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1 **Behavioural characteristics and sex differences of a treatment-resistant**
2 **depression model: chronic mild stress in the Wistar-Kyoto rat**

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14
15 **Abstract**

16 Depression affects 20% of the general population and is a leading cause of disability
17 worldwide, with a strong female prevalence. Current pharmacotherapies have
18 significant limitations, and one third of patients are unresponsive. Male Wistar-Kyoto
19 rats exposed to chronic mild stress (CMS) were recently proposed as a model to study
20 antidepressant resistance. However, sex differences and interindividual vulnerability to
21 stress are yet unexplored in this model. We aimed to investigate these in the context
22 of the behavioural impact of CMS in the sucrose preference test, elevated plus maze
23 (EPM), forced swim test (FST), open field test and daily locomotor activity rhythms, in
24 male and female WKY rats exposed or not to a 4-week CMS protocol. CMS-exposed
25 animals were clustered through K-means into subgroups based on the EPM and FST
26 results. In both sexes, one subgroup behaved similarly to non-stressed animals and
27 was labelled stress-non vulnerable; the second exhibited less open arms exploration
28 in the EPM and higher immobility in the FST and was named stress-vulnerable.
29 Vulnerable males presented phase delay in daily locomotor activity following CMS, but
30 no significant rhythm could be determined in females. CMS-exposed males of both
31 groups showed hyperlocomotion in reaction to novelty and slower weight gain through
32 the course of CMS, while CMS-exposed females showed smaller sucrose intake.
33 Unexpectedly, CMS did not affect sucrose preference. Our findings strengthen the
34 view that in models of psychiatric pathologies based on stress exposure it is important

1 to consider the effect of sex and to differentiate the non vulnerable and vulnerable
2 subpopulations.

3

4 **Keywords**

5 Wistar-Kyoto rat; sex differences; depression model; chronic mild stress; vulnerability.

6

1. Introduction

Depression is a psychiatric disease which presents a major challenge to public health; according to the World Health Organisation, it is the primary cause of disability worldwide, with a prevalence of 10 to 20% of the general population [1]; notably, it is particularly prevalent in women, with a female/male ratio of 1.7:1 [2]. Depression is a disease significantly related to stress, considering that the experience of stressful events is an important risk factor for depressive episodes [3]. This is further amplified by genetic predispositions increasing vulnerability to stress [4]. Moreover, depressive patients show a dysregulation of the main stress response system, the hypothalamic-pituitary adrenal (HPA) axis, including weakened negative feedback processes, resulting in chronically elevated levels of blood cortisol [3]. A major concern regarding depression is that pharmacotherapies remain completely ineffective in about one third of the patients, whose disease is then labelled 'treatment-resistant depression' (TRD) [5]. One of the reasons why TRD remains difficult to overcome is poor knowledge of the underlying pathological brain processes, as well as the mechanisms through which other therapies such as deep brain stimulation or ketamine work. Nevertheless, a link between genetic mediated vulnerability to stress and resistance to conventional antidepressant treatment has been pointed out in humans, and experimentally observed in mice and non-human primates [6]. To further understand the physiological mechanisms of depression that ultimately lead to TRD, and study new therapeutic strategies, it is necessary to develop valid animal models [6].

As stress is a fundamental risk factor for depression, most valid animal depression-like models rely on stress exposure, based for example on social defeat [7]. The most commonly used stress-based model, however, relies on the exposure of rodents to a chronic mild stress (CMS) protocol, which includes several weeks of exposure to factors such as temporary restriction to access to water and/or food, modification of the light/dark cycle and wet bedding [8]. Recently, Paul Willner and collaborators [9] demonstrated that male Wistar-Kyoto (WKY) rats exposed to a CMS procedure showed impairments reminiscent of a depressive-like phenotype in comparison to non-stressed control animals [9]. These deficits were improved following subacute ketamine treatment or deep brain stimulation of the ventromedial prefrontal cortex, whereas the conventional antidepressant drugs imipramine, citalopram and

1 venlafaxine were ineffective, conferring value of this model as one to study
2 antidepressant resistant phenotype.

3 Nonetheless, important aspects of this model were left unexplored and are
4 consequently addressed in the present study. First of all, numerous studies have raised
5 the question of stress-related sex differences in the WKY strain, observing sex-
6 dependent differences in tests evaluating anxiety-like behaviours when compared to
7 other strains [10]; furthermore, a higher physiological response to stress in females
8 has been indicated [11,12]. However, the question still remains overall unclear and
9 poorly explored. Thus, the first aim of the present study was to focus on sex
10 differences, an urgent need, as stated by Millard and colleagues [13]. Second of all,
11 there exists strong interindividual vulnerability to stress due to genetic and epigenetic
12 differences [14], so that in this type of experimental procedure there is the need to
13 differentiate vulnerable from non vulnerable animals. Furthermore, although the WKY
14 strain of rats is labelled as “inbred”, substrains with different characteristics can be bred
15 from it [15], so that it is possible the remaining genetic heterogeneity in the strain may
16 influence vulnerability to the CMS procedure. Therefore, the second aim of the study
17 was to evaluate if interindividual differences in the response to CMS may be identified
18 in male and female WKY rats. Finally, important behavioural aspects possibly
19 influenced by chronic stress exposure remain yet unexplored, the investigation of
20 which might increase our knowledge of the impact of CMS on WKY rats; therefore, in
21 addition to the already performed exploration of anhedonia, body weight gain, and
22 anxiety-like behaviour [9], our third goal was to explore new behavioural traits such as
23 reaction to novelty, coping strategies in the face of an unescapable stressful situation,
24 as maladaptive coping strategies to stressful life events correlate with depressive
25 symptoms and are associated with more severe depression [16,17], as well as daily
26 locomotor activity rhythm, as alterations of daily activity rhythm are important
27 components of depression [18,19].

28

1 **2. Materials and methods**

2 **2.1 Animals**

3 This study, authorised by the French authorities (APAFIS #25270), was carried out
4 with 70 WKY rats, 40 females and 30 males (Charles River, Sulzfeld, Germany). They
5 arrived at the laboratory at the age of 4 to 5 weeks. They were housed in cages of 5
6 individuals for 1 week after their arrival, so they could acclimatise to the new housing
7 conditions. They were then assigned to the different conditions; rats destined to the
8 CMS protocol were housed individually while control animals were housed in pairs;
9 males and females of the CMS condition were housed in the same room (3 meters
10 apart), and male and female controls were housed together (3 meters apart) in a
11 separate room. They were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.)
12 with *ad libitum* access to food and water – except animals of the CMS condition
13 according to the protocol (see below) –, with controlled temperature ($23 \pm 1^\circ\text{C}$) and
14 hygrometry of about 55 %. One week after their arrival all rats were provided
15 maintenance food (Altromin 1324) and remained on this diet during the entire study.
16 Groups were composed as follows: males of the control condition (n = 6); males of the
17 CMS condition (n = 24); females of the control condition (n = 8); females of the CMS
18 condition (n = 32).

19 **2.2 Experimental design**

20 The timeline of the study is described in **Figure 1**.

21 On week 3, rats were allowed to consume a 0.5 % sucrose solution for 24 h in order
22 to prevent neophobia during subsequent sucrose preference test (SPT). On week 4,
23 they underwent home-cage locomotor activity testing (pre-CMS evaluation). On week
24 5, they were subjected to SPT and were weighed in order to obtain baseline measures.
25 Then the stressors began to be applied in a sequence of events (see **Table 1**) which
26 was repeated every week up until week 9, when the 4th and final intra-CMS SPT and
27 weight measures were collected. Finally, animals underwent post-CMS behavioural
28 testing during 3 weeks (week 10 to 12), including 24-h locomotor activity testing (post-
29 CMS evaluation), then open field (OF) the day following the end of locomotor
30 evaluation. The forced swim test (FST) sessions were conducted 48 h after the OF,
31 and the elevated plus maze (EPM) the day after the second FST session; the three
32 latter tests were performed between 9:00 AM and 1:00 PM (i.e., light phase) when
33 CORT secretion, an indicator of the activity of the stress response HPA axis, is minimal

1 and stable [20]. To fit this schedule, females were tested in two subgroups of 20
 2 individuals, and males in two subgroups of 15 individuals.

3 **2.3 CMS protocol**

4 The CMS protocol lasted for four weeks and consisted of the following events (see
 5 **Table 1**): positioning of a metal plate in the bottom of the cage to isolate rats from the
 6 bedding; stroboscopic illumination (120 flashes/min) during the dark phase; food
 7 and/or water deprivation; reduction of space in the cage; intruder session; intermittent
 8 illumination (light on and off every 2 h) during the dark period; loud white noise (74 +/-
 9 1 dB); soaking of the bedding (600 ml water poured into the cage).

10 **Table 1.** Weekly schedule during the CMS protocol

Week day	Morning	Afternoon
Day 1	New cage with clean bedding provided (09 AM). Start of SPT (11 AM).	Ongoing SPT
Day 2	End of SPT, weighing of the rats (11 AM). Regular water bottle returned, and metal plate placed on the bedding (12 AM).	Removal of metal plate (6 PM). Start of stroboscopic light (8 PM).
Day 3	End of stroboscopic light (6 AM). Food removed (9 AM).	Food returned (5 PM). Reduction of space (6 PM).
Day 4	Return to normal space (9 AM). Water removed (10 AM).	Water returned (5 PM). Start of intruder session (6 PM).
Day 5	End of intruder session (9 AM).	Start of intermittent illumination (7PM).
Day 6	Loud white noise ON (9 AM).	Loud white noise OFF (5 PM). Reduction of space (6 PM).
Day 7	Return to normal space (9 AM). Food and water removed (10 AM).	Food and water returned (5 PM). Soaking of the bedding (6PM).

11

12 **2.4 SPT**

13 Sucrose preference was evaluated once weekly for 24 h. The regular water bottle was
 14 removed, and replaced by two new bottles, one containing 200 mL of water and one
 15 containing a 200 mL of 0.5 % sucrose solution. Bottles were placed at 11:00 AM on

1 Day 1 and removed at 11:00 AM the following day. The position of the water and
2 sucrose bottles was alternated from one week to the other. Bottles were weighed
3 before and after the test and the percentage of sucrose preference was assessed using
4 the following formula [100 x (Weight difference of sucrose bottle / Weight difference of
5 sucrose bottle + weight difference of water bottle)]. Then, sucrose preference
6 measures obtained each week of the CMS procedure were indexed to the pre-CMS
7 baseline measure. The consumption of sucrose and water were also calculated for
8 each SPT in g consumed per g of body weight.

9 **2.5 Body weight**

10 Throughout the entire study, from their arrival until the end of the experimental
11 procedure, animals were weighed once a week. After the start of the CMS protocol,
12 the body weight was calculated as a percentage of the baseline (pre-CMS) measure
13 to evaluate body weight gain during the 4 weeks of CMS.

14 **2.6 Behavioural tests**

15 **2.6.1 Locomotor activity in the home cage**

16 Locomotor activity was assessed in a novel home cage by means of two infrared light-
17 beams perpendicular to the width of the cage, placed 4.5 cm above cage floor level
18 and 28 cm apart along the length of the cage. The consecutive interruptions of both
19 light beams were counted as one cage crossing. During both pre- and post-CMS
20 sessions, rats were transported from the housing room to the test room, placed in their
21 respective testing cage, and recording was started at 10 AM for a 24-h duration. The
22 activity during the first hour is influenced by some novelty due to the changes of room
23 and cage, as well as the novel sawdust bedding [21] and is usually larger than the
24 baseline diurnal activity. To assess locomotor reactivity to novelty, the first 10 min were
25 analysed (identical duration to that used in the OF, which was also used to evaluate
26 locomotor response to novelty; see 2.5.2). Moreover, we analysed the pattern of daily
27 locomotor activity rhythm. Two-hour intervals of locomotor activity values were fitted
28 by a non-linear least-squares COSINOR regression analysis [22] using the SigmaPlot
29 Software (Systat Software Inc., version 12.5), with the following fitting equation:

30
$$Y = A + B \left(2\pi * \frac{X - C}{24} \right)$$

1 where Y is the pooled number of crossings of the cage, A the MESOR (Midline
2 Estimating Statistic Of Rhythm) or the mean level of the oscillation, B the amplitude of
3 the oscillation, C the acrophase (time of the maximal value of the oscillation), X the
4 time (h), with a period set at 24 hours. Only significant best-fit parameters ($p < 0.05$)
5 were included in this study if the residues of the fit respected the analysis of variance
6 (ANOVA) rules.

7 **2.6.2 OF**

8 The test was performed in a square device (70 x 70 cm) comprising 50-cm high walls.
9 A paper sheet comprising a mosaic of squares of equivalent sizes (14 x 14 cm, giving
10 5 x 5 squares) was placed under the clear Plexiglas floor of the OF in order to assess
11 locomotor activity by numbering the crossings of the different squares. A crossing was
12 counted when all 4 paws passed the delimitation between 2 adjacent squares. The
13 number of rearings was also counted. The OF was cleaned with water and 70% ethanol
14 between each rat. Rats were transported from their housing room to the room adjacent
15 to the test room 1 hour before the start of the test. The test started when the rat was
16 gently placed facing the wall in one corner of the OF, and lasted for 10 minutes. The
17 test session was recorded by means of a camera situated above the OF.

18 **2.6.3 FST**

19 The FST was performed as previously described by Gardner and colleagues [23] in a
20 Perspex cylinder filled with water (21–23°C) high enough so that the rats' tail could not
21 touch the bottom of the cylinder. The test took place over two days; on each day rats
22 were brought to the room and immediately tested. On day 1, the animals underwent a
23 15–minutes period of swimming as habituation, and the following day were tested over
24 6 minutes. The test session was recorded by means of a camera placed on the side of
25 the cylinder, and the amount of time spent immobile (4 limbs immobile, with no
26 swimming or struggling) was subsequently manually scored and expressed as the
27 percentage of total time.

28 **2.6.4 EPM**

29 The apparatus was made of black Plexiglas, elevated 73 cm above the floor, and
30 consisted of four arms (50 cm × 10 cm), with two opposing closed arms comprising 40
31 cm–high walls and two opposing open arms comprising 1.5 cm–high borders, fixed to
32 a central platform (10 x 10 cm) forming a cross shape. Light intensity was 10 lx in open
33 arms, 7 lx at the center of the maze, and 2.5 lx in closed arms. Rats were first brought

1 to a room adjacent to the testing room; after an hour, they were placed in a new
2 sawdust-free cage and brought into the testing room. The test began following 5
3 minutes of habituation to the room. Testing began when rats were placed on the central
4 platform of the maze with their head facing one of the closed arms; it lasted for 5
5 minutes and was recorded by means of a camera situated above the maze. The maze
6 was cleaned with water and 70 % ethanol between each rat. The collected data
7 included the number of entries and time spent in each of the four arms. Analysed data
8 was the time spent in the open arms in percentage of the total time spent in the four
9 arms.

10 **2.7 Statistical analyses**

11 First, on the basis of EPM and FST performances, CMS-exposed animals were
12 subcategorized in clusters (see Supplementary Materials and **Figure S1**). For
13 statistical analyses, according to the EPM and FST, following clustering, performance
14 of each cluster was compared to this of their respective control using pairwise
15 Student's t-test. Other data were subjected to analysis of variance (ANOVA) with
16 Group and Sex as between-subject factors and, when appropriate, with Week or Period
17 (pre- vs post-CMS) as within-subject factors. In some cases, justified in the text, data
18 for males and females were analysed separately in additional ANOVAs with Group as
19 the only between-subject factor. The ANOVAs were completed by post hoc
20 comparisons using the Newman–Keuls multiple range test. To assess the impact of
21 CMS on the rhythms of locomotor activity, amplitude, mean and acrophase of rhythms
22 were compared between the pre- and post-CMS condition for each group using a
23 paired t-test. The threshold for rejecting the null hypothesis was 0.05 throughout.

24

1 **3. Results**

2 **3.1 Identification of “CMS-non vulnerable” and “CMS-vulnerable” animals**

3 For each sex, the performance (% time in open arms in the EPM and % immobility in
4 the FST) of clusters 1 and 2 revealed by the K-means algorithm (see Supplementary
5 Materials and **Figure S2**) were compared to that of control animals (**Figure 2**). In both
6 sex, there was no significant difference between cluster 1 and controls in both tests
7 [Females: EPM: $t(17)=-0.95$, $p > 0.3$; FST: $t(17)=1.20$, $p > 0.2$; Males: EPM: $t(15)=0.38$,
8 $p > 0.7$; FST: $t(15)=0.41$, $p > 0.6$], whereas there was a significant difference between
9 cluster 2 and controls in both tests [Females: EPM: $t(24)=-5.22$, $p < 0.0001$; FST:
10 $t(24)=2.63$, $p=0.01$; Males: EPM: $t(17)=-5.04$, $p < 0.001$; FST: $t(17)=2.52$, $p=0.02$].
11 Overall, these results indicated that, whatever the sex, performance of animals of
12 cluster 1 was similar to that of control animals so that for each sex cluster 1 was
13 hereafter referred to as “CMS-non vulnerable”; animals of cluster 2 presented a lower
14 proportion of time in the open arms of the EPM and a higher proportion of time in
15 immobility in the FST when compared to controls, so that for each sex cluster 2 was
16 hereafter referred to as “CMS-vulnerable”. The difference in proportion of vulnerable
17 vs non vulnerable animals between males (46% vs 54%) and females (38% vs 62%)
18 was not significant $X^2(1,53) = 0.34$, $p > 0.5$).

19 **3.2 Sucrose preference test**

20 **3.2.1 Sucrose preference**

21 At baseline (**Figure 3A**), all groups showed preference measures significantly above
22 50% (at least $p < 0.00001$). The ANOVA indicated no significant effect of Sex ($F(1,61)$
23 $= 0.035$, $p > 0.8$), no significant effect of Group ($F(2,61) = 1.59$, $p > 0.2$), and no
24 significant Sex x Group interaction ($F(2,61) = 0.02$, $p > 0.9$). Overall, these analyses,
25 indicated that at baseline, all groups presented similar preference for sucrose over
26 water. In order to evaluate the evolution of the sucrose preference during the 4 weeks
27 of the CMS procedure, data were indexed to baseline (**Figure 3B-C**). Analysis
28 indicated no significant effect of Group ($F(2,61) = 2.03$, $p > 0.1$), but a significant effect
29 of Sex ($F(1,61) = 6.31$, $p = 0.01$) and a significant effect of Week ($F(3,183) = 13.94$, p
30 < 0.000001). There was no significant interaction of Group x Sex ($F(2,61) = 0.12$, $p >$
31 0.8), Group x Week ($F(6,183) = 0.78$, $p > 0.5$), Week x Sex ($F(3,183) = 2.50$, $p > 0.05$)
32 or Group x Sex x Week ($F(6,183) = 0.50$, $p > 0.8$). The post hoc conducted on the
33 Week effect indicated significant differences between all weeks except between week

1 2 and week 3 ($p = 0.04$ for week 2 vs week 4, and $p < 0.01$ minimum for all other
2 comparisons). Overall, these analyses indicated that the preference for sucrose
3 (**Figure 3B-C**) increased throughout the 4 weeks of CMS in control and CMS-exposed
4 rats and that this increase was initially higher in female than in male.

5 **3.2.2 Sucrose intake**

6 Analysis indicated a significant effect of Sex ($F(1,61) = 83.64$, $p < 0.000001$), a
7 significant effect of Group ($F(2,61) = 5.96$, $p < 0.01$) and a significant Sex x Group
8 interaction ($F(2,61) = 6.72$, $p < 0.01$). In addition, there was a significant effect of Week
9 ($F(4,244) = 5.32$, $p < 0.001$), and significant Week x Sex ($F(4,244) = 2.60$, $p = 0.04$),
10 Week x Group ($F(8,244) = 2.64$, $p < 0.01$) interactions, whereas there was no
11 significant Week x Sex x Group ($F(8,244) = 0.87$, $p > 0.5$) interaction. As the Sex X
12 Group interaction was significant, analyses were performed separately by sex. In
13 females (**Figure 4A**), there was a significant effect of Group ($F(2,34) = 11.14$, $p <$
14 0.001) and of Week ($F(4,136) = 4.22$, $p < 0.01$), and a significant Group x Week
15 interaction ($F(8,136) = 2.22$, $p = 0.03$). Post hoc analysis indicated that while there was
16 no between-Group difference in terms of baseline intake ($0.48 < p < 0.73$), sucrose
17 intake of control animals significantly differed from that of both CMS groups during the
18 first two weeks of CMS (week 1: $p = 0.01$ controls vs non vulnerable, $p < 0.01$ controls
19 vs vulnerable ; week 2: $p < 0.01$ controls vs non vulnerable, $p < 0.01$ controls vs
20 vulnerable). While a significant difference was maintained with CMS-vulnerable
21 animals throughout CMS (week 3: $p = 0.03$, week 4: $p = 0.04$), the difference was no
22 longer significant with CMS-non vulnerable animals as from week 3 ($0.059 < p < 0.1$).
23 In males (**Figure 4B**), there was a significant effect of Week ($F(4,108) = 4.23$, $p < 0.01$)
24 but no significant effect of Group ($F(2,27) = 0.03$, $p > 0.9$) and no significant Group x
25 Week interaction ($F(8,108) = 1.45$, $p > 0.1$). Post hoc analysis of the Week effect
26 indicated a significant decrease between baseline and all weeks of the CMS protocol
27 ($p = 0.02$ for baseline vs week 1, $p = 0.05$ for baseline vs week 2, $p < 0.01$ for baseline
28 vs week 3, $p = 0.02$ for baseline vs week 4), whereas there was no difference across
29 the 4 weeks of CMS ($0.10 < p < 0.93$ among all comparisons).

30 **3.2.3 Water intake**

31 Analysis indicated a significant effect of Week ($F(4,244) = 43.47$, $p < 0.000001$) and a
32 significant Week x Sex interaction ($F(4,244) = 5.68$, $p < 0.001$). However, there were
33 no significant effect of Sex ($F(1,61) = 0.002$, $p > 0.9$), nor Group ($F(2,61) = 1.66$, $p >$

1 0.1), and no further significant interaction, whether it was Sex x Group ($F(2,61) = 0.46$,
2 $p > 0.6$), Week x Group ($F(8,244) = 1.52$, $p > 0.1$), or Week x Sex x Group ($F(8,244) =$
3 0.55 , $p > 0.8$). Post hoc on the Week x Sex interaction indicated that: i) In females
4 (**Figure 4C**) there was a significant difference between baseline intake and weekly
5 intake during the CMS procedure ($p < 0.0001$), and between intake at week 1 vs week
6 2 ($p < 0.01$) and week 4 ($p < 0.0001$) of the CMS procedure, and, ii) in males (**Figure**
7 **4D**), there was a significant difference between baseline intake and intake at week 2
8 ($p < 0.001$), week 3 ($p < 0.01$) and week 4 ($p < 0.0001$) of the CMS procedure, between
9 intake at week 1 and each of the following weeks of the CMS procedure ($p < 0.0001$
10 for each comparison), and between week 3 and week 4 of the CMS procedure ($p =$
11 0.04). Overall, these analyses indicated that females consumed more sucrose than
12 males. CMS-exposed females also consumed less sucrose than controls as from week
13 1 of CMS but this difference was no longer significant for CMS-non vulnerable females
14 as from week 3. Males presented a slight decrease in their sucrose intake after the
15 baseline, regardless of group. In both sexes, water intake decreased with time.

16 **3.3 Weight gain**

17 Analysis indicated a significant effect of Sex ($F(1,61) = 13.47$, $p < 0.001$) but no
18 significant effect of Group ($F(2,61) = 0.91$, $p > 0.4$) and no significant Sex x Group
19 interaction ($F(2,61) = 1.46$, $p > 0.2$). In addition, there was a significant effect of Week
20 ($F(3,183) = 822.60$, $p < 0.000001$) and significant Group x Week ($F(6,183) = 5.86$, $p <$
21 0.0001), Sex x Week ($F(3,183) = 12.57$, $p < 0.000001$) and Group x Sex x Week
22 ($F(6,183) = 4.81$, $p < 0.001$) interactions. Analyses were thus performed by sex. In
23 females (**Figure 5A**), the analysis showed no Group effect ($F(2,34) = 1.5$, $p > 0.2$), but
24 a significant Week effect ($F(3,102) = 292$, $p < 0.0001$). Even if a significant interaction
25 between Group and Week was present ($F(6,102) = 2.78$, $p = 0.02$), the subsequent
26 post hoc showed no difference between groups at each week ($0.182 < p < 0.972$),
27 though a significant difference between each week ($p < 0.01$ at least) within each
28 group. In males (**Figure 5B**), the analysis indicated no Group effect ($F(2,27) = 0.87$, p
29 > 0.4) but both Week ($F(3,81) = 638$, $p < 0.00001$) and Week X Group ($F(6,81) = 9.10$,
30 $p < 0.00001$) effects were significant. Post hoc analysis on the interaction showed a
31 significant difference between controls and both CMS groups at week 4. It also
32 indicated that in each group, all weeks were different from each other's ($p < 0.001$ at
33 least). Overall, these analyses indicated that while all groups naturally gained weight

1 during the course of the experiment, CMS decreased weight gain at the end of the
2 CMS procedure in males of both CMS-exposed groups whereas it did not affect weight
3 gain in females.

4 **3.4 Locomotor response to novelty**

5 **3.4.1 In a new home-cage (first ten minutes of recording during the pre- and the** 6 **post-CMS tests)**

7 Analysis of the number of cage crossings indicated a significant effect of Group
8 ($F(2,61) = 4.91, p = 0.01$), no significant effect of Sex ($F(1,61) = 2.28, p > 0.1$), and no
9 significant Group x Sex interaction ($F(2,61) = 1.05, p > 0.3$). There was no significant
10 effect of Period ($F(1,61) = 0.79, p > 0.3$), no significant Period x Sex interaction ($F(1,61)$
11 $= 3.49, p > 0.06$) and Period x Group ($F(2,61) = 1.63, p > 0.2$) interactions. However,
12 there was a significant Period x Sex x Group interaction ($F(2,61) = 4.65, p = 0.01$).
13 Therefore, analyses were performed in both sexes separately.

14 In females, analysis indicated a significant effect of Period ($F(1,34) = 4.29, p = 0.05$)
15 but no significant effect of Group ($F(2,34) = 0.85, p > 0.4$) and no significant Period x
16 Group interaction ($F(2,34) = 0.47, p > 0.6$). Visualisation of **Figure 6A** indicates that
17 female rats were generally less active during the post-CMS locomotor activity
18 assessment, in all subgroups, when compared to the pre-CMS. In males, analysis
19 indicated no significant effect of Period ($F(1,27) = 0.43, p > 0.5$) but a significant effect
20 of Group ($F(2,27) = 4.59, p = 0.02$) in addition to a significant Period x Group interaction
21 ($F(2,27) = 5.05, p = 0.01$). Post hoc analysis indicated, at the post-CMS time point, a
22 significant difference between animals of the control group and the CMS-vulnerable
23 group ($p < 0.001$). At this time point, the differences between the CMS-non vulnerable
24 and the two other groups did not reach significance ($p = 0.08$ for each comparison)
25 (**Figure 6B**). Overall, these analyses revealed in females a general decrease of
26 reactivity during the post-CMS session; in males, they revealed that following CMS
27 both CMS-exposed subgroups had an increased reactivity to novelty, which was
28 exacerbated in CMS-vulnerable animals.

29 **3.4.2 In the OF**

30 Analyses of the numbers of crossings and rearings during the 10-min test indicated no
31 significant effect of Group (crossings: $F(2,61) = 2.06, p > 0.1$; rearings: $F(2,61) = 1.46,$
32 $p > 0.2$), no significant effect of Sex (crossings: $F(1,61) = 2.59, p > 0.1$; rearings:
33 $F(1,61) = 0.80, p > 0.3$), and no significant Group x Sex interaction (crossings: $F(2,61)$

1 = 2.35, $p = 0.06$ 0.1; rearings: $F(2,61) = 2.12$, $p > 0.1$). Visualisation of the figures
2 nonetheless suggested the presence of significant differences in males, so that data
3 of each sex were separately analysed. In females, no Group effect was evidenced for
4 the number of crossings and rearings ($F(2,34) = 0.28$, $p > 0.7$; $F(2,34) = 0.08$, $p > 0.9$;
5 **Figures 6C-D**). In males, a significant Group effect was revealed for both variables
6 (crossings: $F(2,27) = 3.62$, $p = 0.04$, **Figures 6C**; rearings: $F(2,27) = 3.82$, $p = 0.03$,
7 **Figures 6D**). Post hoc analysis indicated a significant difference between CMS-
8 exposed animals and controls (in crossings: $p = 0.04$ for controls vs non vulnerable,
9 and $p = 0.02$ for controls vs vulnerable; in rearings: $p = 0.04$ for controls vs non
10 vulnerable, and $p = 0.02$ for controls vs vulnerable), the former not differing from each
11 other ($p > 0.4$ minimum). Overall, these analyses indicated that while females
12 presented no between-group differences, CMS-exposed males of both subgroups
13 presented a significantly higher number of rearings and crossings showing a greater
14 exploratory response in a novel environment.

15 **3.5 Daily rhythms of locomotor activity**

16 **3.5.1 In females**

17 In the control (**Figures 7A**) and CMS-non vulnerable (**Figures 7B**) groups, there was
18 a significant daily rhythm of locomotor activity before CMS exposure (cosinor, $p = 0.03$
19 and $p < 0.001$, respectively); however, no significant rhythmic activity was found after
20 CMS exposure (cosinor, $p > 0.5$). In the CMS-vulnerable group (**Figure 7C**) there was
21 a significant daily rhythm of locomotion both in the pre-CMS (cosinor, $p < 0.001$) and
22 post-CMS (cosinor, $p < 0.001$) periods. When we compared the rhythmic parameters
23 between periods, only amplitude of rhythm was significantly different between the pre-
24 CMS (9.8 ± 0.7) and post-CMS (7.2 ± 0.6) periods, indicating a reduced amplitude after
25 CMS exposure ($t(34) = 2.74$; $p < 0.001$). No difference was found in the mean values
26 of daily rhythms between pre-CMS (5.4 ± 1.0) and post-CMS (4.3 ± 0.9) ($t(34) = -0.78$;
27 $p > 0.4$), nor in the acrophase (pre-CMS, 13.3 ± 0.7 vs. post-CMS, 14.9 ± 0.8 ; $t(34) = -$
28 1.48 , $p > 0.1$).

29 **3.5.2 In males**

30 In all three groups (**Figure 8A-C**), there was a significant daily rhythm of locomotor
31 activity at both the pre-CMS (cosinor $p = 0.01$ for controls, and $p < 0.001$ for CMS-
32 exposed animals) and post-CMS (cosinor $p < 0.01$ for controls, and $p < 0.001$ for CMS-
33 exposed animals) periods. There was a significant reduction in amplitude between the

1 pre- and the post-CMS period in all groups (control: 8.6 ± 1.02 vs 5.1 ± 0.4 ; $t(10) =$
2 3.11 ; $p = 0.01$; CMS-non vulnerable: 8.3 ± 0.9 vs $4.3 \pm 0.$; $t(22) = - 3.94$; $p < 0.001$;
3 CMS-vulnerable: 7.7 ± 0.6 vs 4.7 ± 0.3 ; $t(22) = 3.92$; $p < 0.001$). In none of the groups
4 were there significant differences concerning the mean value between the two time
5 points (control: 4.3 ± 1.4 vs 2.1 ± 0.6 ; $t(10) = 1.42$; $p > 0.1$; CMS-non vulnerable: $3.4 \pm$
6 1.3 vs 2.5 ± 0.5 ; $t(22) = - 0.63$; $p > 0.5$; CMS-vulnerable: 4.9 ± 0.94 vs 2.7 ± 0.5 ; $t(22)$
7 $= 1.98$; $p > 0.05$). However, concerning the acrophase, whereas there was no
8 difference between the two periods in the control (11.8 ± 1.2 vs 12.07 ± 1.1 ; $t(10) = -$
9 0.11 ; $p > 0.9$) and CMS-non vulnerable (13.7 ± 1.4 vs 14.4 ± 0.8 ; $t(22) = 0.44$, $p > 0.6$)
10 groups, a significant difference was found in the CMS-vulnerable group (13.2 ± 0.7 vs
11 15.8 ± 0.7 ; $t(22) = - 2.52$; $p = 0.01$), indicating a phase delay (2.56 h) of the daily rhythm
12 of locomotor activity after CMS exposure (**Figure 8C**).

13 Overall, these analyses and observation of figures 7 and 8 indicated that all groups
14 presented a significant rhythmic pattern of locomotor activity pre-CMS, but while such
15 a pattern was still present in males during the second testing period, only females of
16 the CMS-vulnerable group still had a significant rhythm of locomotor activity at the
17 second period. Rhythms at the second testing period consistently presented a reduced
18 amplitude regardless of the group, and CMS-vulnerable males showed a phase delay
19 in their daily rhythm of locomotor activity.

20

1 **4. Discussion**

2 Our goal was to improve knowledge about the impact of CMS on WKY rats through
3 the study of interindividual differences and the exploration of differences linked to sex
4 on never studied behavioural features including the reaction to novelty, coping
5 strategies in the face of an unescapable stressful situation, as well as daily activity
6 rhythm.

7 Regarding inter-individual differences, k-means clustering based on behavioural
8 results in the EPM and FST, identified two distinct clusters in both male and female
9 WKY rats exposed to CMS. Interestingly, in both tests one behaved similarly to the
10 control group and was thus called “CMS-non vulnerable”. In contrast, the second
11 cluster presented significant behavioural differences in both tests compared to the so-
12 called “CMS-non vulnerable” group and to the control group. In both males and
13 females, CMS-vulnerable animals presented a significantly smaller percentage of time
14 in the open arms of the EPM, and a higher percentage of immobility in the FST,
15 suggesting higher anxiety-like-related behaviour and a more passive coping strategy
16 in the face of a stressful situation [24] in these animals compared to controls and CMS-
17 non vulnerable rats. Higher anxiety-like behaviour in the EPM and immobility in the
18 FST are often reported as a consequence of chronic stress in both male and female of
19 different strains [25–30] including the WKY, at least in males [9]. However, these
20 studies do not consider interindividual differences in vulnerability to stress. Our study
21 shows that in both sexes, only some individuals were affected, while others showed a
22 non vulnerable profile. Thus, even in this strain, considered as inbred, not all animals
23 responded in the same way to CMS, raising the question of what factors influence
24 individual vulnerability to stress. The two main causal factors may be genetic
25 differences and/or environmental factors. Concerning the former, phenotypic and
26 genotypic variability among WKY rats from the same colony have already been
27 reported, to the point where selective breeding allows the generation of distinct WKY
28 substrains [15]. Concerning environmental factors, it has been shown that rodent
29 maternal care, such as pup licking and grooming, very often differs in amount between
30 pups of the same litter [31], and leads to epigenetic modifications altering vulnerability
31 to stress [31–33]. In parallel, clinical studies point out stressful life events [34–36], in
32 particular early life events [35,36], as major risk factors for vulnerability to depression.
33 As Strekalova and colleagues pointed out, interindividual variability in the susceptibility

1 to stress is an important factor of any study exploring the behavioural and physiological
2 consequences of stress exposure; some animals show vulnerability, i.e. present lasting
3 consequences, while others are non vulnerable, so that a stratification of CMS-
4 exposed animals into such categories is recommended [14]. Our study shows that
5 using EPM and FST performance as segregation criteria, male and female WKY rats
6 can be separated into CMS-non vulnerable and CMS-vulnerable groups. These
7 behavioural characteristics are of great interest, as in humans, stressful life events are
8 a particularly important causal factor for depression onset [37] in conjunction with
9 dysfunctional processing and reaction to the situation, i.e. maladaptive coping,
10 constituting an essential core of vulnerability [35]. Comorbid anxiety is equally relevant,
11 as elevated anxiety is associated with greater severity of depression [38,39] and has
12 been suggested to be core feature of depression [39].

13 Our data also indicated that only CMS-vulnerable males displayed a phase delay of
14 their daily locomotor activity rhythms. It is a known fact that glucocorticoids can
15 modulate rhythms and shift circadian phases [40], and phase shifts have previously
16 been observed as a consequence of CMS in male Wistar rats, although as phase
17 advances [41]. In parallel, depressed patients often present sleep and circadian rhythm
18 disturbances [18] including phase delays of activity rhythms and hormonal release [19].
19 Unfortunately, as in females the daily rhythm of locomotor activity was not significant
20 at the second time point for controls and CMS-non vulnerable animals, analysis is
21 limited. The absence of a significant rhythm in those two groups may be due to the
22 great variability of activity onset in females compared to males, as it is strongly
23 modulated by the estrus cycle [42], the phase of which was not controlled for in the
24 present study. Nonetheless, CMS-vulnerable females, who did present a post-CMS
25 rhythm, did not display a phase delay. This difference with males could be the result of
26 a differential impact of stress on clock genes, which regulate circadian rhythms,
27 between females and males that would lead to greater impairments in the latter. A
28 study conducted on Sprague-Dawley rats found sex specific effects of acute stress on
29 the expression of clock genes, increasing the expression of the Per2 gene in the
30 prefrontal cortex and hypothalamic paraventricular nucleus in females but not males
31 [43]. However, this study did not consider locomotor rhythms and the authors
32 emphasise that additional investigations of sex differences in the impact of acute and
33 chronic stress on molecular clock rhythms are needed.

1 While the behaviour of CMS-vulnerable and non vulnerable animals differed in the
2 EPM and FST, the chronic stress regimen affected other physiological or behavioural
3 characteristics similarly in both groups, albeit in a sex-dependent manner. First, CMS
4 impaired body weight gain in males only, in line with studies conducted in other strains
5 such as Wistar [44,45], and Sprague-Dawley [46], and consistent with Willner's study
6 on male WKY rats [9]. The fact that deficits in body weight gain were observed in male
7 rats of both CMS subgroups suggests that despite presenting no impairments in terms
8 of behavioural performance in the EPM, FST, and phase of the locomotor activity
9 rhythm, CMS-non vulnerable male rats may still present CMS-induced metabolism
10 impairments. This has previously been observed in the Wistar rat, in which the CMS-
11 exposed group classified as "resilient" - albeit based on sucrose consumption or
12 preference results - presented similar body weight gain deficits as the vulnerable group
13 [47,48]. In contrast, CMS exposure did not affect body weight gain in females, which
14 is coherent with previous studies in female Wistar [44,49] and Sprague-Dawley [46,50]
15 rats. Furthermore, chronic administration of corticotropin releasing factor, which
16 stimulates glucocorticoid release and which production is increased with CMS
17 exposure [51], reduces body weight gain in male but not female Wistar rats, a
18 differential metabolic response attributed to sex hormones, further suggesting a
19 protective role against stress-induced metabolism impairments in females [52].

20 Secondly, we observed CMS-enhanced locomotor reaction to novelty in males, but not
21 females. Indeed, only the former presented higher exploratory behaviour, in the OF
22 and in a new home-cage during the first 10 minutes of actigraphy testing, although in
23 the last case the CMS-non vulnerable group was below but near the significance
24 threshold. The fact that locomotor activity during the remainder of the day, in both the
25 light and dark periods, was similar in both CMS-exposed and control rats (data not
26 shown), and that there was no inter-group difference prior to CMS, suggests that this
27 enhanced activity is both provoked by the novelty of the situation, and is due to CMS
28 exposure. Although some studies report a decrease in locomotor activity in the OF
29 following CMS [53,54], impairments such as increased locomotor activity of CMS-
30 exposed males has been observed previously in the OFT in Wistar [55] and Sprague-
31 Dawley [56] rats. Furthermore, Strekalova and colleagues pointed out that rats
32 exposed to CMS can display non-specific hyperlocomotion and general behavioural
33 invigoration as a response to the slightly stressful procedure of behavioural testing [14]

1 which may explain the difference observed in the present study. A similar sex-specific
2 pattern of enhanced locomotion in males and no changes in females following CMS
3 has been observed previously in Sprague Dawley rats [57].

4 Unexpectedly, CMS did not decrease the sucrose preference in either females or
5 males. In fact, all groups, including controls, presented an increase of preference with
6 time. According to Willner, several research groups have reported similar findings,
7 which were branded as 'anomalous' [58]. However, studies exploring the
8 consequences of CMS on the outcome of the SPT face significant reproducibility
9 issues across laboratories [59]. Based on preliminary results, and recommendations
10 of using the minimal concentration of sucrose in the solution [59], our study employed
11 a 0.5% sucrose solution, rather than the usual 1%, but it is possible that further
12 reduction of the concentration may lead to other results [59]. The length of the test may
13 also play a role on behavioural results; although we also explored shorter test periods,
14 i.e., 6 or 2 hours (data not shown), a duration as short as a single hour may yield
15 differences between groups [60]. It is also possible that longer periods of habituation
16 to the sucrose solution could be necessary; although in the context of sucrose
17 consumption, Mariusz Papp suggests as many as 7 to 9 weeks of weekly evaluation
18 may be required before a stable and reliable consumption is reached [61]; in our case,
19 preference, which gradually increased with time, may also only stabilize after several
20 weeks of exposure. Nonetheless, regarding fluid *intake* per body weight, CMS-females
21 did present a significantly smaller sucrose intake than controls, which was not the case
22 for males. If sucrose intake reflects a pleasurable activity, this may suggest that
23 although CMS females did not exhibit anhedonia per se, i.e. a decrease in their
24 preference for a palatable solution, CMS exposure could still impact their incentive for
25 an appetitive behaviour. Additionally, we observed an overall higher sucrose intake in
26 females compared to males; this has been previously observed in Wistar rats [44], in
27 a study in which the authors proposed this phenomenon was not related to hedonistic
28 behaviour but rather a sex steroid mediated difference in taste threshold of sucrose,
29 decreased in females compared to males, therefore leading to increased intake [62].

30 Although both sexes seemed to respond differently to the CMS protocol depending on
31 the test, as previously discussed, the proportion of CMS-non vulnerable and vulnerable
32 individuals was similar in both sexes, therefore suggesting that females did not present
33 increased vulnerability to chronic stress. While the sex-dependent response observed

1 in the present study is consistent with previous findings [63], our results are not in line
2 with a postulate that in rodents, males have better habituation than females to a chronic
3 stress situation [63]. This hypothesis is based on observations that in chronically
4 restrained Sprague Dawley rats, corticosterone release was observed for a longer
5 period and in larger quantity in females [64], and by the observations by Paré and
6 colleagues that female WKY rats exposed to chronic restraint stress, but not males,
7 present impaired responses in a passive avoidance task and reduced feeding
8 behaviour [12]. These studies however employed chronic restraint and not CMS as the
9 stress regimen. In humans, the question of increased vulnerability to stress in women
10 has been raised [65]. This “differential vulnerability hypothesis” could explain the higher
11 prevalence of depression in women [2]. While some authors do suggest increased
12 vulnerability to stressful life events in women [66], or increased heritability of resilience
13 in men [67], the hypothesis of an increased vulnerability to stress in women is
14 challenged [68], and such studies propose that higher female prevalence in stress
15 related diseases is due to increased exposure rather than vulnerability to stress
16 [65,68]. Nonetheless, further studies exploring the proportion of vulnerable vs non
17 vulnerable animals between males and females are needed in the preclinical domain
18 for a more in-depth comparison with clinical data.

19 **Limitations of the study**

20 The present study is not without limitations. Firstly, the absence of CMS effects on
21 sucrose preference prevented vulnerable segregation based on this parameter. The
22 estrous cycle was not controlled in female rats, which could have contributed to
23 conclude on the absence of activity rhythm in females following CMS. Given the WKY’s
24 spontaneous anxiety- and depressive-like behaviour, it would have been interesting to
25 measure their basal anxiety-like and stress coping behaviours, and their progression
26 with the ongoing CMS protocol. However, the EPM and FST cannot be performed
27 several times, as repeated exposure in the former modifies behaviour, which no longer
28 represents anxiety-like behaviour, through aversive learning [69,70], and given the
29 stressful nature of the latter, repeated testing may in effect chronically stress the
30 controls, as it has been shown to increase immobility in Wistar rats [71]. There is
31 therefore a need for more depression-related behavioural tests that can be repeated
32 to assess individual development.

33

1 **Conclusion**

2 Overall, our results show that CMS differentially impacts WKY rats depending on their
3 vulnerability to stress and depending on their sex. The stress protocol leads to
4 behavioural impairments relevant in the context of depression, such as anxiety-like
5 behaviour and maladaptive coping strategy in a subset of animals of both sexes, and
6 a phase delay of daily locomotor activity rhythms in vulnerable males. We also
7 observed weight gain impairments and hyperlocomotion in reaction to novelty in all
8 CMS-exposed males, and a reduction in sucrose intake in all CMS-exposed females.
9 Our study suggests CMS-exposed WKY rats to be a good model of depression-like
10 behavioural manifestations based on construct and face validity, and reinforce the
11 importance of investigating differences of sex and inter-individual vulnerability to stress
12 in such studies.

13

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5

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5

1 **FIGURE CAPTIONS**

2 **Figure 1.** Experimental design.

3 **Figure 2.** Performance in the EPM (proportion of time spent in open arms) in females
4 (A) and males (B), and performance in the FST (time spent immobile) in females (C)
5 and males (D). Populations are as follows: females: control (n=8), cluster 1 (n=11),
6 cluster 2 (n=18); males: control (n=6), cluster 1 (n=11), cluster 2 (n=13). \$p < 0.05,
7 \$\$\$p < 0.001 control vs cluster 2 (t-tests).

8 **Figure 3.** Performance in the SPT for females of the control (n=8), CMS-non
9 vulnerable (n=11) and CMS-vulnerable (n=18) groups, and males of the control (n=6),
10 CMS-non vulnerable (n=11) and CMS-vulnerable 2 (n=13) groups, including baseline
11 sucrose preference (A), and evolution of sucrose preference during the 4 weeks of
12 CMS in females (B) and males (C). The dashed line marks the value of absence of
13 preference (50 %). $\alpha\alpha\alpha p < 0.0001$ vs 50 %. Statistics for B and C: main effect of sex
14 and main effect of Week. For details, see text.

15 **Figure 4.** Performance in the SPT for females of the control (n=8), CMS-non
16 vulnerable (n=11) and CMS-vulnerable (n=18) groups, and males of the control (n=6),
17 CMS-non vulnerable (n=11) and CMS-vulnerable 2 (n=13) groups, including the
18 evolution of sucrose and water consumption in females (A and C, respectively) and
19 males (B and D, respectively) from the baseline measure to week 4 of CMS. \$p < 0.05,
20 \$\$\$p < 0.01: CMS-vulnerable vs controls; £p < 0.05, ££ p < 0.01: CMS-non vulnerable
21 vs controls; *p < 0.05, **p < 0.01, ***p < 0.0001 vs other time points.

22 **Figure 5.** Body weight gain in females of the control (n=8), CMS-non vulnerable (n=11)
23 and CMS-vulnerable (n=18) groups (A), and males of the control (n=6), CMS-non
24 vulnerable (n=11) and CMS-vulnerable (n=13) groups (B) during the 4 weeks of CMS.
25 *p < 0.05 vs other time points; £p < 0.05 CMS-non vulnerable vs control, \$p < 0.05
26 CMS-vulnerable vs control.

27 **Figure 6.** Locomotor response to novelty for females of the control (n=8), CMS-non
28 vulnerable (n=11) and CMS-vulnerable (n=18) groups, and males of the control (n=6),
29 CMS-non vulnerable (n=11) and CMS-vulnerable 2 (n=13) groups. Number of cage
30 crossings in the first 10 min of the locomotor activity test pre- and post-CMS in females
31 (A) and males (B) either pre-CMS (plain bars) or post-CMS (striped bars). Total

1 number of crossings (**C**) and total number of rearings (**D**) in the OF. *p < 0.05 pre- vs
2 post-CMS; £p < 0.05 CMS-non vulnerable vs control; \$p < 0.05, \$\$ p < 0.001 CMS-
3 vulnerable vs control.

4 **Figure 7.** Daily locomotor activity rhythm in females before and after the CMS period
5 in the control group (n=8) (**A**), the CMS-non vulnerable group (n=11) (**B**), and the CMS-
6 vulnerable group (n=18) (**C**). The light and grey areas indicate the light and dark phase,
7 respectively. For each graph, the curves represent the significant rhythms of daily
8 locomotor activity. * p < 0.05 pre- vs post-CMS. (amplitude)

9 **Figure 8.** Daily locomotor activity rhythm in males before and after the CMS period in
10 the control group (n=6) (**A**), the CMS-non vulnerable group (n=11) (**B**), and the CMS-
11 vulnerable group (n=13) (**C**). The light and grey areas indicate the light and dark phase,
12 respectively. For each graph, the curves represent the significant rhythms of daily
13 locomotor activity. The dashed lines represent acrophase while the arrow indicates
14 phase delay. *p < 0.05 pre- vs post-CMS period.