Chronic social stress and the circadian system
Ota, Simone Marie
Chapter 6

Continuous social defeat for ten days did not induce permanent behavioral alterations in adult mice
Simone Marie Ota, Peter Meerlo, Deborah Suchecki
Abstract
Social stress has been associated with development of many psychiatric disorders, such as anxiety and depression. Social defeat stress has been used as a model to induce depressive-like behavior in animals. As shown in our previous studies, mice exposed to social defeat stress present suppression of locomotor activity. But this suppression is mostly observed during the days that stress is applied and is not persistent after some days of recovery. Therefore, the aim of this study was to observe whether chronic social defeat stress would have persistent on social investigation behavior and changes in sucrose preference over time in adult mice. For this purpose, C57BL/6 male mice were used as experimental animals and Swiss mice were used as aggressors. The social defeated group was submitted to 10 days of continuous social defeat while the control group cohabitated the cage with other mice from the same line, without physical contact. Four days after the defeat phase, social investigation was observed. Sucrose preference and weight were assessed during habituation, social defeat and recovery. There was no significant effect of social defeat neither in sucrose preference nor in social investigation compared to control group. However, during the social defeat phase, the animals in general presented lower sucrose preference compared to the other phases. Curiously, the social defeat group gained weight throughout the experiment, but the control group did not. In conclusion, continuous social defeat for 10 days did not cause permanent changes on behavior in adult mice, although manipulations may have caused stress on the control group.
1. Introduction

Stressful events, such as job loss, divorce and widowhood, have been reported to trigger the development of depression (Mandal et al., 2011; Aseltine and Kessler, 1993; Umberson et al., 1992, respectively). Furthermore, chronic rather than acute stress seems to be a stronger predictor (Mcgonagle and Kessler, 1990) as several studies report associations between chronic work stress and depression (Wang and Patten., 2001; Lunau et al., 2013; Gherardi-Donato et al., 2015; Hoven et al., 2015). Griffiths and colleagues (2014) observed that defeat (understood as failed social struggle) and entrapment (inability to escape from a situation) predict increased levels of depression and state anxiety 12 months later, irrespective of initial symptoms levels. Despite the evidence, it is still difficult to establish causal relationships.

To study the relationship and try to determine the causal role between chronic social stress and depression, animal models, such as the social defeat, have been used. Chronic social stress is an important risk factor for the development of depression in humans (Johnson and Sarason, 1978; Kessler, 1997; Hammen, 2005). In general, exposure to social defeat stress induces depressive-like behaviors such as reduced social interaction (Favoretto et al., 2017, Krishnan et al., 2007, Macedo et al., 2018, Meierlo et al., 1996; Venzala et al., 2012), decreased weight gain (Carnevali et al., 2012; Meierlo et al., 1996; Venzala et al., 2012) and lower sucrose preference (Favoretto et al., 2017, Krishnan et al., 2007, Macedo et al., 2018, Miczek et al., 2011; Venzala et al., 2012). This model is based on the resident-intruder paradigm and simulates episodes of social stress. The resident is trained to display aggressive behavior and it is expected that it becomes the dominant and the intruder becomes subordinate, to emulate the social hierarchy and subordination seen in some human conditions (Koolhaas et al., 1997). However, many studies with chronic stress in mice are focused on the effects in adolescents and behavioral tests are conducted right after stress procedures. In our studies, we have observed that intermittent social stress for 10 days suppressed locomotor activity in mice during stress days (Ota et al., 2018, Ota et al. – in preparation, see chapters 3 and 4). Therefore, in this study, we investigated whether chronic social stress for 10 days could induce persistent depressive-like behavior, not only during or acutely after defeat, but also after some days of recovery, in adult C57BL/6 mice.

2. Material and methods

2.1 Animals and housing

Fourteen three-month-old C57BL/6 male mice were used as experimental animals and eleven five-month-old Swiss mice, previously trained, were used as aggressors. The animals were obtained from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) of Universidade Federal de São Paulo. All animals were maintained in 12:12 light/dark cycle and controlled temperature (23 ± 2°C), with free access to water and food.
2.2 Experimental procedure

The timeline of the experiment consisted of three blocks of 10 days: Habituation, Social Defeat and Recovery, as shown in Figure 1. The animals were divided into Control or Social Defeated group, based on their body weight and sucrose preference. Weighing occurred before the sucrose preference test, three times during each block, every four days. The mean of the three measurements was calculated for each phase of the protocol. During Habituation, the animals were left in their cages, except for weighing. During the Social Defeat block, mice were placed in a new cage, either with an aggressor (Social defeated group) or a naïve mouse (Control group), as described below. Then, 24 h after the last defeat, the animals returned to their own home cage and were left undisturbed until testing in the Recovery block.

![Timeline of the experiment. Diamond represents sucrose preference test and weighing, circle represents social investigation test.](image)

2.3 Social Defeat

Social defeat stress was conducted similarly to that proposed by Golden and colleagues (2011). The intruder mice (Social Defeated group) were placed in the aggressor’s home cage with a perforated Plexiglas divider between them, which allowed olfactory and visual, but no physical, contact. After five minutes, the partition was removed, and the resident threatened and attacked the intruder. This phase also lasted five minutes, after which the partition was replaced, and the intruder remained in the resident’s cage until the next day, when it was exposed to another aggressive animal. This procedure was repeated throughout the 10 days of social defeat. The animals in the Control group were kept in similar cages, together with a naïve animal of the same strain and age but separated by a divider to avoid physical contact. Control animals were also exposed to a new cage mate throughout the 10 days of the protocol.

2.4 Sucrose Preference

Before onset of the experimental procedure, the animals were presented with two drinking bottles, one containing a 1% sucrose solution and another containing water, for 72 h, to stimulate sucrose intake and balancing of sucrose preference between groups. On testing days, which occurred every four days, the mice were placed back in their own cage for 1 h (at ZT 6) and the two bottles were presented again. The intake in each block (Habituation, Social Defeat and Recovery) was calculated as the mean of three tests. For every test, the position of the bottles was alternated to avoid preference.
2.5 Social Investigation

The animals were placed in the social investigation arena for ten minutes the day before the test for habituation. In the test day, animals were placed in the middle of the arena, with a cage with a naïve animal (same strain and age) and an empty one in opposite sides. Number and time of investigation of each cage were observed and the percentages (investigation of cage with naïve/ investigation of cage with naïve + investigation of empty cage) compared between groups.

2.6 Data Analysis

Data from body weight and sucrose consumption were analyzed with a two-way ANOVA for repeated measures, with the between-subjects factor GROUP (Control x Social Defeated) and within-subjects factor TIME (Habituation, Social Defeat, Recovery). The Newman-Keuls test was used as a post-hoc test for the sucrose consumption data. Social investigation data were analyzed with Student’s t test independently for each parameter evaluated. For all statistical tests, the level of significance was considered as $p \leq 0.05$.

3. Results

3.1 Body weight

The animals were weighted every four days, and because there was no difference among the days in each block, we averaged the data for each block. Statistical analysis showed an effect of time ($F_{2,24} = 15.60, p < 0.01$) and interaction between factors TIME and GROUP ($F_{2,24} = 7.47, p < 0.01$). The post-hoc analysis revealed that the Social Defeated group was heavier during defeat ($p < 0.03$) and Recovery ($p < 0.01$) compared to Habituation and it was also heavier during recovery than during Social Defeat ($p < 0.01$).
3.2 Sucrose Preference Test

The animals were tested for sucrose preference three times during Habituation, Social Defeat and Recovery days, but since there was no difference among the days in each block, we averaged the three measurements per block. The two-way ANOVA for repeated measures showed an effect of TIME ($F_{2,24} = 4.88, p = 0.02$). The Newman-Keuls test revealed that during the Social Defeat block, the animals in general presented lower sucrose preference compared to Habituation ($p = 0.02$) and Recovery ($p = 0.03$) blocks.

![Sucrose preference graph](image)

Figure 3: Sucrose preference. In general, the animals displayed lower sucrose preference during period of social defeat compared to the other periods. The results are represented by mean ± s.e.m. *Different from Habituation and Recovery.

3.3 Social Investigation Test

Student’s t-test revealed no significant difference between groups in the percentage frequency of investigation for Control (46.15% ± 10.53%) and Social Defeated (56.79% ± 10.50%); ($t(12) = 1.87 , p=0.08$). No significant difference was observed in the percentage of time of investigation between Control (53.67% ± 14.30%) and Social Defeated (62.38% ± 9.01%); $t(12) = 1.40 , p = 0.18$. 

4. Discussion

The results of the present study showed that continuous social defeat for 10 days in adult mice did not produce significant alterations in behavior compared to control animals exposed to an unfamiliar cage mate without physical contact. This finding contradicts what is found in the literature. Chronic social defeat is reported to induce anxiety and depressive-like behavior in rats (Carnevali et al., 2012; Meerlo et al., 1996; Miczek et al., 2011) and mice (Krishnan et al., 2007; Venzala et al., 2012). One possible explanation for the unexpected results is the small number of animals in our study and large intragroup variation. Besides, Golden and collaborators (2011) reported a 30-40% rate of animals resilient to the social defeat. However, observing the distribution of frequency and time of social investigation in our study, the control group seemed to investigate the naïve animal less, although there was no significant difference between the groups. Furthermore, the control group did not gain weight during the experiment, indicating that this group might have also been stressed by the manipulation.

Some differences between ours and other protocols may explain the lack of social defeat effects. In this study, defeat started when animals were fully adults (three-month old), and although studies in rats show effects of defeat on social exploration (Meerlo et al., 1996), Golden and collaborators (2011) state that the negative effects are more pronounced when mice are defeated during early adulthood (7-8 weeks). We do not believe that the strain of the aggressive animal (Swiss, not CD1) had any influence on the results, since they were trained to reach high scores of aggression. On the other hand, control animals were
paired with age matched mice, and that 12-week old mice present more aggressive behavior than 8-week olds (Kawai et al., 2003). Therefore, even when physically separated, control mice might have been exposed to visual and auditory aggressive displays by their cage mates. Furthermore, control animals might also have been stressed because the rotation of cage mates caused social instability. It has been shown that rats from different cages, when housed together, present higher levels of ACTH and corticosterone than rats from social stable groups (Suchecki and Tufik, 2000). Lower body weight and worse fur condition was observed by Boleij and colleagues (2014) in response to changes in group composition twice a week for seven weeks. It was hypothesized by Meshalkina and Kalluef (2016) that mice from the same strain, more similar in size could fight more fiercely to determine the winner, leading to a stronger social stress. If this is the case, our control mice could have experienced social stress, although there was no experimentally induced physical interaction during the defeat phase for the animals in the control group.

The results observed for sucrose preference may be due to the already low level of preference in the beginning of the experiment. Krishnan and colleagues (2007) observed that sucrose preference for controls was higher than 80%, while in our study the preference was around 64% for both groups before the onset of social defeat and dropped to 43% during the stress period. Nevertheless, decrease in the sucrose intake after maintaining the control mice with another individual in the same cage was also observed in another study (Venzala et al., 2012). One might argue that single housing could induce depressive-like behaviors (Chourbaji et al., 2005). However, in our study, mice were already individualized in the habituation phase and the reduction in sucrose consumption occurred during the social defeat phase, without difference of intake between recovery and habituation phase, indicating that social defeat and housing with an unknown mouse induced this depressive-like behavior, with no permanent effect after the end of stress.

Regarding the social investigation test, some differences are observed in our protocol compared to other studies. In our protocol, the animals were habituated to the empty arena one day before and on the following day, they were placed in the arena with an empty cage and one cage containing the naïve target animal at the same time, while in other studies, the animals were exposed to the arena with an empty cage and some minutes later, the naïve animal was introduced (Favoretto et al., 2017, Golden et al., 2011, Krishnan et al., 2007, Macedo et al., 2018). In the studies from Krishnan and colleagues (2007) and Golden and colleagues (2011), the naïve animal was from the same strain as the aggressors (CD1), whereas in our study, the naïve animal was from the same strain as the intruder (C57BL/6). The question of whether the use of the same strain as the aggressor for the target mouse could affect the results was also discussed by Meshlakina and Kalluef (2016). They argue that the use of the “heavier winner” strain could result in a conditioned avoidance rather than a lack of interest for social interaction. On the other hand, Venzala and collaborators (2012) also used a naïve animal from the same line as the intruder and observed less interaction by
the defeated group compared to control animals.

Finally, some studies do not provide neither the time of day when defeats nor behavioral tests start. This detail is relevant once Bartlang and colleagues (2012) showed that social defeat during the night, but not during the day, affects time spent in social contact zone. Furthermore, it was reported that male rats travelled more in the center of the open field during the dark phase than in the light phase, suggesting that evaluation of some behaviors are affected by the time of testing (Verma et al., 2010). However, Verma and colleagues (2010) did not observe differences in sucrose consumption between light and dark phases.

In conclusion, chronic social defeat in the light phase did not induce permanent depressive-like behavior in adult C57BL/6 mice compared to control animals maintained with an unfamiliar mouse in the same cage.
References


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