perforated Plexiglas cage (10 × 6.5 × 35 cm) was positioned on one wall of the arena. During the second session (social session), an unfamiliar male mouse of the OF1 strain was introduced into the cage as a social stimulus. While one could argue that the probe mouse used in the social interaction test resembles the aggressor and might induce social aversion, this is unlikely. Previous experiments have shown consistent levels of social interaction, irrespective of the strain used<sup>51</sup>. Before each session, the arena was cleaned with a 5% alcohol solution to minimize odour cues. Between sessions, the experimental mouse was temporarily removed from the arena and returned to its cage for 2 minutes.

The analysis of arena occupancy during object and social sessions involved monitoring the horizontal position of the mice, facilitated by commercial video tracking software (EthoVision XT 11, Noldus). Traditional metrics of arena occupancy, including the duration spent in the interaction zone and corners, were assessed. The initial metric is frequently employed as a score for social preference-avoidance, determined by assessing the duration spent in a 6.5 cm wide corridor encircling the cage. Corners were defined as two squares of comparable areas located on the opposite wall of the arena. The social interaction ratio was calculated by examining the time an

experimental mouse spends in the interaction zone when a social target is present, divided by the time spent in the interaction zone when the target is not present. A ratio of 1 indicates an equal amount of time spent in the presence and absence of a social target. Following the typical behaviour of control OF1 mice, those with a ratio below 1 are categorized as susceptible, while those with a ratio equal to or greater than 1 are classified as resilient<sup>52</sup>.

**2.4.4. Drinking in the Dark (DID).** Following the basic paradigm of Rhodes et al.<sup>53</sup>, the experiment is composed of two distinct phases. The initial phase involves habituation, during which mice are taken from their cages and individually housed for two hours per day over one week. This period aims to acclimate them to both the cages and the sipper tubes (containing a ball bearing at the end to prevent leakage and consistently utilized throughout the test) filled with water.

The second phase of the protocol commences three hours after lights out. During this stage, the mice are placed in individual cages and exposed to 10 ml graduated tubes containing a 20% (v/v) ethanol solution. This procedure lasts for two hours. After this period, the mice are returned to their grouped cages, with access to food and water bottles ad libitum. This process is replicated on days 2 and 3, and on day 4, the procedure extends for 4 hours, simulating a binge-drinking situation. Additionally, immediately after each day, the volume of ethanol consumed is recorded. In our case, we adhered to the protocol for one more week, replicating the same process previously explained. A fresh ethanol solution was prepared daily.

**2.4.5. Tissue sampling and biochemical analyses.** Mice were euthanized via cervical dislocation and brains were extracted. The striatum and hippocampus were precisely dissected following the Paxinos and Franklin atlas of a coronal brain matrix<sup>54</sup>. These tissue samples were stored at -80°C until RNA extraction.

**2.4.6. Quantitative Real-Time PCR.** Total RNA from the striatum and hippocampus was isolated using the Total RNA Isolation Kit (Nzytech, Lisbon, Portugal). First-strand cDNA was synthesized using the first-strand cDNA Synthesis Kit (Nzytech), and 2 µg (in the case of hippocampus samples) or 0,8 µg (in the case of striatum samples) of RNA were reverse-transcribed to DNA. Quantitative real-time PCR (qPCR) analysis was performed using the SYBR green method (Quantimix Easy kit, Biotools, Madrid, Spain) in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The relative expression of each gene was normalized using Rpl13, Rpl37, Rn18s, and Hprt1 as housekeeping genes, and the data were analysed by the Livak method. The primer sequences used, the experimental conditions and additional details are shown in Table 1.

## 2.5. Statistics

Body weight, the total mean caloric intake during HFD sessions and the data from the DID test, were analysed using a repeated-measures ANOVA with one within-subject variable (Weeks, Period of HFD intake and Days, respectively) and two between-subject variables (Stress, with 3 levels –EXP, Resilient and Susceptible–, and Diet, with 3 levels – SD, HFD1x2, and HFD2x3). The data of the SIT and



Gene	Primer Forward/Reverse	T <sub>a</sub> (°C)	Product length (bp)	%CG	Self complementarity y	Self 3' complementarity y
<i>Ccl2</i>	5'-GGCTCAGCCAGATGCAGTTAA-3'	61	76	52.38	7.00	4.00
	5'-CCTACTCATTGGGATCATCTTGCT-3'			45.83	5.00	1.00
<i>Hmgb1</i>	5'-CGGAGAACTTCAGACCGGA-3'	60	178	55.00	4.00	2.00
	5'-CCCATGTTTAGTTGATTTCCAGC-3'			41.67	4.00	2.00
<i>Hprt1</i>	5'-TGCTCGAGATGCATGAAGG-3'	53	196	50.00	6.00	0.00
	5'-TATGTCCCCCGTTGACTGAT-3'			50.00	3.00	2.00
<i>Il6</i>	5'-TAGTCCTTCTACCCCAATTTCC-3'	60	76	47.83	4.00	0.00
	5'-TTGGTCCTTAGCCACTCCTTC-3'			52.38	3.00	0.00
<i>Mfn1</i>	5'-CACAGTCGAGCAAAAGTAGTGG-3'	61	71	50.00	4.00	0.00
	5'-GTGAGCTTACCAGTGCAAA-3'			50.00	6.00	2.00
<i>Mfn2</i>	5'-CATTCTTGTGGTCGGAGGAG-3'	61	63	55.00	3.00	0.00
	5'-AAGGAGAGGGCGATGAGTCT-3'			55.00	3.00	3.00
<i>Rn18s</i>	5'-CTCAACACGGGAAACCTCAC-3'	61	110	55.00	2.00	0.00
	5'-CGCTCCACCAACTAAGAACG-3'			55.00	3.00	2.00
<i>Rpl13</i>	5'-GGTGCCCTACAGTTAGATACCAC-3'	61	137	52.17	3.00	0.00
	5'-TTTGTTCGCTCCTTGGGTC-3'			52.38	2.00	1.00
<i>Rpl37</i>	5'-ACCGCAGATTCAGACATGGATT-3'	61	197	45.45	4.00	1.00
	5'-AGCGTAGGATCCCAGAGCAA-3'			55.00	6.00	0.00
<i>Tnfα</i>	5'-AGGCACTCCCCAAAAGATG-3'	65	192	55.00	3.00	0.00
	5'-TGAGGGTCTGGCCATAGAA-3'			55.00	5.00	2.00
<i>Ucp2</i>	5'-ACAGCCTTCTGCACTCTG-3'	60	76	57.89	4.00	1.00
	5'-GGCTGGGAGACGAAACT-3'			57.89	2.00	1.00

Table 1. Primer sets used for qPCR analysis. *Ccl2*: Chemokine (C–C motif) ligand 2; *Hmgb1*: High mobility group box 1; *Hprt1*: Hypoxanthine phosphoribosyltransferase 1; *Il6*: Interleukin 6; *Mfn1*: Mitofusin 1; *Mfn2*: Mitofusin 2; *Rn18s*: 18S ribosomal RNA; *Rpl13*: Ribosomal protein L13; *Rpl37*: Ribosomal protein L37; *Tnfα*: Tumor necrosis factor alpha; *Ucp2*: Uncoupling protein 2 (mitochondrial, proton carrier).

neuroinflammation markers were analysed using a two-way ANOVA, with two between-subject variables: Stress (with 3 levels –EXP, Resilient and Susceptible) and Diet (with 3 levels –SD, HFD1x2, and HFD2x3). Post-hoc comparisons were performed with a Bonferroni test. A significance level of  $p < 0.05$  was adopted throughout the study. The data were statistically analysed using the IBM SPSS Statistics v28 program, and graphical representations were created using the Prism program (Graphpad).

### 3. Results

#### 3.1. Social interaction test (SIT)

The percentage of defeated mice which showed resilient phenotype (SIT ratio equal to or  $> 1$ ) was 57% and 50% of the defeated groups fed with STD or HFD2x3 respectively (Fig. 2a). However, 70% of the defeated mice fed with HFD1x2 were classified as resilient. Importantly, mice in the HFD1x2 group had already been exposed to an HFD session immediately after finishing the last episode of SD

when they performed the SIT, which was not the case for the HFD2x3 group. Results of ANOVA for the SIT ratio (Fig. 2b) showed an effect of the variable Stress [ $F(2,89) = 21.903$ ;  $p < 0.001$ ]. Defeated mice with a susceptible phenotype presented an SIT ratio lower than those categorized as resilient and non-stressed ( $p < 0.001$ ).

#### 3.2. Body weight and mean of total of Kcal intake in HFD sessions.

Results obtained in the ANOVA for the percentage of body weight gain revealed an effect of the variable Week [ $F(3,240) = 9.048$ ;  $p < 0.001$ ], Diet [ $F(2,80) = 3.271$ ;  $p < 0.043$ ], Stress [ $F(2,80) = 7.303$ ;  $p < 0.001$ ], and the interactions Week  $\times$  Diet [ $F(6,240) = 6.106$ ;  $p < 0.001$ ] and Diet  $\times$  Stress [ $F(4,80) = 2.709$ ;  $p < 0.036$ ]. Mice fed on the STD and HFD1x2 decreased their weight gain on the fourth week with respect to the second ( $p < 0.001$  in the case of STD) and the third week ( $p < 0.01$  and  $p < 0.001$  for STD and HFD 1x2, respectively). In the case of mice fed on HFD2x3, the percentage of weight gain significantly decrease in the third and fourth week with respect to the second ( $p < 0.001$ ) and the first week ( $p < 0.001$  only for the third week) (see Figures 3a and 3b).



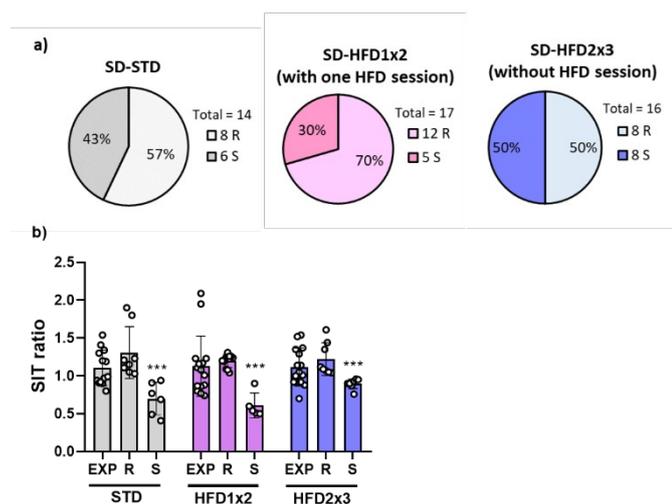


Fig. 2. SIT Ratio. (a) Percentages of resilient vs. susceptible mice. Among the defeated mice, those with a SIT ratio below 1 are categorized as susceptible, while those with a ratio equal to or greater than 1 are classified as resilient. The pie chart illustrates the proportions of resilient and susceptible mice among the defeated groups across the three experimental conditions. Percentages are shown for the following groups based on the SIT ratio: SD-STD group, SD-HFD1x2 (after one HFD session), and SD-HFD2x3 (prior to any HFD sessions). (b) SIT Ratio. Data represent the effects of SD and diet on the SIT ratio in male mice, expressed as mean  $\pm$  SEM. \*\*\* $p$ <0.001 compared to the corresponding EXP and R groups.

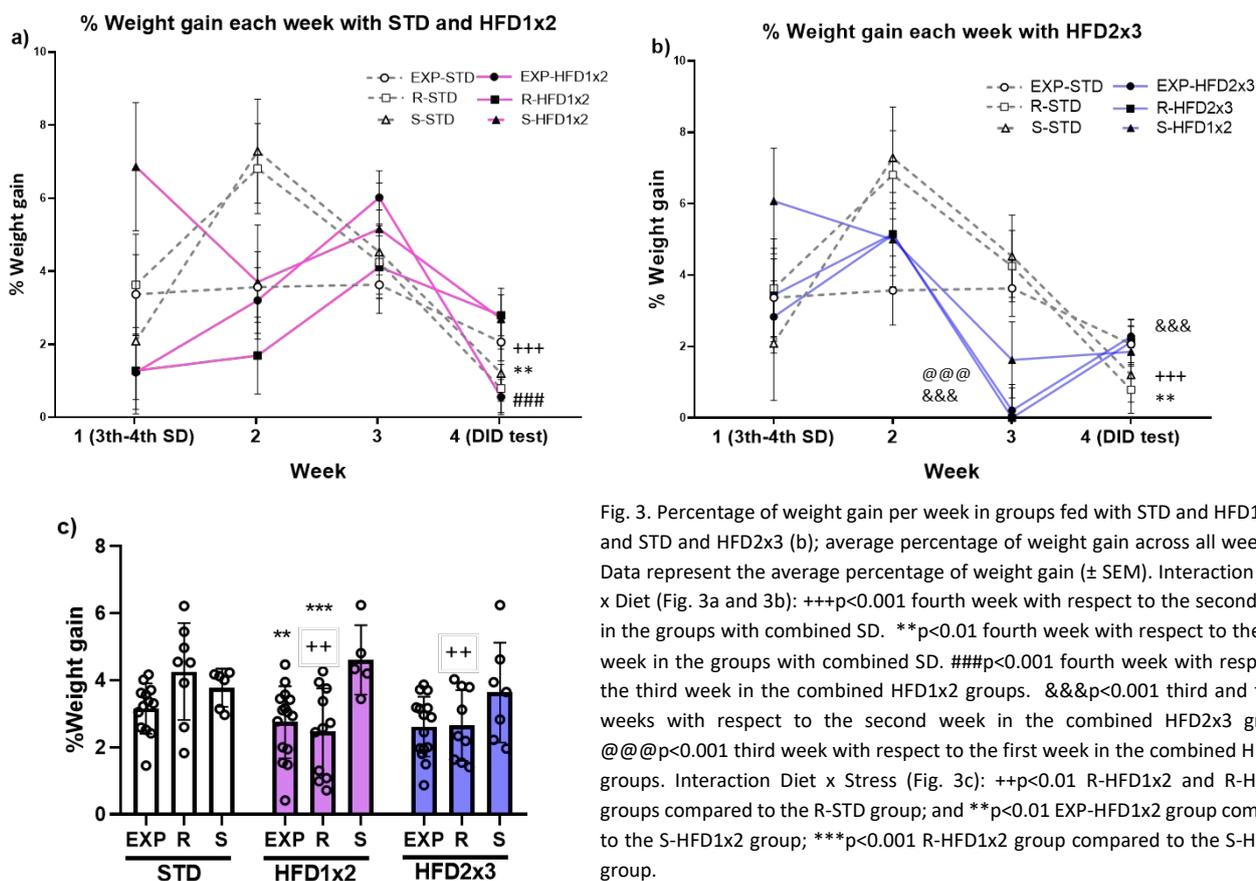


Fig. 3. Percentage of weight gain per week in groups fed with STD and HFD1x2 (a) and STD and HFD2x3 (b); average percentage of weight gain across all weeks (c). Data represent the average percentage of weight gain ( $\pm$  SEM). Interaction Week  $\times$  Diet (Fig. 3a and 3b): @@@ $p$ <0.001 fourth week with respect to the second week in the groups with combined SD. \*\* $p$ <0.01 fourth week with respect to the third week in the groups with combined SD. ### $p$ <0.001 fourth week with respect to the third week in the combined HFD1x2 groups. &&& $p$ <0.001 third and fourth weeks with respect to the second week in the combined HFD2x3 groups. @@@ $p$ <0.001 third week with respect to the first week in the combined HFD2x3 groups. Interaction Diet  $\times$  Stress (Fig. 3c): ++ $p$ <0.01 R-HFD1x2 and R-HFD2x3 groups compared to the R-STD group; and \*\* $p$ <0.01 EXP-HFD1x2 group compared to the S-HFD1x2 group; \*\*\* $p$ <0.001 R-HFD1x2 group compared to the S-HFD1x2 group.

Mice on the STD showed higher weight gain than mice on the HFD1x2 on week 2 ( $p$ <0.01). On week 3, mice fed on HFD2x3 presented lower weight gain than mice in the STD and HFD1x2 groups ( $p$ <0.001). No differences in weight gain were observed during fourth week depending on the diet.

On the other hand, resilient mice fed with the STD displayed greater weight gain than those fed with any of the HFD schedules ( $p$ <0.01). Among those mice fed with HFD 1x2, susceptible mice showed greater weight gain than EXP ( $p$ <0.01) and resilient ( $p$ <0.001) mice (see Figure 3c).

The ANOVA of the HFD Kcal intake (Fig. 4) showed an effect of the variable DID [ $F(1,55)=94.286$ ;  $p$ <0.001], Diet [ $F(1,55)=21.078$ ;  $p$ <0.001] and an effect of the interaction DID  $\times$  Diet [ $F(1,55)=26.967$ ;  $p$ <0.001]. Mice exposed to HFD2x3 ingested more calories than those exposed to HFD1x2 during the pre-DID and DID periods (PND 60-67 and 74-81, respectively) ( $p$ <0.01 and  $p$ <0.001, respectively). Additionally, both HFD groups ingested more calories during the DID period than in the pre-DID period ( $p$ <0.01 and  $p$ <0.001 respectively). In addition, to detect the development of an HFD binge pattern, an ANOVA of Kcal intake during each administration prior to the DID procedure (5 administrations for HFD1x2 and 7 for HFD2x3) was conducted. For the HFD1x2 schedule (Fig. 5a), the ANOVA revealed a significant effect of the variable Day [ $F(4,112)=7.910$ ;  $p$ <0.001]. On the first day of HFD1x2 administration (PND 56), mice consumed fewer Kcal compared to subsequent HFD administrations ( $p$ <0.01 for days 61, 63, and 70;  $p$ <0.001 for day 68). Therefore, there was a significant increase in Kcal intake between the first and subsequent



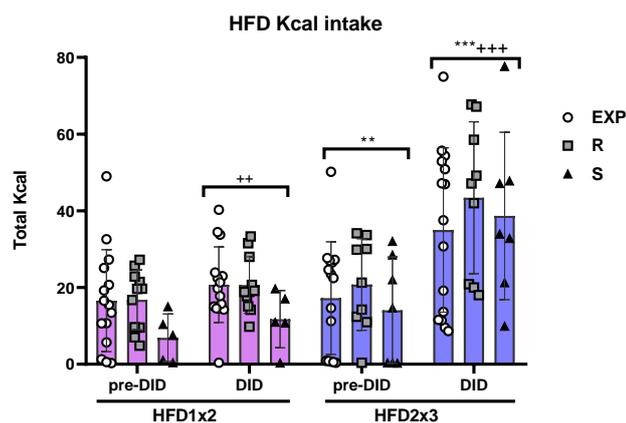


Fig. 4. HFD Kcal intake. Data represent the HFD Kcal intake ( $\pm$  SEM) per periods (pre-DID and DID). \*\* $p<0.01$  HFD2x3 groups compared to HFD1x2 groups during the pre-DID period; \*\*\* $p<0.001$  HFD2x3 groups compared to HFD1x2 groups during DID period; ++  $p<0.01$  HFD1x2 groups during the DID period compared to the pre-DID period; +++  $p<0.001$  HFD2x3 groups during the DID period compared to pre-DID.

administrations, indicating the development of a binge pattern after the first HFD administration.

For the HFD2x3 schedule (Fig. 5b), the ANOVA also revealed a significant effect of the variable Day [ $F(6,168)=6.559$ ;  $p<0.001$ ]. The Kcal intake on PND 69 was significantly higher than on days 57, 60, 62, 64, and 67 ( $p<0.01$  for all days, except  $p<0.05$  for day 60). Thus, in the case of HFD2x3, the development of a binge pattern was delayed until the 6th HFD administration.

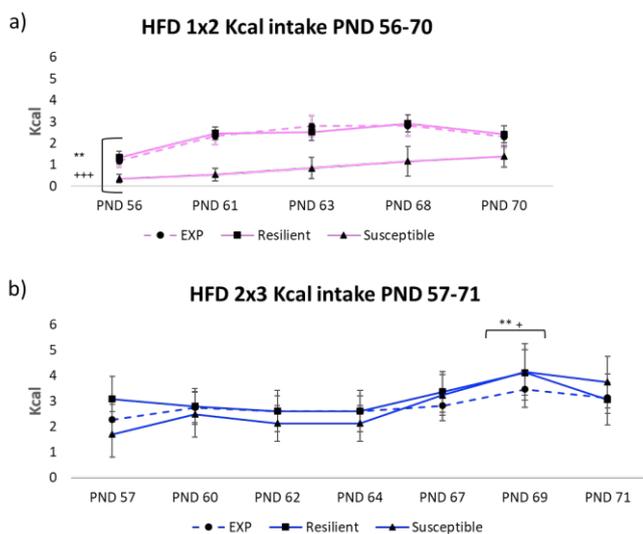


Fig. 5. Development of a binge pattern after HFD administration. Data represent the HFD Kcal intake ( $\pm$  SEM) per HFD administration before the beginning of the DID. (a) \*\* $p<0.01$  with respect days 61, 63 and 70 +++  $p<0.001$  with respect day 68; (b) \*\*  $p<0.01$  with respect days 57, 60, 62, 64 and 67, +  $p<0.05$  with respect day 60.

### 3.3. Drinking in the dark (DID)

The ANOVA for the gr/kg of ethanol intake during the DID session revealed an effect of the variable Day [ $F(7,504)=12.217$ ;  $p<0.001$ ], Diet [ $F(2,72)=17.491$ ;  $p<0.001$ ] and the interaction Stress  $\times$  Diet

[ $F(4,72)=4.973$ ;  $p<0.001$ ]. As expected, all mice increased their ethanol consumption on the 4th and 8th days (binge drinking days). On the 4th day, animals ingested more ethanol compared to the 2nd ( $p<0.001$ ) and 3rd days ( $p<0.001$ ). On the 8th day, mice consumed more ethanol compared to the 6th ( $p<0.001$ ) and 7th day ( $p<0.05$ ). Furthermore, mice exhibited elevated ethanol consumption on the 5th day (after having been abstinent from ethanol over the weekend) compared to the 2nd ( $p<0.01$ ) and 3rd days ( $p<0.001$ ).

Susceptible mice fed with the ST diet (S-STD) presented higher total ethanol consumption than their non-stressed counterparts (EXP-STD,  $p<0.01$ ) and susceptible mice exposed to HFD (S-HFD1x2 and S-HFD2x3,  $p<0.001$  in all cases). In addition, R-HFD1x2 mice consumed less ethanol than R-STD ( $p<0.05$ ). All results from the DID sessions are displayed in Figures 6 a, b and c.

Interestingly, a negative correlation between the percentage of weight gain and the ethanol intake during the DID sessions was observed only among the groups fed on HFD ( $r = -0.340$ ,  $p<0.012$ ). In these groups, the Kcal intake of HFD before DID positively correlated with ethanol intake during DID ( $r = 0.315$ ,  $p<0.023$ ) (see Figure 7).

### 3.4 Social defeat and a high-fat diet alter the gene expression levels of neuroinflammation markers

For the of Il-6 gene expression in the hippocampus, the ANOVA revealed a significant effect of the variable Stress [ $F(2,35)=4.237$ ,  $p<0.023$ ], Diet [ $F(2,35)=10.296$ ,  $p<0.001$ ], and the interaction Stress  $\times$  Diet [ $F(4,35)=4.230$ ,  $p<0.007$ ] (Fig. 8a). Both resilient and susceptible defeated mice fed on the Standard Diet (R-STD and S-STD) showed higher IL-6 levels in the hippocampus than their corresponding exploration group (EXP-STD) ( $p<0.01$  for susceptible and  $p<0.001$  for resilient group). Standard Diet resilient mice showed higher levels of hippocampal IL-6 than resilient mice fed on HFD1x3 or HFD2x3 ( $p<0.001$  in both cases). Although the expression levels of IL-6 in the striatum were lower in the groups treated with any of the HFD administrations [ $F(2,28)=3.607$ ,  $p<0.04$ ], these differences did not reach statistical significance.

For the  $Tnf\alpha$  gene expression in the hippocampus, the ANOVA revealed a significant effect of the variable Stress [ $F(2,35)=6.903$ ,  $p<0.003$ ], Diet [ $F(2,35)=4.483$ ,  $p<0.018$ ], and the interaction Stress  $\times$  Diet [ $F(2,35)=2.875$ ,  $p<0.037$ ] (Fig. 8b). Resilient defeated mice fed on the Standard Diet showed higher  $Tnf\alpha$  gene expression in the hippocampus than their corresponding exploration group (EXP-STD) ( $p<0.001$ ). Standard diet resilient mice showed higher expression levels of hippocampal  $Tnf\alpha$  than resilient mice fed on HFD1x3 or HFD2x3 ( $p<0.01$  in both cases). In the striatum, the ANOVA of the gene expression levels of  $Tnf\alpha$  revealed a significant effect of the variable Stress [ $F(2,28)=12.911$ ,  $p<0.001$ ], Diet [ $F(2,28)=35.482$ ,  $p<0.001$ ], and the interaction Stress  $\times$  Diet [ $F(4,28)=14.371$ ,  $p<0.001$ ]. Resilient and susceptible mice fed on standard diet (R-STD and S-STD) showed higher gene expression levels of  $Tnf\alpha$  than their non-stressed groups (EXP-STD) ( $p<0.001$  in both cases). These groups, R-STD and S-STD, also presented higher gene expression levels of  $Tnf\alpha$  than resilient or susceptible mice fed on HFD1x2 or HFD2x3 ( $p<0.001$  in all cases).



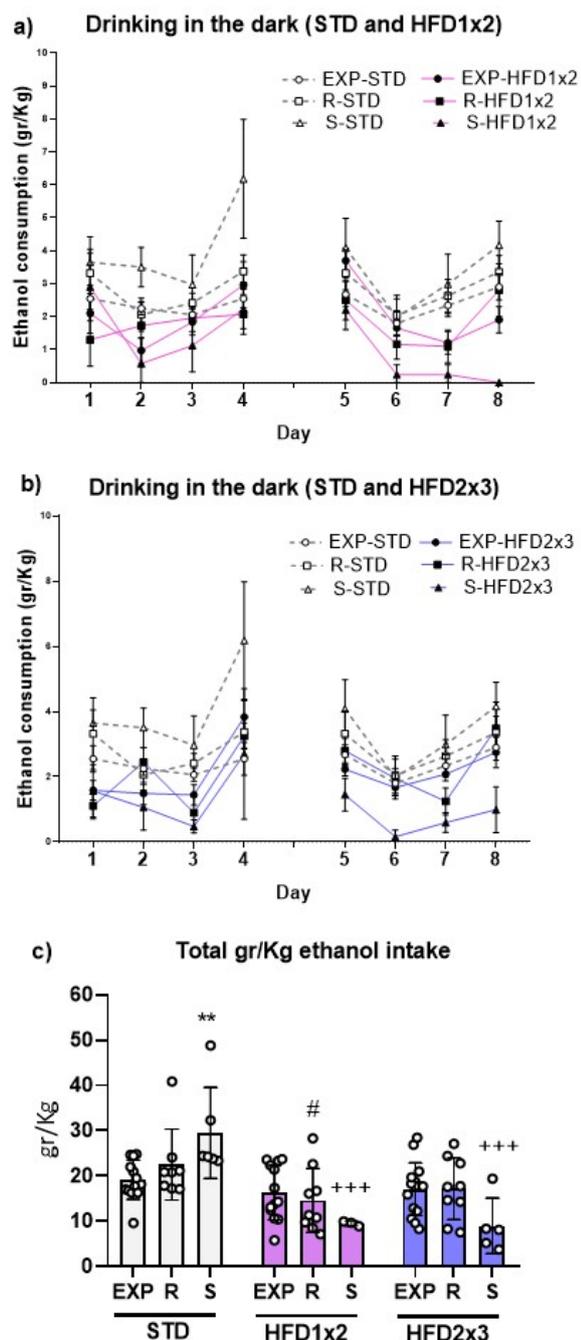


Fig. 6. Total ethanol consumption (gr/Kg) in DID. Effect of SD and HFD intake in male mice on ethanol consumption during DID. The dots represent means and the vertical lines  $\pm$  SEM of the gr/kg of 20% ethanol consumption. (a) STD- and HFD1x2-fed groups. (b) STD- and HFD2x3-fed group. (c) Total gr/Kg ethanol consumption during the eight sessions of the DID. \*\*  $p < 0.01$  with respect to EXP-STD group and +++  $p < 0.001$  with respect to S-STD; #  $p < 0.05$  with respect to the R-STD group.

The analysis of *Ccl2* gene expression levels in the hippocampus did not reveal significant differences (Fig. 8c). For the striatum, the ANOVA revealed a significant effect of the Diet variable [ $F(2,28)=4.287$ ,  $p < 0.024$ ], with animals in the HFD2x3 group

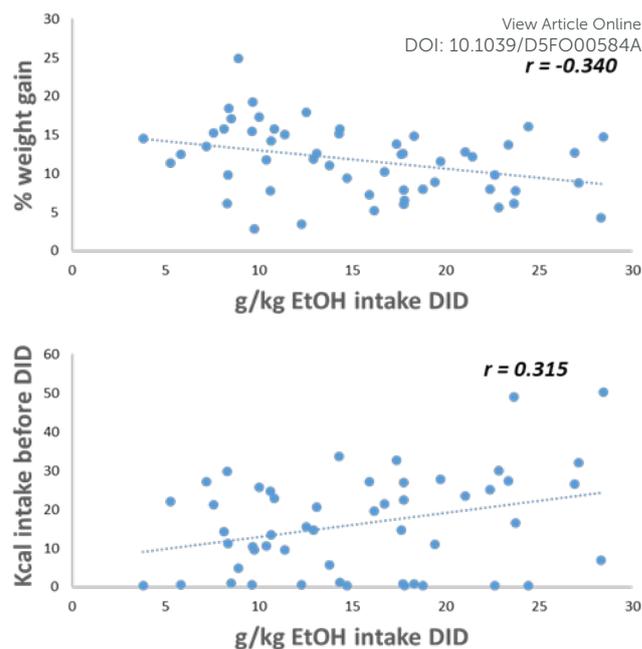


Figure 7. Pearson's coefficient demonstrated that in mice fed with HFD, the g/kg of ethanol intake during the DID test negatively correlates with the percentage of weight gain but positively correlates with the Kcal intake before the test.

expressing higher levels of this gene expression compared to the HFD1x2 group ( $p < 0.05$ ).

The ANOVA of the *Hmgb1* gene expression levels in the hippocampus (Fig. 8d) indicated a significant effect of the variable Diet [ $F(2,35)=5.890$ ,  $p < 0.006$ ], with animals on the HFD2x3 diet displaying higher expression levels of *Hmgb1* compared to those fed with STD or HFD1x2 ( $p < 0.01$  in both cases). In the striatum, the ANOVA revealed a significant effect of Diet [ $F(2,28)=10.537$ ,  $p < 0.001$ ], and a significant interaction Stress  $\times$  Diet [ $F(4,28)=4.424$ ,  $p < 0.007$ ]. The R-STD and S-STD groups exhibited higher expression of *Hmgb1*



		SIT		DID		Body weight	
		Hippocampus	Striatum	Hippocampus	Striatum	Hippocampus	Striatum
<b><i>Il6</i></b>	<i>r</i>	-0,202	0,233	<b>0,319</b>	0,114	<b>0,396</b>	0,129
	p value	0,168	0,129	<b>0,027*</b>	0,46	<b>0,005*</b>	0,405
<b><i>Tnfa</i></b>	<i>r</i>	<b>-0,286</b>	-0,261	<b>0,389</b>	<b>0,595</b>	0,125	0,247
	p value	<b>0,049*</b>	0,079	<b>0,006*</b>	<b>0,001*</b>	0,396	0,099
<b><i>Ccl2</i></b>	<i>r</i>	-0,207	0,013	0,011	-0,078	<b>0,286</b>	0,017
	p value	0,163	0,934	0,939	0,627	<b>0,05*</b>	0,917
<b><i>Hmgb1</i></b>	<i>r</i>	-0,159	0,007	0,23	-0,197	0,05	-0,038
	p value	0,275	0,966	0,112	0,201	0,733	0,805
<b><i>Mfn1</i></b>	<i>r</i>	-0,12	-0,046	-0,125	<b>0,487</b>	<b>-0,347</b>	<b>0,295</b>
	p value	0,412	0,759	0,393	<b>0,001*</b>	<b>0,015*</b>	<b>0,047*</b>
<b><i>Mfn2</i></b>	<i>r</i>	-0,136	-0,101	0,189	0,267	-0,009	<b>0,381</b>
	p value	0,356	0,503	0,199	0,073	0,954	<b>0,009*</b>
<b><i>Ucp2</i></b>	<i>r</i>	-0,178	-0,03	0,012	<b>0,375</b>	0,263	<b>0,315</b>
	p value	0,222	0,84	0,937	<b>0,009*</b>	0,068	<b>0,031*</b>

Table 2. Correlations between the expression levels of genes related to mitochondrial biogenesis, dynamics and the mitochondrial function and the SIT ratio, total ethanol intake (g/kg) during DID, and the percentage of weight gain. *Il6*: Interleukin 6; *Tnfa*: Tumor necrosis factor alpha; *Ccl2*: Chemokine (C-C motif) ligand 2; *Hmgb1*: High mobility group box 1; *Mfn1*: Mitofusin 1; *Mfn2*: Mitofusin 2; *Ucp2*: Uncoupling protein 2 (mitochondrial, proton carrier).

compared to their non-stressed counterparts (EXP-STD), and defeated mice fed with the HFD ( $p < 0.001$  in all cases). Additionally, we tested possible correlations between the gene expression levels of neuroinflammation markers and the SIT ratio, total ethanol intake (g/kg) during DID, and the percentage of weight gain (see Table 2). The main results in the striatum showed a positive correlation between ethanol intake during the DID test and the expression levels of *Tnfa* ( $r = 0,595$ ,  $p < 0,001$ ) and *Hmgb1* ( $r = 0,487$ ,  $p < 0,001$ ). The percentage of weight gain also showed a positive correlation with the expression levels of *Hmgb1* ( $r = 0,295$ ,  $p < 0,047$ ) in the striatum. In the hippocampus, ethanol intake during the DID test showed a positive correlation with the expression levels of *Il6* ( $r = 0,319$ ,  $p < 0,027$ ) and *Tnfa* ( $r = 0,386$ ,  $p < 0,001$ ). We also observed a positive correlation between the percentage of weight gain and the expression levels of *Il6* ( $r = 0,396$ ,  $p < 0,005$ ); in contrast, we detected a negative correlation between the percentage of weight gain and the expression levels of *Hmgb1* ( $r = -0,347$ ,  $p < 0,015$ ). The rest of the correlations are depicted in Supplementary Material.

### 3.5 Social defeat and a high-fat diet alter the expression levels of genes related to mitochondrial biogenesis, dynamics and the mitochondrial function

The analysis of *Mfn1* gene expression levels in the hippocampus (Fig. 8e) revealed a significant effect of Diet [ $F(2,35) = 5.021$ ,  $p < 0.012$ ]. In the hippocampus, animals in the HFD2x3 group showed lower *Mfn1* expression levels compared to those in the standard diet ( $p < 0.01$ ).

The analysis of *Mfn1* gene expression levels in the striatum did not reveal any significant effect.

In the case of the *Mfn2* gene expression in the striatum (Fig. 8f), the results showed a significant effect of Stress [ $F(2,28) = 4.538$ ,  $p < 0.02$ ], Diet [ $F(2,28) = 9.373$ ,  $p < 0.001$ ] and a significant interaction Stress x Diet [ $F(4,28) = 3.240$ ;  $p < 0.026$ ]. R-STD mice exhibit higher expression levels of *Mfn2* compared to their non-stressed counterparts (EXP-STD) and the resilient mice fed on HFD (R-HFD1x2 and R-HFD2x3) ( $p < 0.01$  in all cases).

Finally, the ANOVA of *Ucp2* gene expression levels in the hippocampus and striatum (Fig. 8g) showed a significant effect of Diet [ $F(2,35) = 8.791$ ,  $p < 0.001$ ] and [ $F(2,28) = 5.848$ ,  $p < 0.008$ ], and a significant interaction Stress x Diet [ $F(4,35) = 3.352$ ,  $p < 0.023$ ] and [ $F(4,37) = 2.957$ ,  $p < 0.032$ ]. Post-hoc analyses indicated that in both structures, resilient mice fed on the Standard Diet showed higher gene expression than the non-stressed group ( $p < 0.01$ ). Moreover, resilient R-STD group also showed higher expression levels of *Ucp2* compared to resilient mice in the R-HFD1x2 and R-HFD2x3 ( $p < 0.01$  in all cases).

Additionally, we tested possible correlations between the expression levels of genes related to mitochondrial biogenesis, dynamics and the mitochondrial function and the SIT ratio, total ethanol intake (g/kg) during DID, and the percentage of weight gain (see Table 2). The main results in the striatum showed a positive correlation between ethanol intake during the DID test and the expression levels of *Mfn2* ( $r = 0,375$ ,  $p < 0.009$ ). The percentage of weight gain also



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showed a positive correlation with the expression levels of *Ucp2* ( $r=0,381$   $p<0,009$ ). The rest of the correlations are depicted in Supplementary Material.

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#### 4. Discussion

The main result of the present study demonstrates, for the first time, that an intermittent intake of an HFD after exposure to an SD can block the long-term increase in alcohol consumption. It is well-known that SD induces a long-lasting increase in ethanol intake, with this effect being, at least partially, mediated by a strong



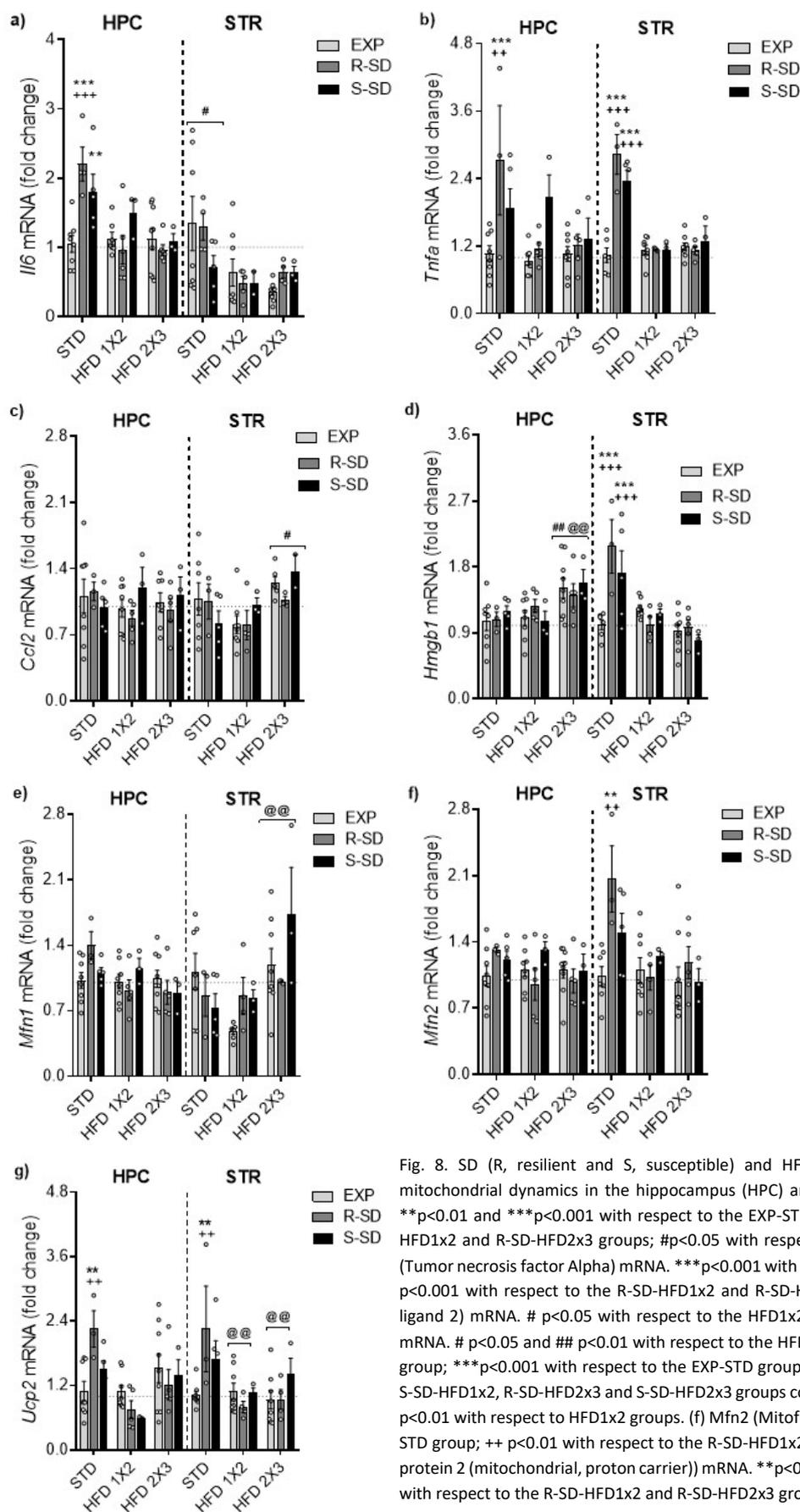


Fig. 8. SD (R, resilient and S, susceptible) and HFD feeding effects on neuroinflammation and mitochondrial dynamics in the hippocampus (HPC) and striatum (STR). (a) *Il6* (Interleukin 6) mRNA. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  with respect to the EXP-STD group; +++  $p < 0.001$  with respect to the R-SD-HFD1x2 and R-SD-HFD2x3 groups; # $p < 0.05$  with respect to the HFD1x2 and HFD2x3 groups. (b) *Tnfa* (Tumor necrosis factor Alpha) mRNA. \*\*\* $p < 0.001$  with respect to the EXP-STD group; ++  $p < 0.01$  and +++  $p < 0.001$  with respect to the R-SD-HFD1x2 and R-SD-HFD2x3 groups. (c) *Ccl2* (Chemokine (C-C motif) ligand 2) mRNA. #  $p < 0.05$  with respect to the HFD1x2 group. (d) *Hmgb1* (High mobility group box 1) mRNA. #  $p < 0.05$  and ##  $p < 0.01$  with respect to the HFD1x2 group; @@  $p < 0.01$  with respect to the STD group; \*\*\* $p < 0.001$  with respect to the EXP-STD group; +++  $p < 0.001$  with respect to the R-SD-HFD1x2, S-SD-HFD1x2, R-SD-HFD2x3 and S-SD-HFD2x3 groups corresponding. (e) *Mfn1* (Mitofusin 1) mRNA. @@  $p < 0.01$  with respect to HFD1x2 groups. (f) *Mfn2* (Mitofusin 2) mRNA. \*\* $p < 0.01$  with respect to the EXP-STD group; ++  $p < 0.01$  with respect to the R-SD-HFD1x2 and R-SD-HFD2x3 groups. (g) *Ucp2* (uncoupling protein 2 (mitochondrial, proton carrier)) mRNA. \*\* $p < 0.01$  with respect to the EXP-STD group; ++  $p < 0.01$  with respect to the R-SD-HFD1x2 and R-SD-HFD2x3 groups; @  $p < 0.05$  with respect to the STD group.



continuous access to an HFD, although an efficient blocker for drug-seeking behaviour, induces deep changes in the metabolic profile of the animals and an increase in the neuroinflammatory response<sup>41,55</sup>. However, we have previously shown that intermittent and limited access to an HFD was capable of blocking the cocaine-induced reinstatement of the preference without provoking metabolic disturbances, increased bodyweight or modifying leptin or ghrelin circulating levels<sup>46</sup>. The results of the present study also demonstrate that intermittent HFD access after SD counteracts the stress-induced increase in several neuroinflammatory markers.

#### 4.1. Intermittent and limited HFD blocks SD-induced increase in ethanol intake

It is known that not all subjects show consequences after being exposed to social stress, such as social defeat. The SIT is a widely accepted and etiologically validated model that categorizes rodents into resilient or susceptible phenotypes by observing depressive-like behaviours, such as social avoidance<sup>14,56</sup>. Among those fed a standard diet, the percentage of resilient and susceptible mice was like that in other studies, with 57% of the defeated mice showing a resilient phenotype (e.g.<sup>13,14</sup>). Similar proportions were observed in the group assigned to be fed with HFD2x3, although a higher percentage of resilient subjects (70%) was observed in the HFD1x2 group. In this group, stressed animals had access to the HFD immediately after the last episode of social defeat and before being tested into SIT, in contrast to the HFD2x3 group, which initiated HFD the following day after its evaluation with the SIT. Thus, it appears that a single exposure to the HFD was sufficient to reduce stress-induced social avoidance. Although the mechanisms are largely unknown, results from human and rodent studies strongly suggest that palatable food intake blunts acute stress responses. This was previously observed in stressed rats, where exposure to a limited access to sucrose decreases stress responses such as anxiety-like behaviours<sup>29</sup>.

An important aspect of this study was the lack of HFD impact on body weight. Chronic exposure to HFD induced obesity, metabolic disorders and neuroinflammation which are associated with depressive and anxious behaviours<sup>41,57,58</sup>. However, neither of the HFD schedules employed in this study induced an increase in body weight, though both patterns of HFD induced a binge pattern, as previously reported<sup>46</sup>. In the case of HFD1x2, the increased in Kcal intake was observed in the second session, although in the HFD2x3 this increase occurred in the sixth session. Even though the Kcal intake of HFD was significantly higher in the HFD2x3 groups, these mice did not show differences in body weight compared to those fed a standard diet. This lack of effect is consistent with findings from prior studies using similar discontinued HFD administration patterns<sup>46,59</sup>. In fact, in the present study, we found that resilient defeated animals fed with a standard diet exhibited greater weight gain than resilient mice fed with an HFD. However, susceptible mice showed greater body weight gain in general than controls and resilient mice, with this increase being more pronounced in susceptible mice fed with an HFD 1x2 schedule. Our results align with a recent report of Ben-Shachar and colleagues<sup>60</sup>, where mice with inherited vulnerability to stress developed significant metabolic disturbances

after exposure to HFD, while resilient mice were protected. In an elegant study, Coccorello and co-workers<sup>60</sup> reported that social defeated mice showed higher HFD intake, larger adipose tissue accumulation, with increased energy expenditure and lipid oxidation, resulting in lower body weight. This study points to the notion that the chronic activation of the stress response can be associated with altered energy homeostasis.

The principal aim of our study was to test the putative value of an intermittent HFD in the increase in ethanol intake induced by SD. Both methods of HFD administrations efficiently blocked the long-term increase in ethanol consumption observed in defeated male mice. Firstly, we confirmed that only susceptible mice fed with a standard diet exhibited elevated ethanol consumption, in accordance with previous research<sup>8-10,17,19,46</sup>. Secondly, defeated animals fed with intermittent exposure to an HFD presented lower ethanol intake, with a more evident effect in susceptible mice. Additionally, the HFD1x2 schedule also reduced ethanol consumption in resilient mice.

Based on our previous studies, the relation between HFD and drug intake mainly depends on the pattern of HFD administration. An intermittent schedule with 2h of access 3 days a week during adolescence, induces an increase in ethanol intake in non-stressed mice, even after switching to the standard diet<sup>62</sup>. However, when administered during the extinction phase of a cocaine-induced CPP, with continuous, and more importantly, intermittent exposure to HFD, efficiently inhibited cocaine seeking behaviour<sup>41,46</sup>. In addition, limited access to HFD protected mice from developing a long-term increase in the reinforcing effects of cocaine induced by stress due to social isolation<sup>59</sup>. Although both isolated and grouped mice developed a binge pattern of HFD, isolated animals significantly increased their fat consumption with respect to their grouped counterparts. This result also supports the hypothesis that palatable food is used as compensation during periods of stress<sup>63,64</sup>.

Therefore, the present results clearly show that limited and intermittent access to HFD blocks the increase in alcohol consumption induced by SD without increasing the weight of the animals. No differences were observed when analysing Kcal intake of HFD among non-stressed, resilient or susceptible mice, though all mice developed a binge pattern of HFD, as previously reported in other studies<sup>42,44,62</sup>.

#### 4.2. Intermittent and limited HFD limits social defeat-induced increases in neuroinflammatory markers and alterations in the expression of genes related to mitochondrial biogenesis and dynamics and the mitochondrial function

The ability of an HFD to block social stress-induced increases in ethanol intake could have several explanations. Circulating leptin levels modulate stress-induced eating, being involved in both satiety signalling and food intake<sup>65</sup>. Since leptin receptors are localized in dopaminergic neurons of the ventral tegmental area, leptin can affect both drugs and food reward<sup>66</sup>. Although a role of leptin cannot be ruled out, we have demonstrated in several studies that the employed HFD administration did not modify circulating leptin levels<sup>42</sup>.

Another possible explanation for the effect of HFD in reducing ethanol consumption may lie in the fact that rodents find HFDs



rewarding and prefer them over the potentially rewarding effects of ethanol<sup>67</sup>. The hedonic properties of HFD can stimulate feeding behaviour resulting in hyperphagia even after energy requirements have been met. It is important to highlight that access to HFD was provided during the period of ethanol consumption assessment. Notably, caloric intake in the HFD groups increased during the DID period. This increase indicates the bidirectional relationship between ethanol and HFD, corroborated by studies in humans<sup>68</sup>. However, on the days that HFD was available, access was allowed after the daily session of DID procedure. Therefore, satiety is not a likely reason for the decrease in ethanol intake. An interesting pair of correlations between the total amount of EtOH consumption during DID and the percentage of body weight gain and Kcal intake could shed some light on these findings. Ethanol intake during DID was higher in those mice ingesting more Kcal of HFD but with lower weight gain. These results agree with the above-mentioned studies showing stress-induced increase HFD intake but decrease weight gain. Therefore, the more stressed mice consumed more Kcal and ethanol, but did not proportionately increase their body weight.

A more probable mechanism underlying the beneficial effects of an HFD could involve the neuroinflammatory response induced by SD. Increased neuroinflammation is a well-characterized phenomenon after SD. Using the same protocol for SD employed in the present study, the neuroinflammation response includes affectation of the brain blood barrier and the activation of microglia, with an increase in pro-inflammatory cytokines and chemokines such as *Il-6*, *Tnf $\alpha$*  or fractalkine (CX3CL1) in several brain regions<sup>22,27,69,70</sup>. In agreement with these results, in the present study, defeated mice fed a standard diet expressed higher levels of proinflammatory cytokine genes, such as *Il-6* and *Tnf $\alpha$* , in the hippocampus and striatum, along with higher levels of *Hmgb1* in the striatum. Several studies suggest that *Hmgb1* is released in the central nervous system in response to stress, resulting in an enhanced proinflammatory environment. Furthermore, stress-induced *Hmgb1* represents a molecular mechanism that modulates microglial activation promoting neuroinflammation and behavioural alterations<sup>71</sup>. Interestingly, both, resilient and susceptible mice showed these increase neuroinflammatory response. We must take into consideration that brain samples were obtained after the end of the DID procedure, therefore all mice were previously exposed to ethanol intake. Ethanol consumption activates the neuroimmune system mainly through TLR4 receptors, leading to increased production of proinflammatory cytokines and chemokines, which contribute to neuroinflammation and brain damage<sup>72,73</sup>. Our results indicate that SD stress increases the expression of cytokines and chemokines released by alcohol overlapping with the upregulation of neuroimmune mechanisms in the brain (see review in<sup>74</sup>). We have previously reported that in socially defeated mice, the levels of the *Il-6* cytokine in the striatum positively correlate with oral self-administration of ethanol intake, confirming the modulatory role of ethanol consumption in neuroinflammation<sup>10</sup>. We have confirmed and extended the results in the present study with a positive correlation between total ethanol intake in the DID and the hippocampal levels of *Il6* gene expression, as well as striatal and

hippocampal levels of *Tnf $\alpha$* . We must note that in the present study we observed increased gene expression of *Il-6*, *Tnf $\alpha$* , and *Hmgb1* both resilient and susceptible animals. In previous studies<sup>8,14</sup>, we observed that only susceptible animals exhibited increased *Il-6* protein levels in the striatum and prefrontal cortex following an oral ethanol self-administration protocol. The more extensive ethanol exposure in those earlier studies<sup>8,14</sup>, along with the measurement of protein levels instead of gene expression, may explain these different results.

In addition to this well-known response, our results also showed a deep affectation of the mitochondrial function in defeated animals. Mitochondria are subcellular organelles linked to numerous essential cellular and physiological processes, including energy production. The brain is particularly energy demanding and stress due to social interactions, sharply increases energy demand in the brain<sup>75</sup>. In fact, although there is not enough evidence yet, mitochondrial function could serve as the link between neuroinflammation and the depressive-like effects of SD<sup>76</sup>. Resilient and susceptible defeated mice fed on the Standard Diet showed an increase in striatal gene expression of *Mnf2*, but only resilient mice also presented an increased gene expression of hippocampal and striatal *Ucp2*. Again, resilient mice did not show protective phenotypes, either for the neuroinflammatory markers or in the mitochondrial function.

*Mnf2* increase in striatum is supported by studies that have reported a disequilibrium between *Mfn1* and *Mfn2* leading to an abnormal transport respiration chain in mitochondria concerning mitochondrial stress. An HFD can re-establish this equilibrium. This group also reported an increased expression of the *Ucp2* gene in the hippocampus and striatum. In this same line, using a model of stress induced by social crowding, an increase in the gene expression of *Ucp2* was reported in the retroperitoneal white adipose tissue of stressed mice<sup>77</sup>. An increase of *Ucp2* in the central nervous system is related to an increment of inflammation and mitochondrial respiration leading to less neural activation and leptin sensitivity but higher gliosis<sup>78</sup>.

Several studies point to an antidepressant effect of leptin<sup>79,80</sup>. Its signalling may be influenced by various situations beyond its plasma levels. In this sense, *Ucp2* modulates leptin sensitivity in the central nervous system. The increase of *Ucp2* in the hippocampus and striatum of the R-STD animals might be associated with a decrease in the sensitivity to leptin within the central nervous system and, therefore, would enhance the anxious behaviour of the animals in this experimental group, which would correlate with a greater urge to drink alcohol. Conversely, the HFD prevents this increase in *Ucp2* induced by SD, supporting correct leptin signalling, alleviating anxious behaviour, and reducing the associated alcohol consumption.

Furthermore, numerous studies have reported that SD increased ROS production and mitochondrial stress in the central nervous system<sup>81</sup>. As a transport uncoupling protein, an increase in *Ucp2* is related to a protective mechanism against increased ROS production and mitochondrial stress. We observed that this upregulation is reversed following HFD feeding. We hypothesized that HFD consumption reduces mitochondrial stress and ROS production,



thereby preventing *Ucp2* upregulation and related neuroinflammation.

Consequently, our results confirmed the limited studies associating mitochondrial dysfunction to chronic social stress and pointed to a mitochondrial dysfunction playing a possible role in stress-induced ethanol intake. The positive correlation between ethanol intake during DID and the striatal expression levels of *Mfn2* confirmed this association.

Interestingly, there was a general correlation among all the genes studied in the striatum and hippocampus (see supplementary material). For instance, hippocampal *Il6* positively correlates with *Tnfα*, *Ccl2*, *Mfn1*, *Mfn2* and *Ucp2*. Similarly, striatal *Tnfα* gene expression positively correlates with *Mfn1*, *Mfn2*, and *Ucp2* gene expression, highlighting the relation between the expression of cytokines and markers of mitochondrial dysfunction.

One of the most noteworthy findings, our study reported that intermittent access to the HFD was able to reverse the neuroinflammatory responses induced by social stress in both the hippocampus and the striatum. Numerous studies have reported elevated levels of inflammatory cytokines associated with continued HFD consumption<sup>82-84</sup>. Moreover, intermittent administration of HFD (2h per day, 3 days a week) during adolescence produced an additive effect on ethanol binge drinking, amplifying neuroinflammatory response, although without any significant effect by itself<sup>55</sup>. Although defeated mice with intermittent access to HFD in our study exhibited a lower weight gain than standard diet defeated mice, it is important to note that animals fed an intermittent HFD2x3 displayed signs of neuroinflammation and mitochondrial dysfunction. Mice fed on an intermittent HFD regimen, irrelevant of the stress exposure, displayed higher expression levels of *Hmgb1* in hippocampus, and *Mfn1*, *Ucp2* and *Ccl2* in the striatum. This highlights the difference when compared with the HFD1x2 protocol that induce fewer changes in gene expression levels, only increasing the *Ucp2* gene expression levels in the striatum.

Interestingly, both HFD patterns prevented the inflammatory effect induced by social stress and alcohol consumption in stressed mice, which showed reduced levels of *Il6*, *Tnfα*, *Ucp2* and *Mfn2* in both the striatum and hippocampus compared to their counterparts fed with STD. The protective effect of HFD on *Hmgb1* gene expression was only observed in the striatum, as *Hmgb1* expression levels were not increased by SD in the hippocampus. Thus, although intermittent HFD administered (2h/day, 3 days a week) induces a slight but detectable increase in neuroinflammation markers, our findings show for the first time that it counteracts the characteristic neuroinflammatory response of a stress condition.

## Conclusions

The results of the present study support our initial hypothesis, which states that intermittent HFD intake could prevent both ethanol consumption increase and neuroinflammation mediated by social stress. Specifically, although both HFD intake patterns tested herein were shown to be effective for this

purpose, we highlight the 1x2 pattern as the most suitable for implementation, due to the minimal fat exposure and lower neuroinflammation levels compared to the 2x3 pattern. Thus, further investigations may focus on the resilience potential of the 1x2 HFD pattern in relation to other social stressors or its effects regarding the increased consumption of other drugs induced by stress. Finally, it would be interesting to perform a similar study using female mice to assess whether the protective effect of intermittent HFD consumption is modulated by sex.

## Abbreviations

<i>Ccl2</i>	Chemokine (C-C motif) ligand 2
CPP	Conditioned place preference
DA	Dopamine
DID	Drinking-in-the-dark
EtOH	Ethanol
EXP	Exploration
HFD	High fat diet
<i>Hmgb1</i>	High mobility group box 1
<i>Il6</i>	Interleukin 6
<i>Mfn1</i>	Mitofusin 1
<i>Mfn2</i>	Mitofusin 2
NAcc	Nucleus accumbens
<i>Rn18s</i>	18S ribosomal RNA
<i>Rpl13</i>	Ribosomal protein L13
<i>Rpl37</i>	Ribosomal protein L37
SA	Self-administration
SD	Social defeat
SIT	Social interaction test
STD	Standard diet
SUD	Substance use disorder
<i>Tnfα</i>	Tumor necrosis factor alpha
<i>Ucp2</i>	Uncoupling protein 2 (mitochondrial, proton carrier)

## Author contributions

M.Carmen Arenas: Conceptualization, Formal analysis, Investigation, Methodology, Software, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. Irene Pérez-Esteban: DaTa curation, formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft. Héctor Cañeque-Rufo: Data curation, Formal analysis, Investigation, Software, Validation, Visualization. Esther Gramage: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Writing – review & editing. Gonzalo Herradón: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing. Marta Rodríguez-Arias: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing.

## Conflicts of interest

The authors declare no conflict of interest.



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## Data availability

Data for this article are available at ZENODO at <https://zenodo.org/me/requests/ee54bbcb-27f1-45db-903c-9796de93ef14>

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## Data availability statement

Data for this article, including changes in weight, social interaction test ratio, and g/kg of alcohol intake in the drinking in the dark test of male mice OF1 exposure to social defeat and exposed to stress and on different types of diet (standard or two types of intermittent high fat diet), as well as expression data of the following genes in the striatum and hippocampus: Il6, Tnfa, Ccl2, Mfn1, Hmgb1, Ucp2, and Mfn2, are available at Zenodo at <https://zenodo.org/records/14775137>

