HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

National and Kapodistrian University of Athens- Medical School-Department of Pharmacology, Neuropsychopharmacology Group

RESEARCH THESIS PROJECT

INVESTIGATION OF THE IMPACT OF GPER1 ACTIVATION ON BEHAVIORAL ASPECTS IN MALE AND FEMALE RATS AFTER CHRONIC MILD STRESS

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2022-2024

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Investigation of the Impact of GPER1 Activation on Behavioral Aspects in Male and Female Rats After Chronic Mild Stress

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Scientific Summary

Estrogens have been shown to be involved in the pathophysiology of depression and anxiety, supporting the observed higher prevalence of these disorders in women than men. Neuro-estrogens are considered to act rapidly in the brain through G protein-coupled estrogen receptor 1, GPER1. Recent studies have shown its implication to anxiety and depression, but its action is yet to be clarified. The aim of this study is to investigate the potential antidepressant and/or anxiolytic effect of GPER1 activation in experimental animals subjected to chronic mild stress (CMS). In the present thesis, female and male Wistar rats were subjected to CMS and subsequently were treated intraperitoneally daily for one week with vehicle, ketamine or G1, a GPER1 agonist. All animals were subjected to open field and light-dark tests. Interestingly, in our study, CMS resulted into a possibly stress-induced animals' hyperlocomotion, especially in females. G1-treated stressed female rats displayed reduced anxiety in both tests. In opposition, GPER1 activation in stressed male rats affected only zone entries, revealing a sex difference. Overall, this study supports that G1 potentially has an anxiolytic effect in a sex- and stress-related manner, prompting further investigation into leveraging GPER1's function to develop novel treatments for depression and anxiety.

Highlights

- Rats subjected to chronic mild stress exhibited hyperlocomotion, possibly induced by stress.
- G1 treatment showed a potential anxiolytic effect in stressed female rats compared to control female rats.
- GPER1 activation exhibited sex differences in open field and light dark tests.

Keywords

GPER1, Estrogen, Chronic Mild Stress, Hyperlocomotion, Anxiety, Depression, Open Field test, Light Dark test

Επιστημονική Περίληψη

Έχει αποδειχθεί πως τα οιστρογόνα εμπλέκονται στην παθοφυσιολογία της κατάθλιψης και του άγχους, υποστηρίζοντας τον παρατηρούμενο υψηλότερο επιπολασμό αυτών των διαταραχών στις γυναίκες συγκριτικά με τους άνδρες. Τα νεύρο-οιστρογόνα θεωρείται ότι δρουν ταχέως στον εγκέφαλο μέσω του υποδοχέα οιστρογόνων συζευγμένου με G-πρωτεΐνη, GPER1. Πρόσφατες μελέτες έχουν δείξει πως επιδρά στο άγχος και την κατάθλιψη, αλλά η δράση του δεν έχει ακόμη διευκρινιστεί πλήρως. Στη μελέτη αυτή θα διερευνηθεί η πιθανή αντικαταθλιπτική και/ή αγχολυτική επίδραση της ενεργοποίησης του GPER1 σε πειραματόζωα που έχουν υποβληθεί σε χρόνιο, ήπιο στρες (CMS). Στην παρούσα διατριβή, θηλυκοί και αρσενικοί αρουραίοι Wistar υποβλήθηκαν σε CMS και έπειτα έγινε χορηγήση ενδοπεριτοναϊκώς, καθημερινά και για μία εβδομάδα, έκδοχο, κεταμίνη ή G1, αγωνιστής του GPER1. Όλα τα ζώα υποβλήθηκαν σε δοκιμασίες ανοιχτού πεδίου και φωτεινού-σκοτεινού κλωβού. Είναι ενδιαφέρον ότι στη μελέτη μας, το CMS οδήγησε σε μια πιθανώς προκαλούμενη από στρες υπερκινητικότητα των ζώων, ειδικά στα θηλυκά. Οι θηλυκοί αρουραίοι που υποβλήθηκαν σε θεραπεία με G1 εμφάνισαν μειωμένο άγχος και στις δύο δοκιμασίες. Αντίθετα, η ενεργοποίηση του GPER1 σε αρσενικούς αρουραίους με στρες επηρέασε μόνο τον αριθμό εισόδων στη κέντρο της πλατφόρμας, αποκαλύπτοντας διαφυλική διαφορά. Συμπερασματικά, αυτή η μελέτη υποστηρίζει ότι ο G1 αγωνιστής έχει δυνητικά αγχολυτική δράση με τρόπο που σχετίζεται με το φύλο και το στρες. Κρίνεται απαραίτητη περαιτέρω έρευνα για τη λειτουργία και δράση του GPER1 στην παθοφυσιολογία των διαταραχών της διάθεσης και για την ανάπτυξη νέων θεραπειών για την κατάθλιψη και το άγχος.

Λέξεις-κλειδιά

Υποδοχέας οιστρογόνων συζευγμένος με G-πρωτεΐνη, Χρόνιο Ήπιο Στρες, Άγχος, Κατάθλιψη, Φύλο

Lay Summary

Mood disorders, such as depression and anxiety, have higher prevalence in women than men. Estrogens have been linked to the pathophysiology of these disorders. In the brain, estrogens can act rapidly through G protein-coupled estrogen receptor 1, GPER1. This study aims to investigate whether this receptor activation results in an antidepressant and/or anxiolytic effect in stressed animals. Female and male rats, after being subjected to chronic mild stress, they were treated daily for one week with a GPER1 activator, ketamine or vehicle and then their anxiety-related behavior was estimated. Interestingly, our results showed that stressed animals were hyperactive, in comparison to control animals. In behavioral tests, stressed female rats that received treatment with GPER1 activator exhibited behaviors of reduced anxiety, revealing a sex difference. Overall, this study supports that GPER1 activation

could have an anxiolytic effect in stressed animals. Future studies should further investigate this possibility, as well as its potential antidepressant action.

Introduction

Mental diseases have emerged as a prominent cause of disability within western societies and are currently among the top ten leading causes of disease burden worldwide (Wittchen et al., 2011; Patel et al., 2018). Mood, stress and eating disorders affect disproportionally women than men (Kornstein, 1997; Marcus et al., 2008; Kokras and Dalla, 2017; Dovey and Vasudevan, 2020). Specifically, regarding depression, its prevalence is twice more frequent in females than in males (Salk, Hyde and Abramson, 2017). Women also encounter gender-specific disorders, including peri-menopausal and postpartum depression, as well as premenstrual dysphoric syndrome (Halbreich and Karkun, 2006). Many studies suggest that an underlying reason for this difference is estrogen fluctuation or plunge that could trigger disorders, such as depression or anxiety (Bloch, 2000; Cohen et al., 2006; Freeman, 2010; Wang et al., 2021). Supporting this, human studies have shown that estrogens' administration during peri-menopausal period improve depressive symptoms (Gregoire et al., 1996; Bloch, 2000; Ahokas et al., 2001; Moses-Kolko et al., 2009).

Also, in preclinical studies, treatment of female rats with estrogen receptor β (ERβ) agonist/estradiol prevented depressive-like behavior, after a hormone-simulated pregnancy (HSP) (Green, Barr and Galea, 2009). In a similar manner, it has been shown that estradiol reverses the anxiety-like behavior caused by ovariectomy in rats, in the elevated plus maze and the open field test (OF) (Zheng et al., 2020). Apart from sex differences in the prevalence of depression and anxiety, sex differences in treatment have been observed; antidepressants present sex differences in pharmacokinetic parameters (Kornstein et al., 2000; Kokras, Dalla and Papadopoulou-Daifoti, 2011; Franceschelli et al., 2015). Currently, the pharmacotherapy of depression and anxiety relies heavily on drugs that modulate monoaminergic neurotransmission. However, the commonly used drugs require several weeks of treatment to take effect, and are ineffective in 35% of patients (Berton and Nestler, 2006). One third of patients are considered to suffer from treatment-resistant depression (TRD), meaning that they do not respond to two or more antidepressant medications. Esketamine is used as fast-acting treatment for TRD (Matveychuk et al., 2020). However, es-ketamine has many potential side effects and an abuse potential (Melo *et al.*, 2015; Yang *et al.*, 2022). Therefore, there is urgent need to discover novel pharmacological targets and new antidepressants and anxiolytics, although research up to date has not yet clarified the exact etiology of these disorders (O'Leary, Dinan and Cryan, 2015; Kuehner, 2017). Given the incidence of mood disorders and anxiety in women, it is considered appropriate to study the role of estrogen in pathophysiology and pharmacotherapy of depression and anxiety.

Both the female and male brain produce local neuro-estrogens, from cholesterol via the enzyme aromatase, and have rapid non-genomic actions that affect neuronal function through synaptic plasticity (Rune and Frotscher, 2005; Arevalo, Azcoitia and Garcia-Segura, 2015). These actions are important for cognitive function and neuroprotection and differ significantly from the classical hormonal actions of

estrogens, which are of little utility for psychiatry (Gillies and McArthur, 2010). Neuro-estrogens preserve their effect by binding to estrogen receptors (ER), which are widely distributed in the brain and present at both neurons and glia. There are two categories of ER: nuclear and membrane embedded/membrane associated (mERs). Classical estrogen signaling occurs through the action of the nuclear receptors, ERα and ERβ (Rettberg, Yao and Brinton, 2014). ERβ's anxiolytic and antidepressant action has already been shown by numerous studies (Lund et al., 2005; Green, Barr and Galea, 2009; Yang et al., 2014). The rapid non-genomic actions of estrogens in the brain are currently believed to be exerted through a novel receptor, that belongs in mERs category and was discovered in 2005, the G protein-coupled estrogen receptor (GPER1; also known as GPR30) (Tang et al., 2014; Alexander, Irving and Harvey, 2017). This receptor is suggested to act through the activation of second messenger pathways (ERK, mTOR, etc.) (Wang et al., 2017). It appears to be necessary for the "micro-regulation" of neuronal circuits, involved in depression and anxiety (Tang et al., 2014; Sellers, Raval and Srivastava, 2015; Alexander, Irving and Harvey, 2017). Also, GPER1 activity is linked to serotonin (Xu et al., 2009) and BDNF (Srivastava, Woolfrey and Evans, 2013), as well as to the regulation of neuroplasticity in hippocampal synapses (Srivastava, Woolfrey and Evans, 2013), which are involved in the action of antidepressants (Pittenger and Duman, 2008). It has been shown that neuro-estrogens increase the density of neural spines in the frontal cortex, via the mTOR- and ERK-pathways (Tuscher et al., 2016), and in the hippocampus in a sex-specific manner (Li et al., 2021). This finding is interesting as our group discovered that the hippocampus-cortical circuit is important for stress response and depression (Kafetzopoulos et al., 2018).

The involvement of GPER1 in stress and anxiety is evident by the fact that GPER-deficient rats and mice of both sexes exhibit altered anxiety-like behavior, in comparison to wild type animals (Kastenberger and Schwarzer, 2014; Zheng et al., 2020). It has been shown that GPER1 may contribute to anxiogenic-like effects that estrogens exert in male and ovariectomized female mice (Kastenberger, Lutsch and Schwarzer, 2012; Kastenberger and Schwarzer, 2014). Supporting this, in human studies serum GPER1 levels were positively correlated with anxiety levels in women, excluding those in postmenopausal period (Fındıklı et al., 2016). However, there are contradictory findings. Studies support that GPER1 activation has an anxiolytic effect in male and in, ovariectomized or not, female rats, which imitate the condition of menopause (Zheng *et al.*, 2020; Wang *et al.*, 2021; Tongta, Daendee and Kalandakanond-Thongsong, 2022). It is worth mentioning that a study showed that the genetic ablation of GPER1 in female and male rats resulted in increased anxiety-like behaviors reported that the effects were more pronounced in females than males. On the other hand, a study presented reduced anxiety-like behavior of GPER1 knock out mice, predominantly in males (Kastenberger and Schwarzer, 2014). This indicates that sex could highly contribute in the anxiogenic or anxiolytic action of GPER1. However, it is also indicated that species, hormonal status and age are important factors for GPER1 function (Zheng et al., 2020).

Simultaneously, GPER1 is also involved in depression, but the studies are still limited and its relation to depressive behavior is to be clarified. In general, it suggested that GPER1 exerts antidepressant action, as it has been shown in aged female rats,

ovariectomized rats and male mice (Dennis et al., 2009; Wang et al., 2019, 2021). Supporting this, GPER1 agonist, G1, is sufficient to desensitize 5-HT_{1A}R signaling in the hypothalamic paraventricular nucleus of the hypothalamus (PVN), as selective serotonin reuptake inhibitors (SSRIs) do (McAllister et al., 2014). This is of importance because 5-HT_{1A}R signaling is involved in hyperactivity of the hypothalamic-pituitary-adrenal axis (HPA), which is a characteristic of depression. Additionally, depressive-like behavior in adolescent mice of both sexes, caused by prenatal exposure to the pesticide dichlorodiphenyltrichloroethane (DTT), was accompanied by reduced levels of GPER1 (Kajta et al., 2017). Contrary to that, a study has shown higher serum GPER1 levels in drug-naïve major depressive disorder (MDD) patients than in the controls and has proposed GPER1 serum levels as a valuable tool in predicting the presence of depression (Findikli et al., 2017).

While it is strongly indicated that GPER1 is involved in the pathophysiology of depression, stress, and anxiety, its mechanism and way of action, depending on sex/gender and other factors, are yet to be clarified. Additionally, it is important to investigate the exploitation of GPER1 modifiers in the treatment of mood and anxiety disorders, by expanding the knowledge about this possible, novel pharmacological target that may relieve depression rapidly and improve the quality of life of patients. This is of importance in males as well as females, as neuro-estrogens play an important role in males, with their levels being six times higher in the hippocampus than in the blood (Hojo *et al.*, 2004). In this context, this thesis project aims to investigate the possible antidepressant and/or anxiolytic effects of GPER1 in male and female rats, which were subjected to a chronic mild stress (CMS) protocol (Willner, 1997; Kafetzopoulos et al., 2018). CMS is a widely accepted model for establishing depressive-like behavior in rodents, as it shows good predictive, face and construct validity. It produces anhedonia, a core symptom of depression, and other symptoms of MDD (Willner, 1997). It consists of a variety of mild stressors, applied sequentially to rats for a prolonged period to imitate daily life stressors that can contribute to manifestation of depression in humans. Research has shown that it causes a number of behavioral, endocrinological, neurochemical and neurobiological changes and affect the two sexes differently (Dalla et al., 2005). Based on current bibliography, we hypothesized that G1 would have an antidepressive-like and anxiolytic effect in rats. To that cause, we examined GPER1 possible effects in animals that were subjected to CMS and then were treated repeatedly with the agonist of GPER1 (G1) or ketamine or vehicle, and then subjected to the behavioral tests of open field (OF) and light-dark (LD).

Methods

Animals

Adult female and male Wistar rats were obtained from Hellenic Pasteur Institute and were used throughout this study. At the start of CMS protocol, female and male rats were approximately 4-5 months of age and weighed 200-250g and 350-450 g, respectively. All male and female rats were singly housed in plastic, transparent

cages (40*25*15 cm). They were housed in two separate rooms, depending on their group (control or CMS). In both rooms, they were housed under controlled conditions 12-h light/dark cycle (lights on at 08:00 h), temperature (20 ± 2 °C) and humidity 40-60%, and free access to food and tap water, unless it was differently indicated by the CMS protocol. During CMS, housing conditions and access to water and food was defined by the protocol. Monitoring of health and welfare conditions took place daily. The body weight of each animal was measured once per week. All animal experiments were reviewed and approved by the local committee (License number: 1348406/16-12-2022) and all studies have been carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. G*power analysis was performed prior to the start of this study aiming to minimize the numbers of animals used and to reduce their suffering. For the analysis, three-way factorial analysis of variance (ANOVA) was used, based on effects sizes previously observed in previous similar experiments, at 80% power and type I error equal to 5%.

Drugs

The drugs used were a GPER1 agonist G1 (0.01 mg/kg, CAS# 881639-98-1, (Dennis *et al.*, 2009; Wang *et al.*, 2019)), ketamine (10mg/kg, (Melo *et al.*, 2015)) or vehicle (0.9% saline with 5% DMSO, CAS# 67-68-5). G1 and ketamine were dissolved in 5% DMSO in 0.9% saline. All drugs were administered intraperitoneally (i.p.), with volume of injection 1ml/kg (Kokras et al., 2015; Kafetzopoulos et al., 2018). Drugs were administered repeatedly for 7 days, daily.

Experimental design

All animals spent four weeks of adaptation to consumption of 1% sucrose solution in water to establish baseline preference levels by performing sucrose preference test (SPT). The adaptation period took place before the beginning of any stressful procedures and consisted of three sessions per week. The mean measurements of the three last tests were used as the baseline sucrose consumption and based of that, rats were assigned to the control and CMS groups alternating from highest to lowest preference, so as the difference of means between the two groups would be minimal. Rats not showing sucrose preference were excluded from the following experiments. Therefore, rats were matched and divided into four groups: Control Females (n=19), Control Males (n=20), CMS Females (n=27) and CMS Males (n=26). The CMS protocol started three days after the last sucrose test of the adaptation period and lasted for ten weekly cycles that consisted of continuous stressors alternating during the day. After the end of CMS, the rats were assigned to treatment groups and were treated with i.p. injections of vehicle, G1 or ketamine daily for one week (Figure 2). The assignment to groups was based on the mean measurements of the three last SPT tests, alternating from highest to lowest preference, so as the difference of means between the groups would be minimal. On the 5th day of treatment, they were subjected to open field test (OF) and on the 6th day on light dark test (LD), 30 minutes after the injection accordingly. Finally, the following day after LD test, all rats were killed by rapid decapitation and their brains were collected and stored to −80 °C.

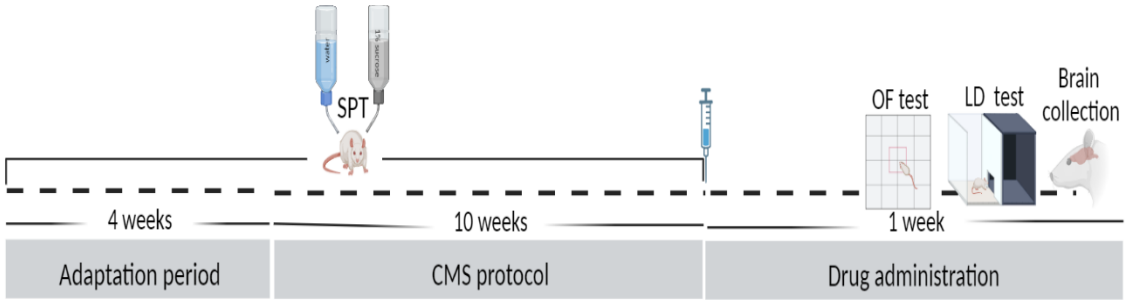

Figure 1. Experimental procedure. Animals spent 4 weeks of adaptation to sucrose consumption and then they were subjected to 10 weeks cycles of CMS protocol. After CMS, they were administered drugs for 7 days. Behavioral tests and euthanasia were performed from day 5th to 7th of treatment.

Figure 2. Drug administration (D.A.) schedule. Animals were treated daily. On the 5th day, after treatment, they were subjected to OF test. On the 6th day, after treatment, they were subjected to LD test. On the 5th day, after treatment, they were euthanized and their brains were collected.

Sucrose preference test

SPT was used to assess anhedonia, a core symptom of depression (Willner, Muscat and Papp, 1992; Kafetzopoulos *et al.*, 2018). SPT was performed three times per week (Monday, Wednesday, Friday) during the adaptation period and once a week (every Thursday based on CMS schedule) after the initiation of CMS protocol. To measure the sucrose preference, animals that were food- and water-deprived for 18 h were presented with two pre-weighed bottles containing 1% sucrose solution or tap water for a period of 1 hour (between 13:00 and 14:00). In each subsequent session, the position of the two bottles was alternated to avoid habituation. Sucrose preference was calculated according to the formula: sucrose preference = (sucrose intake / (sucrose intake + water intake))*100 (Bessa *et al.*, 2009; Kafetzopoulos *et al.*, 2018).

Chronic mild stress

A CMS protocol, that has been previously described for male rats (Willner, 1997) and modified by (Dalla *et al.*, 2005) and (Kafetzopoulos *et al.*, 2018), was employed to male and female rats. Specifically, male (N=26) and female (N=27) rats were subjected to chronic exposure of a series of mild stressors, according to a specific hourly schedule (Table 1). The stressors were: stroboscopic illumination in dark (120 flashes/ min), confinement, tilted cage (at 35 degrees), exposure to white noise, soiled cage (250 ml of tap water into the sawdust bedding) followed by cage cleaning, overcrowding (each rat was housed in a group of 3-5 depending on their sex, different combinations of rats each time), water deprivation followed by presentation of empty bottle, overnight illumination combined with food deprivation

followed by exposure to inaccessible food, combination of food and water deprivation and reversed light cycle. The CMS protocol lasted for ten weeks.

Table 1. CMS hourly schedule

Day / Hour	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY SUNDAY
9 – 10	Stroboscopic illumination	Empty bottle presentation	Inaccessible food	Overcrowding	Cage cleaning	Reversed light cycle
10 - 11		Overcrowding	Stroboscopic illumination		Confinement	
11 - 12					Tilted cage	
12 – 13						
13 – 14	Confinement			SPT		
14 – 15	Tilted cage	Noise	Confinement	Noise		
15 – 18						
overnight	Water deprivation	Food deprivation & overnight illumination	Water & food deprivation (18 h)	Soiled cage	Reversed light cycle	

Open field test

On the 5th day of treatment, the rats were subjected to OF, to assess anxiety- and stress-like behaviors. All rats were injected and acclimated to the test room for 30 minutes before the test accordingly. The latency to escape from the center of the open field and the time spent in the center served as indices of anxiety (Kokras *et al.*, 2012). An open field apparatus, a square arena of dimensions 43.2 × 43.2 cm^2 and surrounded by tall Perspex walls (Med Associates, St Albans City, VT, USA), was used. The apparatus includes infrared beams and the manufacturer's software were used to automatically register movements and exploration (Kafetzopoulos *et al.*, 2018; Kokras *et al.*, 2018). All rats were acclimated to the test room for 30 minutes before the test, and then they were placed in the center and allowed to explore the area for 10 min.

Light dark test

On the 6th day of treatment, the rats were subjected to LD (Kokras *et al.*, 2012), to assess anxiety-like behavior. All rats were injected and acclimated to the test room for 30 minutes before the test accordingly. The OF apparatus was used. An appropriate, Plexiglass chamber was inserted to equally divide the apparatus into two compartments, one transparent, brightly illuminated and a second opaque and dark formed by the inserted chamber, as provided by the manufacturer. The two compartments were communicating through an opening measuring approximately 105 mm height and 85 mm width. All rats were acclimated to the test room for 30 minutes before the test, and then they were placed in one corner of the illuminated compartment facing away from the opening towards the dark compartment and

allowed to explore the area for 10 min. LUX of the room was measured in order the transition of light between the two compartments to be smooth.

Statistics

Behavioral results (OF, LD) were analyzed using a three-way ANOVA with sex (male:female), treatment (vehicle:G1:ketamine) and stress status (control:CMS) as independent factors. Differences between groups were then determined by Bonferroni's post hoc analysis. Statistical significance was set at $p < 0.05$. Results are reported as means ± standard error of mean (SEM). Percentage of body weight change (%) was calculated using this formula: Weight change (%) = (Weight $_{\text{end of CMS}}$- Weight $_{\text{beginning of CMS}}$)/ Weight $_{\text{beginning of CMS}}$ *100, where Weight $_{\text{beginning of CMS}}$ and Weight $_{\text{end of CMS}}$ are the mean of two body weight measurements before the start of the protocol and the last two weeks of CMS, respectively. Differences between CMS and controls groups were determined by unpaired t test with Welch's correction.

Results

Chronic mild stress was not sufficient to induce anhedonia, but impacted body weight

Female and male rats were subjected to CMS protocol for 10 weeks. CMS is a widely accepted model for establishing depressive-like behavior in rodents and has been shown to induce anhedonia assessed by SPT. However, it is difficult to establish the desired phenotype, for reasons which remain unclear. In our study, SPT did not reveal statistically significant difference between CMS and control group. The time-wise extension of the protocol to 10 weeks did not affect the sucrose preference of the subjects. CMS animals, though, displayed reduced body weight compared to control animals ($t_{(38.82)}$=4.074, p=0.0002 for females and $t_{(37.35)}$=5.828, p<0.0001 for males), depicted by the % weight change (Figure 3). Reduced body weight after CMS has been described and can be considered as a possible marker of stress load (Strekalova *et al.*, 2022), and it is proven to be independent of CMS-induced decreases in sucrose intake (Pothion *et al.*, 2004). Nevertheless, CMS has been shown to evoke neurobiological changes or if the procedures are insufficient enough it may provoke other behavioral alterations, such as hyperactivity (Strekalova *et al.*, 2022). To examine this, we decided to continue accordingly to the experimental design and subject the animals to treatment and behavioral testing.

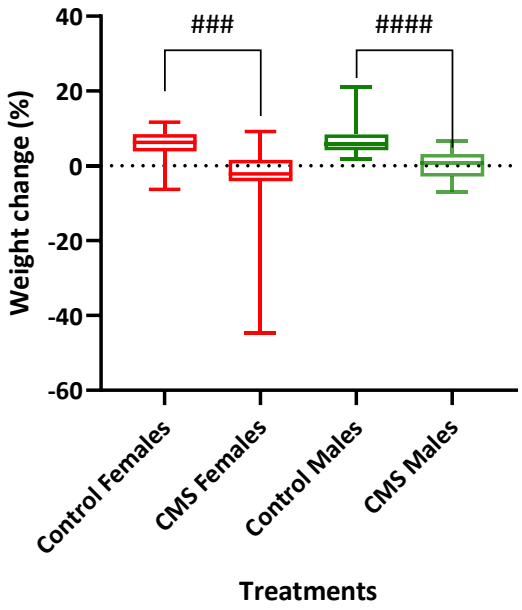

Figure 3: Percentage of body weight change of female and male rats during CMS period. Body weight change (%) was calculated for each group for the 10 week-period of CMS. The sample sizes of each group as shown in the diagram are: n=19, n=27, n= 20, n=26. Data are represented as mean ± SEM. The sign # denote the level of significance of stress status. Female and male rats that were subjected to CMS presented negative percentage of weight change, meaning weight loss. These changes were statistically significant, in comparison to control rats gained weight, shown by the positive percentage of weight change (###, p<0.001 for females and ####, p<0.0001 for males).

The effect of CMS and semi-chronic G1 and ketamine treatment in OF test

Female and male rats were subjected to 10 min open field test, after exposure to chronic mild stress or control conditions and daily treatment for one week with vehicle, G1 or ketamine. Total horizontal distance and counts, horizontal distance, percentage of distance and time in center, total vertical counts and zone entries were examined.

Regarding total horizontal distance travelled (Figure 4A), three-way ANOVA tests revealed main effects of sex and stress ($F_{(1,80)}$=21.904, p<0.0001 and $F_{(1,80)}$=18.523, p<0.0001). Also, ANOVA revealed main effects for sex and stress for total horizontal counts (Figure 4B) ($F_{(1,80)}$=30.320, p<0.0001 and $F_{(1,80)}$=18.566, p<0.0001) and main effect of stress for horizontal distance travelled in the center (Figure 4C) ($F_{(1,80)}$=13.174, p<0.0001). Post-hoc Bonferroni tests revealed that there are sex differences in stressed animals. In more detail, in ketamine-treated animals, sex had an effect in horizontal activity by increasing both the distance travelled (Figure 4A) and the number of movements (Figure 4B) of stressed female rats in comparison to stressed males (p<0.0001 for both parameters). In accordance to that, ketamine-treated, stressed, female rats travelled more distance in the center of the open field platform in comparison to male, stressed rats (p=0.012) (Figure 4C). Vehicle-treated and G1-treated, stressed female rats exhibited an increased number of horizontal counts in comparison to vehicle-treated and G1-treated stressed males, respectively (p=0.050, p=0.024, respectively) (Figure 4B). This, overall, shows that female rats had increased activity, and especially horizontal counts, versus male rats. Simultaneously,

in stressed females, ketamine treatment resulted in increased horizontal distance travelled in relation to vehicle treatment ($p=0.003$) (Figure 4A) and in increased horizontal counts in relation to vehicle and G1 treatment ($p=0.019$ and $p=0.002$, respectively) (Figure 4B). These results could be explained by a contingent ketamine-induced increase of locomotion, presented in existing bibliography (Crawford *et al.*, 2020). However, ketamine-treated, female animals travelled more distance after CMS versus control conditions ($p=0.001$) (Figure 4A). Finally, in G1-treated rats of both sexes, CMS increased the total horizontal distance travelled ($p=0.021$ for males, $p=0.007$ for females) compared to control conditions (Figure 4A). Regarding total horizontal counts (Figure 4B) and distance travelled in the center (Figure 4C), exposure to CMS increased both compared to control conditions in female, G1-treated rats ($p=0.009$ and $p=0.011$, respectively for each parameter), and in female ketamine-treated rats as well ($p<0.0001$ and $p=0.019$, respectively for each parameter). While increased horizontal activity in the center of the OF arena is a sign of reduced anxiety, total increased horizontal activity could also be associated with reduced anxiety, as the animals that explore the arena more are likely less anxious. Nevertheless, the observed increase of horizontal activity could be explained by other factors, such as a contingent CMS-induced agitation (Strekalova *et al.*, 2005) or sex differences in ambulation (Ramos *et al.*, 1997a). Additionally, CMS affected more parameters examined in OF test in G1-treated females than males, reinforcing sex differences. Overall, based on these results, we could speculate that G1-treatment in interaction with stress background potentially has an anxiolytic effect, especially in females, as CMS-subjected animals present a less anxious profile than control animals. However, there are no statistical differences compared to vehicle treatment to strengthen this hypothesis.

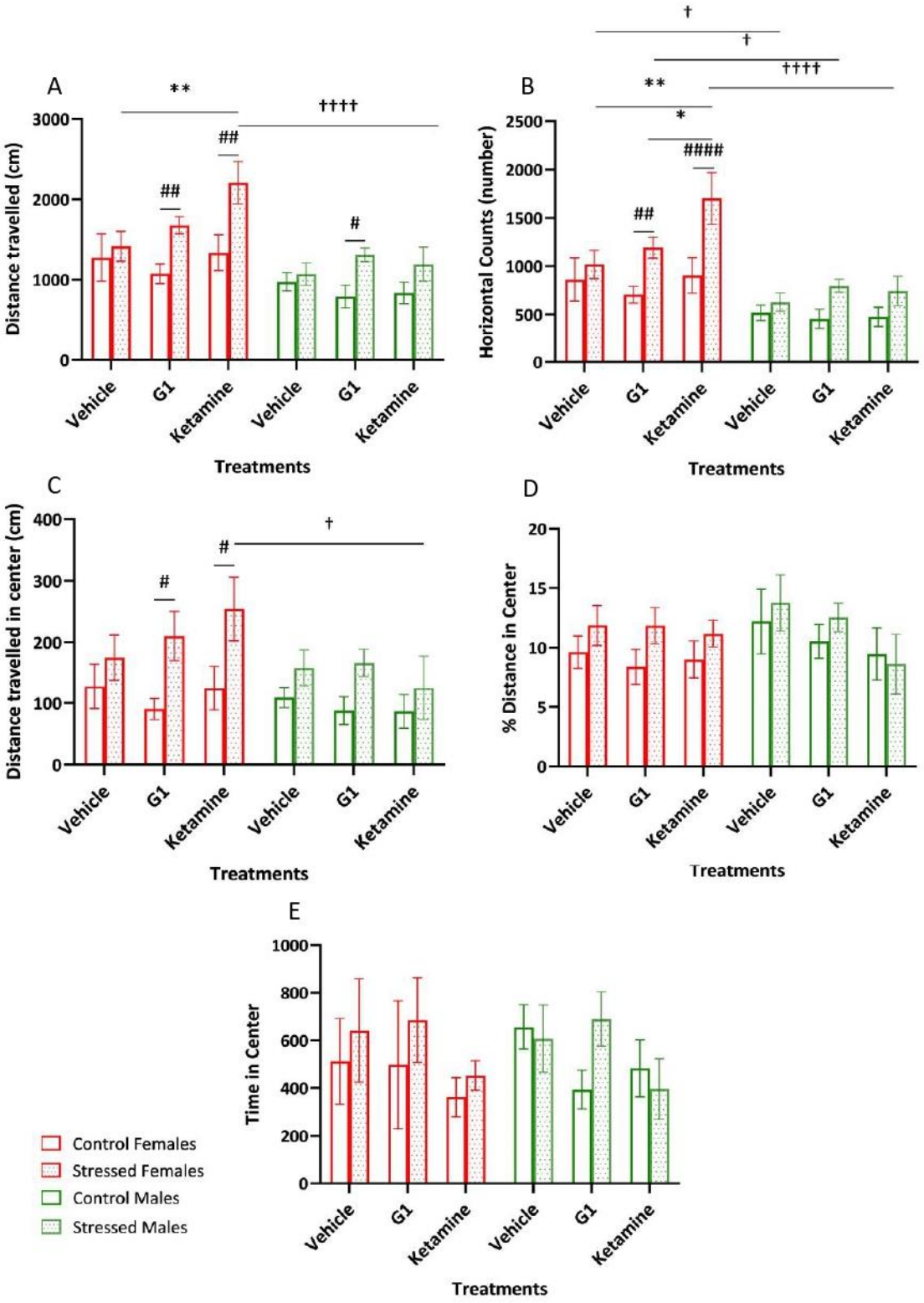

Figure 4. Horizontal activity by female and male rats during a 10 min open field test. To study horizontal activity, total horizontal distance travelled (A), total horizontal counts (B), horizontal distance travelled in the center (C), percentage of distance travelled in the center (D) and time in time center (E) were examined. Animals were subjected to 8-weeks-long CMS or control conditions. Then, they were treated with i.p. injections of vehicle, G1 or ketamine daily for one week and subjected to behavioral testing. Each group includes n=5-8 for control animals, n= 8-11 for stressed animals. Data are represented as mean ± SEM. The signs (*, # and +) denote the level of significance of treatment, stress

status and sex respectively in the interaction sex*treatment*stress status, as resulted by multiple comparisons with Bonferroni corrections. (A) Within stressed animals, ketamine-treated females travelled more horizontal distance than ketamine-treated males (++++, p<0.0001). Ketamine increased the travelled distance in comparison to vehicle in stressed female rats (**, p<0.01). Exposure to CMS of female and male, G1-treated rats increased the total horizontal distance travelled (##, p<0.01 for females and #, p<0.05 for males) compared to exposure to control conditions. Stressed, female rats travelled more horizontal distance when they were treated with ketamine compared to when they were treated with vehicle (##, p<0.01). (B) Vehicle, G1 and ketamine increased horizontal counts of stressed female rats in comparison to stressed males (+, p=0.05, +, p<0.05 and ++++, p<0.0001, respectively. Female, stressed rats treated with ketamine display higher number of ambulatory counts than those treated with G1 (*, p<0.05) or vehicle (**, p<0.001). Additionally, G1- and ketamine-treated, female animals had higher numbers of horizontal counts when they were subjected to CMS compared to control conditions (##, p<0.01 and ####, p<0.0001 respectively). (C) Female, ketamine-treated, stressed rats displayed lower distance travelled in the center of the open field compared to the male ones (+, p<0.05). Females treated with G1 and ketamine travelled more ambulatory distance in the center when they were previously subjected to CMS in comparison to control conditions (#, p<0.05 for both G1 and ketamine treatments). (D, E) The percentage of distance travelled in the center and the time spent in the center did not differ between the examined groups.

Regarding vertical activity (Figure 5A), a three-way ANOVA revealed main effects of sex, treatment, stress status and sex*treatment interaction for total vertical counts in the OF ($F_{(1,80)}$=14.239 p<0.0001, $F_{(2,80)}$=15.752 p<0.0001, $F_{(1,80)}$=8.165 p=0.005 and $F_{(1,80)}$=3.492 p=0.035,respectively). Post-hoc Bonferroni tests indicated that there is a sex difference in vehicle- and G1-treated, stressed animals. In more detail, females had higher number of vertical counts than males in both treatment groups (p=0.002 and p=0.001, respectively). In general, a higher number of vertical counts indicates increased rearing behavior and is associated with increased exploratory behavior. Therefore, we can speculate that stressed females are more exploratory than stressed males. Additionally, ketamine-treated female rats, after being subjected to either CMS or control conditions, displayed lower number of vertical counts than vehicle-treated females (p=0.047 and p<0.0001, respectively). Regarding G1 administration, there is no statistical difference with vehicle, but it is worth mentioning that female, stressed animals treated with G1 had higher number of vertical counts than corresponding control group (p=0.004). This result could be due to increased variability within the vehicle-treated, control, female group. Also, G1 treatment resulted in higher number of vertical counts in stressed, female rats than ketamine-treated stressed females (p<0.0001).

With regards to zone entries (Figure 5B), a three-way ANOVA revealed main effect of stress for zone entries in the OF ($F_{(1,80)}$=14.690, p<0.0001). Multiple comparisons with post-hoc Bonferroni corrections showed that G1-treated, female and male rats display higher number of entries in the center when they were previously subjected to CMS compared to control conditions (p=0.009 and p=0.01, respectively). Given that the latency to escape from the center of the open field and the time spent in the center serve as indices of anxiety, it would be expected that stressed animals perform less zone entries. However, the increased center entries maybe could be explained by the prementioned agitation caused by CMS.

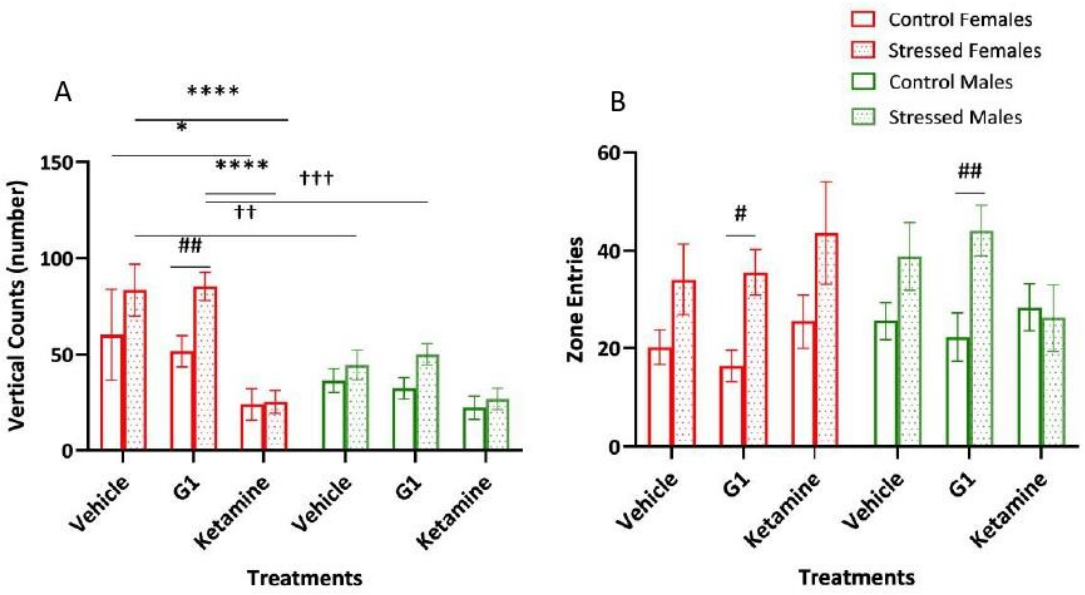

Figure 5. Vertical counts (A) and zone entries (B) by female and male rats during a 10 min open field test. Animals were subjected to 8-weeks-long CMS or control conditions. Then, they were treated with i.p. injections of vehicle, G1 or ketamine daily for one week and subjected to behavioral testing. Each group includes n=5-8 for control animals, n= 8-11 for stressed animals. Data are represented as mean ± SEM. The signs (*, # and +) denote the level of significance of treatment, stress status and sex respectively in the interaction sex*treatment*stress status, as resulted by multiple comparisons with Bonferroni corrections. *(A)* Female, stressed animals display more activity when treated with vehicle (++, p<0.01) or G1 (+++, p<0.001) than stressed males. Moreover, stressed female animals treated with G1 had higher number of vertical counts than corresponding control G1-treated group (##, p<0.01). Female animals of both stress statuses perform fewer vertical counts, when treated with ketamine in comparison to vehicle (*, p<0.05 for control and ****, p<0.0001 for stressed animals). Also, stressed female animals treated with ketamine are less active than the corresponding group treated with G1 (****, p<0.0001). (B) Females and males treated with G1 display higher number of center entries when they were previously subjected to CMS in comparison to control conditions (#, p<0.05 for females and ##, p<0.01 for males).

The effect of CMS and semi-chronic G1 and ketamine treatment in LD test

Female and male rats were subjected to 10 min light dark test, after exposure to chronic mild stress or control conditions and daily treatment for one week with vehicle, G1 or ketamine. Percentage of distance travelled in the light compartment of the platform, zone entries and total, horizontal and resting time in the light compartment were examined.

Regarding the percentage of distance travelled in the light compartment (Figure 6A), a three-way ANOVA revealed main effect of treatment for percentage of distance travelled in the light compartment during the LD ($F_{(2,76)}$=3.172 p=0.048). Multiple comparisons with Bonferroni corrections indicated a sex difference in G1-treated, stressed animals, where females had higher percentage of distance travelled in the light compartment than males (p=0.027). Simultaneously, the G1-treated, stressed female group travelled a higher percentage of distance in light than corresponding control group (p=0.025). These results combined suggest that G1 in a chronic stress background probably resulted in an anxiolytic-like effect in females, as distance travelled in the light is positively associated with reduced anxiety. Additionally, in

female, stressed animals, ketamine treatment resulted in lower percentage of travelled distance in light compared to G1 treatment (p=0.008). As for the zone entries (Figure 6B), a three-way ANOVA revealed no main effects for transitions between the two compartments during the LD test, where either CMS or treatment affected significantly anxiety-like behavior. Finally, regarding total and resting time spent in the light compartment (Figures 6C,6E), three-way ANOVA test revealed a main effect of treatment for total time spent in light compartment during the LD test ($F_{(2,76)}$=3.372, p=0.040) and for resting time in the same compartment $F_{(2,76)}$=4.622, p=0.013), respectively. Also, three-way ANOVA resulted in main effects of sex and stress status for horizontal time spent in light compartment during the LD test ($F_{(1,76)}$=10.630 p=0.002, $F_{(1,76)}$=5.645 p=0.020, respectively) (Figure 6D). Animals previously subjected to CMS spent more ambulatory time in the light compartment compared to animals living in control conditions. This is in accordance to the CMS-induced, increased activity observed in the OF test and could be explained by a contingent CMS-induced agitation that has been observed in some studies (Strekalova *et al.*, 2005). However, total or resting time in this compartment did not differ between these two groups. In addition, female rats spent in general more horizontal time in the light compartment than males ($F_{(1,76)}$=10.630 p=0.002). This is also in line to our OF's results where females presented increased activity compared to males, which could be an intrinsic sex difference in ambulation (Ramos *et al.*, 1997a). Post-hoc Bonferroni tests indicated that stressed females treated with G1 spent more ambulatory time in light than stressed males treated with G1 (p=0.037). This is in accordance with the observed sex difference regarding the percentage of distance travelled in the light compartment by G1-treated, stressed animals. Additionally, ketamine-treated, control, female rats spent less resting time in light compared vehicle-treated control, female rats (p=0.031). Neither G1 or ketamine treatment were sufficient to reveal a potential anxiolytic action.

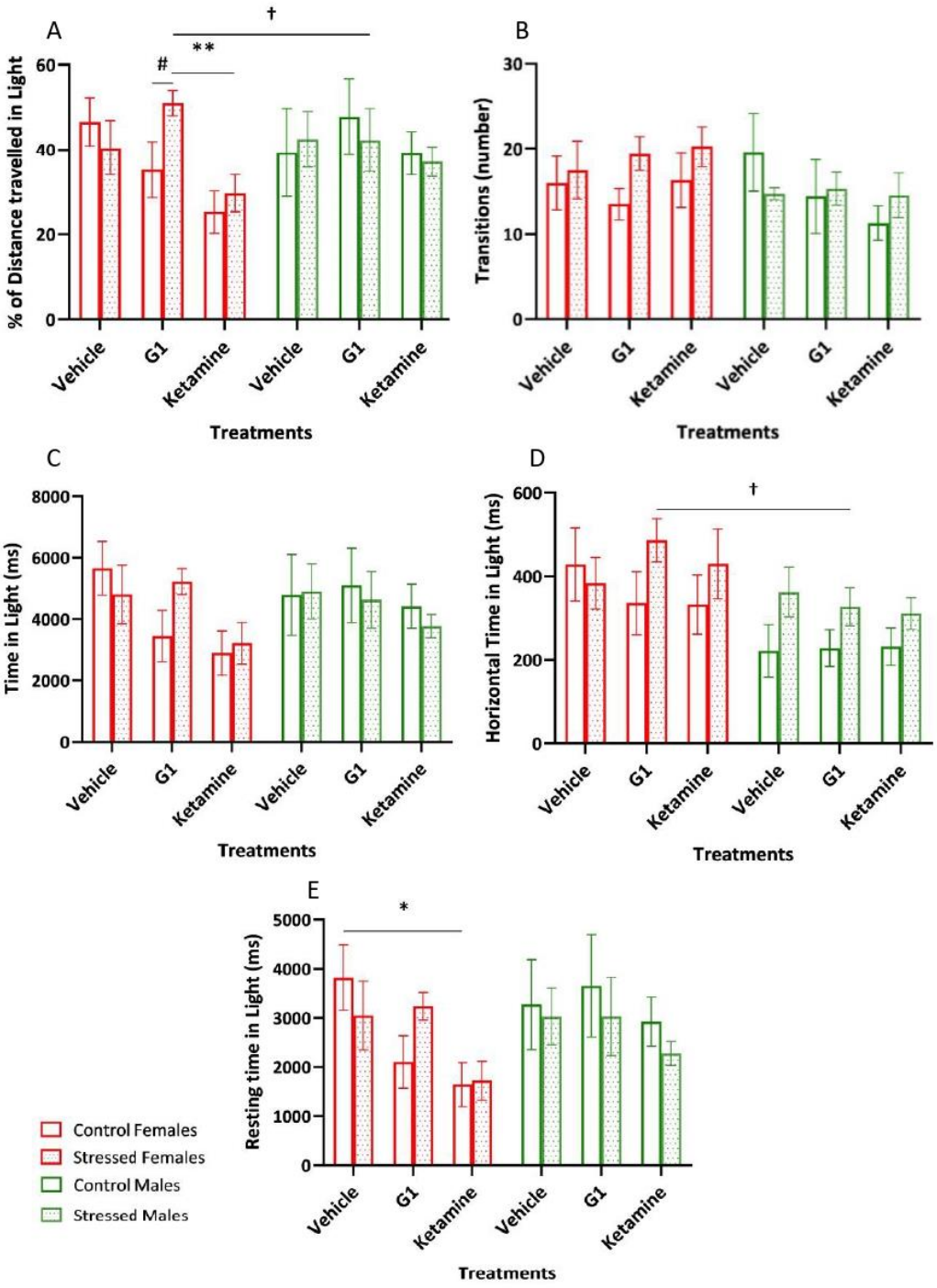

Figure 6. Percentage of distance travelled in light (A), transitions between the two compartments (B), total time spent in light (C), horizontal time spent in light (D) and resting time spent in light (E) by female and male rats during a 10 min light dark test. Animals were subjected to 8-weeks-long CMS or control conditions. Then, they were treated with i.p. injections of vehicle, G1 or ketamine daily for one week and subjected to behavioral testing. Each group includes n=5-8 for control animals, n= 7-11 for stressed animals. Data are represented as mean ± SEM. The signs (*, # and +) denote the

level of significance of treatment, stress status and sex respectively in the interaction sex*treatment*stress status, as resulted by multiple comparisons with Bonferroni corrections. (A) Female, G1-treated, stressed rats spent more time in the light compartment of the LD field compared to males of the corresponding group (+, $p<0.05$). Female, G1-treated, stressed rats, also, travelled more distance compared to the corresponding control group (#, $p<0.05$). Ketamine treatment resulted in less distance travelled in light by female, stressed rats travel less distance in light than G1 treatment (**, $p<0.01$). (B) There is no difference regarding the zone entries during LD test between the examined groups. (C) There is no difference regarding the time spent in light during LD test between the examined groups. (D) Female, G1-treated, stressed rats spent more time in the light compartment of the light dark field compared to G1-treated, stressed males (+, $p<0.05$). (E) Ketamine treatment resulted in less resting time spent in light by female animals previously subjected to control conditions than vehicle-treated controls (*, $p<0.05$).

Discussion

The aim of the present study was to investigate GPER1's role in the stress response in female and male rats subjected to CMS. The CMS protocol, that we followed , has been previously used by our team, and it was sufficient to decrease sucrose preference in Wistar male rats (Kafetzopoulos *et al.*, 2018). However, in this study, it was not sufficient to induce anhedonia. While CMS is a valid model for the establishment of depressive-like behavior and most of the published studies achieved anhedonia, the procedure involves labor-intensive experiments and most importantly, it is difficult to establish the desired phenotype (Willner, 1997). There are studies that have failed to observe a significant alteration in sucrose intake or preference and others showing anomalous findings. Additionally, there is a pervasive issue of under-reporting negative results and of unpublished literature (Willner, 2005). Simultaneously, a study has confirmed a depressive-like phenotype in forced swim test (FST), but not in SPT (Marco *et al.*, 2017), while most of studies use SPT to confirm this phenotype. It is important to note that factors such as the stressors used during the protocol, sex, strain, (Antoniuk *et al.*, 2019) and time of SPT (D'Aquila, Newton and Willner, 1997) can impact sucrose consumption. In our study, these factors were chosen to be the same as in (Kafetzopoulos *et al.*, 2018) with the exception of sex. Of note, most existing studies use only male rats, but we chose to incorporate both sexes to also examine sex differences. Nevertheless, inter-strain and inter-individual differences, because of factors such as genomic makeup or endocrine status, can also affect the sucrose consumption and the manifestation of depressive-like behavior after CMS. In fact, male and female Wistar rats display individual patterns of liquid consumption throughout the day (Kurre Nielsen, Arnt and Sánchez, 2000; Strekalova et al., 2022). These differences could explain why in our study the same protocol was not sufficient. In addition, it has been proven that some rats show resiliency to developing exhibit depression- or anxiety-like behaviors despite their exposure to CMS. In those cases, SPT did not reveal significant difference between the control groups and the rats that were classified as resilient (Raya et al., 2018; Tang et al., 2019; Zurawek et al., 2019). Individual vulnerability to stress-induced anhedonic states in rats has been shown to be reflected by altered expression of numerous receptors such as 5-HT1A, D2 and glucocorticoid receptors and by elevated secretion of CRH. Also, distinct features between resilient and susceptible rats involve general proteomic changes in the

hippocampal region, response to dopamine agonists, turnover and binding ability of beta-adrenergic receptor as well as neuroanatomical features and interactions between the hippocampus and prefrontal cortex (Strekalova et al., 2022). Differentiating between resilient and susceptible animals would enable the exploration of epigenetic and post-translational mechanisms of resilience. Future studies should focus on elucidating these underlying mechanisms, as they could be exploited for therapeutic purposes. In the context of our research, exploring whether the absence of induced anhedonia is attributable to resilience factors would be a compelling future investigation. However, chronic stress protocols of insufficient intensity or/ and duration may evoke other behavioral alterations, such as increased signs of anxiety and hyperactivity (Strekalova and Steinbusch, 2010; Spasojevic et al., 2016). Of note, we observed reduced body weight in CMS animals, which can be considered as a possible marker of stress load (Strekalova *et al.*, 2022), and it has been shown to be independent of CMS-induced decreases in sucrose intake (Pothion *et al.*, 2004). Apart from behavior, CMS has been shown to evoke neurobiological changes (Dalla et al., 2005; Hill et al., 2012). Therefore, we decided to continue accordingly to the experimental design and subject the animals to treatment and behavioral testing.

To examine the effect of GPER1, CMS and control animals were administered repeatedly for 1 week with G1, as well as vehicle and ketamine, acting as control and fast-acting antidepressant treatment, respectively. Then, the animals were subjected to OF and LD tests. In OF, animals presented higher horizontal activity regarding the total distance traveled and the counts, when they were previously subjected to CMS compared to control conditions, regardless of sex or treatment. This could be explained in the context of hyperlocomotion induced by chronic stress, in rats that has been previously reported in male rats and mice (Strekalova et al., 2005; Spasojevic et al., 2016). This, however, contradicts the well-established behavior of locomotor inhibition as symptom of depression and stress. In fact, a study showed that mice subjected to CMS, regardless if anhedonia was induced, displayed increased open field activity (Strekalova and Steinbusch, 2010). In our case, this is mostly observed in females, which is in accordance to the in general higher ambulation of female rats of different strains compared to males (Ramos et al., 1997b). Also, stress in G1-treated animals had impact on more parameters examined in the two behavioral tests in females than in males, reinforcing sex differences. Therefore, while CMS was insufficient to induce anhedonia, it caused hyperactivity. Additionally, CMS animals, regardless of sex or treatment, traveled more distance in the center of the platform and showed higher number of zone entries and vertical counts, which are considered signs of reduced anxiety. Other studies have also displayed anxiolytic-like features in rodents subjected to CMS, using tests such as elevated plus/O-maze and LD (D'Aquila, Brain and Willner, 1994; Strekalova *et al.*, 2005; Strekalova and Steinbusch, 2010). However, this is not confirmed in OF (Spasojevic *et al.*, 2016) and anxiolytic-like effect of CMS was not confirmed by time spent in the center, therefore the increased distanced traveled in the center could be an artifact of the hyperlocomotion/agitation observed in stressed animals. In our study, besides the main effect of stress, zone entries were further increased in G1-treated, stressed animals compared to the G1-treated, control groups. Also, G1-treated, stressed, females traveled more distance in the center and performed higher number of vertical counts. This reveals a potential

anxiolytic-like effect of G1 in a stress background, but not an overall anxiolytic effect, as there is no statistical difference compared to vehicle treatment. In agreement with this, studies have showed that the genetic ablation of GPER1 resulted in increased anxiety-like behaviors, more pronounced in female rats (Zheng *et al.*, 2020). With regards to ketamine treatment, in our study it was not sufficient to exert the antidepressant effect, shown in SPT (Tornese *et al.*, 2019; Matveychuk *et al.*, 2020). This could potentially be attributed to the abovementioned hyperactivity. Moreover, ketamine treatment might have contributed to female rats' hyperactivity, which is supported by existing literature presenting ketamine-induced locomotion and female sensitivity (Franceschelli et al., 2015; Crawford et al., 2020).

In general, LD findings were in agreement with the OF results. Animals previously subjected to CMS spent more ambulatory time in the light compartment compared to the control group. This is probably an artifact due to the stress-induced hyperlocomotion, and not a sign of less anxiety, as the total of resting time in this compartment did not significantly differ between these groups. Regarding G1 treatment, stressed, female rats presented increased percentage of distance traveled in the light compartment compared to control group, hinting a stress effect in G1-treated rats. However, the transitions between compartments during the test didn't reveal any main effects, indicating that none of the factors affected significantly anxiety-related behaviors. Additionally, G1-treated, stressed females exhibited more horizontal time in the light compartment compared to males, reinforcing the sex difference in anxiety-related behavior with G1 treatment observed in OF. Interestingly, a study has shown that GPER1-deficient, male mice display an anxiolytic-like phenotype in LD (Kastenberger and Schwarzer, 2014). Therefore, sex and gender could be factors explaining the anxiogenic/anxiolytic action of GPER1 observed in literature. It is worth mentioning that studies showing an anxiogenic effect has been performed in mice, while studies displaying an anxiolytic effect, including ours, have been performed in rats. So, the species may explain the difference in action as well. As for ketamine, in control female animals resulted in less resting time spent in the light compared to vehicle, supporting the abovementioned ketamine-induced locomotion. However, it was not sufficient to reveal the expected anxiolytic effects, therefore longer periods of treatment should be considered in future studies.

In conclusion, this study suggested a stress-induced hyperlocomotion contrary to the expected anhedonic behavior. Additionally, they indicate differential effects of G1 and ketamine treatments on anxiety-related behaviors, specifically in stressed female rats, revealing sex differences. G1 treatment interaction with stress resulted in reduction of anxiety-like behaviors, while ketamine showed contrasting outcomes. Although our study indicates at a potential anxiolytic effect of G1 treatment in stress background, we acknowledge the significant limitations. Taking into account literature regarding its implication in anxiety and depression, it could be of importance to repeat this experiment to examine its effect in a framework of achieved anhedonia, as the observed stress-induced agitation affects the results. Notably, though, sex differences were evident in behavioral responses, highlighting the importance of considering sex and gender in evaluating treatment outcomes for anxiety-related behaviors in rats.

Acknowledgments

I am deeply grateful to everyone who contributed to the completion of my MSc thesis. Foremost, I would like to express my sincere thanks to Professor Dalla Christina, whose guidance was invaluable in navigating this thesis and for giving me the opportunity to be involved in a neuropsychopharmacological project. As neuropsychopharmacology is one of my main scientific interests, this means a lot to me. Additionally, I want to express my appreciation to Dr. Kokras Nikolaos for his insightful feedback and expertise, which significantly enriched my work. Of course, I would like to express my gratitude towards the PhD candidate of the lab Pavlidi Pavlina, for all her help, scientific inputs and countless hours we spent together. Finally, I would like to communicate my heartfelt appreciation to the Serveta Lida, Sageorgi Maria, Rapti Anastasia, Dhimarko Katerina and Kalampokis Dimitris. Working with them in this project truly made my thesis journey an enjoyable experience, as they all contributed greatly both morally and practically.

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RESEARCH PROPOSAL

Effect of GPER1 activation on chronic stress resilient and non-resilient male and female rats

Project summary

Chronic mild stress (CMS) evokes behavioral, endocrinological and neurobiological changes in rats. In our study, CMS induced a rather unexpected phenotype characterized by lack of anhedonia, hyperactivity, and some anxiolytic-like features. In the literature, other studies have also failed to observe a significant alteration in sucrose preference or display anomalous findings, while the underlying reasons are not clarified. Additionally, our study indicates a potential anxiolytic effect of G1 treatment in stress background, but this worths further exploration. Taking into account GPER1 implication in anxiety and depression, it is of importance to repeat this experiment with some changes to examine its effect in a framework of achieved anhedonia. Apart from behavioral alterations, we also aim to deepen our knowledge about neurochemical and neurobiological changes caused by CMS by examining molecular markers of stress response in the brain. Additionally, given the importance of CMS model in depression research, we also aim to understand the neurobiological and neurochemical effect of chronic stress that could contribute to explaining anomalous findings of CMS and possibly features of resiliency to stress. To examine the neurochemical and neurobiological effects, monoamines and amino acid neurotransmitters concentrations as well as brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD-95), total and phosphorylated mammalian target of rapamycin (mTOR) levels will be determined in stress-related brain regions of chronically stressed rats exhibiting and not exhibiting anhedonia. This project will shed light in GPER1 potential anxiolytic and/or antidepressant effect, as well as in the neurobiology of resilience to chronic stress.

Project description

Specific aims

1. To investigate the behavioral, neurobiological and neurochemical effect of G1 treatment impact in chronically stressed animals exhibiting anhedonia. This will shed light in the potential antidepressant and/or anxiolytic effect of GPER1 activation. To this cause, we will:
    - Examine the neurochemical effect of G1 treatment on the levels of the neurotransmitters NA, DA, DOPAC 3-MT, 5-HT, 5-HIAA, glutamate and GABA.
    - Study the neurobiological impact on the proteins of BDNF, PSD-95, mTOR and phosphorylated p-mTOR.

2. To investigate the neurobiological and neurochemical alterations between animals displaying depressive-like symptomatology and those displaying lack of anhedonia. To this cause, we will stratify CMS animals to resilient and susceptible cohorts based on SPT results. Simultaneously, stratification will be performed in CMS animals of the previous project by re-analyzing the already collected data of SPT. Results will be compared within the experiments and between experiments, in order to determine resilience features as well as clarify CMS discrepancies and whether CMS affects the endophenotype (neurobiologically and neurochemically) regardless of the phenotype.
3. To examine whether sex differences observed in behavioral tests are reflected in the neurobiological and neurochemical level.

Introduction and significance

Mental disorders are a prominent cause of disability within western societies, with depressive disorders to be the third most prevalent between them (Wittchen *et al.*, 2011). As depressive and anxiety disorders are more prominent in women than men, it is of importance to study the role of estrogen in pathophysiology and pharmacotherapy of depression and anxiety. GPER1 is considered a potential pharmacological target, as it is necessary for the "micro-regulation" of neuronal circuits and it is involved in the pathophysiology of depression, stress, and anxiety (Srivastava, Woolfrey and Evans, 2013; Alexander, Irving and Harvey, 2017; Tang *et al.*, 2019). Studying GPER1's action is of importance in males as well as females, as neuro-estrogens play an important role in males, with their levels indicatively being six times higher in the hippocampus than in the blood (Hojo et al., 2004). GPER1 leads to sex-dependent changes (Kastenberger and Schwarzer, 2014; Li et al., 2021). Also, among other factors, GPER1 activity is linked to serotonin (Xu et al., 2009), BDNF (Srivastava, Woolfrey and Evans, 2013). It is, also, linked to the regulation of neuroplasticity in hippocampal synapses (Srivastava, Woolfrey and Evans, 2013), and to PSD-95, a postsynaptic component of synaptic plasticity (Akama et al., 2013). Furthermore, it has been shown that neuro-estrogens increase the density of neural spines in the frontal cortex, via the mTOR- and ERK-pathways (Tuscher et al., 2016). However, its mechanism of action and potential antidepressant or anxiolytic effect, depending on sex/gender and other factors, are yet to be clarified. Our results showed that G1 treatment interaction with stress showed a potential anxiolytic effect in stressed female rats not displaying anhedonia compared to control female rats. Despite the limitations, implication of GPER1 in stress-related behaviors, such as depression and anxiety, as well as prominent sex differences, are clear in the study. Therefore, we aim to investigate the behavioral, neurochemical and neurobiological effect of GPER1 activation in resilient and non-resilient to chronic stress, female and male rats.

Additionally, our study displayed a rather unexpected CMS-induced phenotype characterized by lack of changes in sucrose preference, hyperlocomotion, and some anxiolytic-like features. CMS protocol is a well-established protocol for the

establishment of depressive-like behavior in rodents, valuable in depression research. The combination of mild stressors applied sequentially for a prolonged period of time lead to anhedonia, a core symptom of depression (Willner, Muscat and Papp, 1992). Apart from behavioral changes, CMS also causes endocrinological, neurochemical and neurobiological changes, affecting differently the two sexes (Dalla et al., 2005). As the hypothalamic–pituitary–adrenal (HPA) axis and the monoaminergic nervous system play an important role in stress, several studies have focuses on the effect of chronic stress upon these two compartments (Armario, 2006). Studies exhibit reduced levels of serotonin (5-HT) in the hippocampus and frontal cortex of chronically stressed rats (Ahmad *et al.*, 2010; He *et al.*, 2012), while results for dopamine (DA) and norepinephrine (NA) are inconsistent. Currently, glutamatergic neurotransmission is also thought to be involved in the neurobiology of anxiety (Simon and Gorman, 2006). Furthermore, CMS alters the expression of BDNF and CREB (Grønli *et al.*, 2006) and affects Akt-mTOR pathway (Zhu *et al.*, 2019; Liao *et al.*, 2021).

However, some studies have failed to induce anhedonia, as CMS did not significantly alter sucrose intake/preference, while other studies have shown anomalous findings (Willner, 2005). Underlying causes for the contradictory results and the insufficiency of CMS protocols reported in some studies are yet to be defined. To this direction, it has been shown that some rats show resiliency to developing exhibit depression- or anxiety-like behaviors despite their exposure to CMS. (Strekalova et al., 2005; Tang et al., 2019; Zurawek et al., 2019). Resiliency has been linked to several molecular, biochemical or neuroanatomical features. Among numerous findings, resiliency has been linked to proteomic changes, altered expression/activity of 5-HT1A, D2 and beta-adrenergic receptors, BDNF and mTOR (Willner, 2005; Sun *et al.*, 2017; Tang *et al.*, 2019; Tornese *et al.*, 2019; Zurawek *et al.*, 2019; Liao *et al.*, 2021). As in our research as well many other studies anhedonia was not achieved, it is apparent that features linked to resiliency and reasons behind CMS insufficiency must be studied. To better understand CMS neurochemical and neurobiological effect, we are aiming to compare animals either classified as resilient or as susceptible. Additionally, to investigate reasons of insufficiency, we will also examine these effects on the animals showing lack of changes in sucrose preference, hyperactivity, and reduced anxiety.

Overall, this project seeks to investigate the behavioral, neurochemical and neurobiological effect of GPER1's activation in anhedonic, female and male Wistar rats. This will help in understanding the mechanism behind its potential antidepressant and/or anxiolytic action. Simultaneously, features linked to resiliency to stress and to CMS insufficiency will also be examined. Taking into account the value of CMS in depression and stress research, increasing our understanding regarding its mechanisms would contribute to this field's research.

Research strategy

This project will be a continuation of the thesis project titled "Investigation of the impact of GPER1 activation on behavioral aspects in male and female rats after

chronic mild stress". In this study, CMS did not induce anhedonia, and GPER1 activation was studied in the context of a possibly stress-induced hyperactivity. To investigate the potential antidepressant and/or anxiolytic effect of GPER1, we will repeat the experiment, aiming to induce anhedonia. In parallel, in this project, we will try to answer questions that were developed during the thesis regarding CMS discrepancies, that are also observed in literature.

To this cause, female and male Wistar rats, housed in transparent cages, will spend four weeks of adaptation to consumption of 1% sucrose solution in water to establish baseline preference levels by performing sucrose preference test (SPT). During adaptation period, three sessions of SPT per week will be performed. Rats will be assigned to the control and CMS groups alternating from highest to lowest preference. Rats not showing sucrose preference were excluded from the following experiments. A slightly changed version of the CMS protocol already used by our team (Kafetzopoulos *et al.*, 2018) will be followed and it will last 4 weeks. Some of the applied stressors will be differentiated. In more detail, while overcrowding stressor will be applied to males, females will be subjected to spatial limitation within one third of their come cage to adjust to sex-dependent differences in behavior. Additionally, 400 ml of tap water will be added into the sawdust bedding instead of 250ml to soil their cage, and caged will be tilted at 45 degrees instead of 30. During CMS, animals will be subjected to SPT once a week. Anhedonia is expected to be achieved in a proportion of animals. Every animal will be examined separately and stratification of CMS animals to resilient and susceptible cohorts will be performed, as proposed in literature (Strekalova et al., 2022). In parallel, we will re-analyze the collected data of SPT during the thesis project to examine whether animals could be classified as resilient or susceptible. This step will clarify whether resilience to stress or insufficiency of CMS to induce anhedonia is the reason of the observed phenotype. After the end of CMS, the rats will be assigned to treatment groups. As previously, they will be treated with i.p. injections of vehicle, G1 or ketamine daily for three weeks. Then, they will be subjected to OF and LD tests. Finally, the following day after LD test, all rats will be killed by rapid decapitation and brains will be collected and stored at −80°C.

Apart from the behavioral effects, we aim to investigate the neurobiological and neurochemical effect of GPER1 activation. Ketamine-treated animals will be used as positive control, as es-ketamine is a fast-acting treatment for TRD. Additionally, we will examine CMS effect between resilient and susceptible rats. This will contribute in clarifying molecular elements of resilience. Attempting to understand if the lack of anhedonia is accompanied by lack of stress-related changes in a non-behavioral level, we will also incorporate the animals that did not exhibit anhedonia, during the thesis project. Rats not exhibiting anhedonia will be compared to susceptible and resilient rats. This will only reveal tendencies, knowing the limitations of these comparisons, however we are hoping this will shed light to the chronic stress mechanisms and to the literature discrepancies. Additionally, sex differences will be examined, as literature and our team's results indicate that CMS and G1 treatment affect the two sexes differently (Dalla *et al.*, 2005; Kastenberger and Schwarzer, 2014; Li *et al.*, 2021). In more detail, brains will be dissected and hippocampus,

hypothalamus and PFC will be isolated. All these brains area of interest for stress-related research (Kafetzopoulos *et al.*, 2018). Brain tissue will be homogenized to be analyzed either by high performance liquid chromatography (HPLC) or Western blot (WB). To study the neurochemical effect on neurotransmission, the monoamines 5-HT and its metabolite 5-HIAA, DA and its metabolites DOPAC and 3-MT, and NE and some metabolites and the amino acids GABA, glutamic acid will be analyzed by HPLC (Kokras et al., 2018). Additionally, BDNF, PSD-95, mTOR and p-mTOR levels in the brain areas will be measured by WB to examine the neurobiological level.

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Budget

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
1.Post-Doc Researcher(s)		**48,000**
2.PhD Candidate(s)		**36,000**
	Total Direct costs for Personnel	**84,000**
Other Direct Costs	**Justification**	
6.1.2 Consumables	WB and HPLC analysis materials	**11,000**
6.1.3 Travel	Transportation accommodation, daily expenses	**1,500**
6.1.4 Dissemination	Conference registration	**1,500**
6.1.5 Use and/or Access to equipment etc.		
6.1.6 Equipment	WB and HPLC equipment	**55,000**
6.1.7 Other Costs	Animal housing	**10,000**
6.1.8 Purchase of animals		**1,800**
	Total "other direct costs"	**80,800**
	Total Direct Costs	**164,800**
	Indirect Costs (Institution overhead, 10%)	**16,480**
	Total Budget	**181,280**