EFFECTS OF CHRONIC DIAZEPAM TREATMENTS ON BEHAVIOR ON INDIVIDUALLY HOUSED RATS

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Abstract – The present study analyzed the effects of chronic treatment with low doses of diazepam on body weight, defecations and urinations, vertical rears, the elevated platform test, and self-grooming in male rats exposed for 21 days to social isolation. The rats were treated for 21 days with diazepam (0.2 mg/kg, i.p) or its vehicle. Social isolation led to decreased body weight and vertical rears, more defecations and urinations, increased reluctance to step down from the test platform, shorter duration of grooming, and longer reluctance to start grooming. Chronic diazepam in individually housed rats produced increase in body weight and vertical rears, decrease in the number of defecations and urinations, and shortening of the time of reluctance to step down from the platform. The number of grooming bouts, their duration, and reluctance to start grooming were not altered by diazepam, but it decreased the percentage of incorrect transitions. The obtained data indicate that chronic diazepam treatment of socially isolated rats changes non-grooming behavior and some grooming behavior parameters.

Key words: Diazepam, social isolation, elevated platform test, grooming analysis algorithm

INTRODUCTION

Chronic stress has been found to affect different behavioral processes (Bowman et al., 2003; D’Aquila et al., 2000). However, final behavioral outcome depends on various factors and their interactions. The character of the stressful stimulus and the stress model, its intensity and duration, and the time period when it occurs are considered to be most relevant. Social interactions are an important source of stress. Social isolation and acute environmental change are risk factors in human depression and represent a lack of social stimuli necessary to modulate adaptive responses to a new situation (Ishida et al., 2003). Benzodiazepine drugs such as diazepam have been widely used as anxiolytics in human medicine. Diazepam acts by enhancing GABA-ergic neurotransmission through an allosteric interaction at the benzodiazepine-GABA[α]-barbiturate-chloride ionophore receptor complex (Carrasco and Van de Kar, 2003). Self-grooming is a particularly important part of the behavioural repertoire of rodents (Berridge and Whishaw, 1992; Van Erp et al., 1994). In rodents, grooming is a complex process, with a rich rite consisting of several stages, including licking the paws, fur, and legs, washing movements over the head, and cleaning of the tail and genitals (Fentress, 1977; Berridge and Aldridge, 2000; Egibar et al., 2003). It has long been known that rodent grooming activity can be generally increased in two opposite situations: in both high and low stress (Kalueff, 2000, 2002; Moyaho and Valencia, 2002). Low-stress comfort grooming is a spontaneous body care ritual which occurs as a transition from rest to activity. On the other hand, stress-evoked grooming is ethologically different from low-stress grooming and is characterized by frequent bursts of rapid short grooming. Since rodent self-grooming is increased by both stress and comfort conditions, the major problem with grooming behavioral analysis is that its traditional cumulative “gross” measures may not be valid for correct data interpretation. For this, Kalueff and Tuohimaa (2004) have designed a grooming analysis algorithm based on the above-mentioned ethological differences between comfort and stress-evoked groomings. This approach is based on differential registration and analysis of grooming behavioral microstructure. In
the present study, we therefore used this approach to assess grooming behavior of rats. Grooming is highly sensitive to various stressors and drugs (Dunn et al., 1988; Gerlai et al., 1998; Choleris et al., 2001). GABA and GABA_A receptors are involved in the regulation of emotions and various forms of behavior, including grooming (Nutt and Malizia, 2001; Barros et al., 1994).

Given the role of GABA in the regulation of anxiety and the grooming, and influence of stress on grooming behavior, we felt that it would be interesting to assess the effects of chronic administration of a low diazepam dose on non-grooming and grooming behavior in rats exposed to social isolation for 21 days.

MATERIALS AND METHODS

Animals

Adult Wistar rat males weighing 280 - 320 g at the onset of experiment were used. They were maintained under standard conditions in a temperature-controlled room (21±1.0°C) under conditions of a 12 h/12 h light/dark cycle. Before exposure to stress, animals housed in groups of four individuals per cage were divided into four groups. The first group consisting of four rats per cage was given the vehicle i.p. for 21 days. The second group of four animals per cage was treated with diazepam i.p. for 21 days. Rats individually housed for 21 days and injected i.p. every day with the vehicle made up the third group. In the fourth group, rats individually housed for 21 days were given diazepam i.p. throughout the entire period of social isolation.

Drugs and treatment

Bensedin® ampullae (Galenika Pharmaceutical Works, Zemun, Serbia) containing 5 mg diazepam/ml of solution prepared in on ethanol-propylene glycol mixture, pH 6.5, as well as the solvent were kind gifts of the producer. The diazepam solution was diluted with a vehicle to a suitable concentration, injection volume being 0.1 ml/100 g b.w. The rats were given daily i.p. injections of either diazepam (0.2 mg/kg b.w.) or the vehicle for 21 days.

Non-grooming behavior

Defecation and urination indices were scored as the conventional emotionality index in all groups of animals by the actimeter test. In addition to grooming and visceral behavior measurements by the actimeter test, we also assessed general vertical exploratory activity as the conventional measure of stress-sensitive behaviour.

Elevated platform test

The tests were performed on day 14 of stress according to Dubovicky and Jezo (2004). Rats were placed on an elevated platform (14 x 14 x 6 cm) and the period of reluctance to step down was recorded. They were tested in three sessions with 1-h intervals between sessions.

Grooming analysis

The actimeter test was used for grooming and visceral behavior measurements. In our case, the actimeter was a glass cylinder with diameter of 20 cm and length of 40 cm. During the testing session, the experimenter remained standing in front of the testing boxes at a distance of 2.0 m. Between the tests, each apparatus was cleaned with a 10% ethanol solution and dried with paper toweling. The tests were performed on day 21 of social isolation or group housing of rats. The observer recorded the duration and number of groomings according to Kalueff and Tuohimaa (2004). Three gross measures of grooming activity were evaluated: the period of reluctance to start grooming, the number of grooming bouts, and the total time the animal spent grooming. The percentages of interrupted bouts and incorrect transitions were assessed in the study.

Statistics

The effects of diazepam treatment were analyzed by ANOVA. When a significant p-value was obtained, the Tukey HSD test was employed to determine differences between the groups. The level of statistical significance was set to 5%.

RESULTS

Figure 1 shows body weight of group housed and individually housed rats on the 7th, 14th, and 21th day of social isolation and the effects of chronic diazepam administration. Individually housed rats exhibited a decrease of body weight measured on days 14 and 21 of the experiment as compared to group-housed rats (p<0.05). Diazepam in a dose of 0.2 mg/kg increased body weight in the control-group housed rats measured on days 7 (p<0.05) and 14 (p<0.01), but no significant differences of body weight were observed in socially isolated rats.
Table 1. Behavioral alterations in non-grooming and grooming activity of individually housed and group-housed rats subjected to long-term treatment with diazepam as obtained by the actimeter test. The values are means ± S.E.M of seven animals. * p<0.05 diazepam vs. vehicle; + p<0.05, ++ p<0.01, +++ p<0.001 animals isolated for 21 days vs. vehicle-receiving group-housed animals.

<table>
<thead>
<tr>
<th>BEHAVIOR PARAMETERS</th>
<th>Control + diazepam</th>
<th>Control + vehicle</th>
<th>Isolation + diazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON-GROOMING BEHAVIORS</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Defecation and urination index</td>
<td>1.00 ± 0.33</td>
<td>1.11 ± 0.35</td>
<td>3.10 ± 0.40</td>
</tr>
<tr>
<td>Number of vertical rears</td>
<td>13 ± 0.76</td>
<td>16 ± 0.96</td>
<td>10 ± 0.86</td>
</tr>
<tr>
<td>Reluctance to stepdown from platform (s)</td>
<td>8.25 ± 0.86 *</td>
<td>12.71 ± 0.61</td>
<td>16.29 ± 1.12</td>
</tr>
<tr>
<td>GROOMING BEHAVIORS</td>
<td></td>
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<tr>
<td>Reluctance to start grooming (s)</td>
<td>78.0 ± 10.08</td>
<td>82.4 ± 16.11</td>
<td>148.80 ± 11.86</td>
</tr>
<tr>
<td>Total number of bouts</td>
<td>1.20 ± 0.11</td>
<td>1.00 ± 0.10</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>Average duration of a single bout (s)</td>
<td>38.2 ± 8.14</td>
<td>62.78 ± 8.56</td>
<td>21.80 ± 0.89</td>
</tr>
<tr>
<td>Total number of transitions</td>
<td>34.1 ± 5.23</td>
<td>37.5 ± 8.19</td>
<td>28.40 ± 0.89</td>
</tr>
<tr>
<td>Percentage of incorrect transitions (% of total T)</td>
<td>0.77 ± 0.40</td>
<td>0.79 ± 0.41</td>
<td>2.19 ± 0.40</td>
</tr>
<tr>
<td>Percentage of interrupted bouts (% of total bouts)</td>
<td>14.28 ± 9.70</td>
<td>20.00 ± 13.33</td>
<td>22.22 ± 1.12</td>
</tr>
<tr>
<td>REGIONAL DISTRIBUTION</td>
<td></td>
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</tr>
<tr>
<td>Forepaws (% of total)</td>
<td>33.93 ± 1.94 *</td>
<td>38.60 ± 2.95</td>
<td>40.90 ± 3.84</td>
</tr>
</tbody>
</table>

Table 1 shows non-grooming and grooming activity of group-housed, socially isolated, and diazepam-treated rats. Individually housed rats performed significantly more defecations and urinations than did the group-housed rats (p<0.001). Diazepam-treated rats showed a decrease in the number of defecations and urinations only in individually housed rats (p<0.05). The most of vertical rears were recorded in group housed rats (p<0.001). However, diazepam exerted a clear anxiolytic effect on socially isolated rats, significantly increasing vertical rears (8 ± 0.72 in vehicle-treated rats vs. 10 ± 0.84 in diazepam-treated animals) (p<0.05). Long-term social isolation markedly affected the behavior of rats in the elevated platform test. Statistical analysis revealed a significantly longer period of reluctance to step down from the platform in socially stressed rats in each individual session compared to that in the group-housed rats (p<0.01). Administration of a low diazepam dose (0.2 mg/kg b.w.) significantly (p<0.05) shortened the period of reluctance to step down from the platform in both groups.

Duration of grooming bouts was shorter and the period of reluctance to start grooming longer in individually housed rats in comparison with those housed in groups of four individuals (p<0.001), but no differences in the number of grooming bouts were recorded. Application of diazepam did not affect the number of grooming bouts, duration of grooming, or length of the period of reluctance to start grooming in either of these groups. Using a detailed grooming ethological analysis, we found that diazepam produced a decrease in the percentage of incorrect transitions (p<0.05), but no interrupted bouts in socially isolated rats. Our results also showed that the regional distribution of grooming actions was markedly affected by stress: to be specific, there were more forepaw grooming actions and fewer tail and genital grooming actions in individually housed rats as compared to vehicle-treated group-housed animals (p<0.05). Diazepam-treated rats of both the socially isolated and the group-housed groups spent significantly (p<0.05) less time grooming the forepaws and more time grooming the tail and genitals.

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**EFFECT OF DIAZEPAM TREATMENTS ON RATS**

Fig. 1. Effects of chronic diazepam treatment on body weight of individually housed and group-housed rats. The animals received diazepam (0.2 mg/kg b.w., i.p) for 21 days. The values are means ± S.E.M of seven animals.

- GROUP-Housed+Vehicle
- GROUP-Housed+Diazepam
- Isolation+Vehicle
- Isolation+Diazepam
DISCUSSION

In the present work, we studied the effects of chronic administration of diazepam, a representative of anxiolytic drugs, in a dose of 0.2 mg/kg on behavioral changes in adult Wistar rat males subjected to individual housing for 21 days. The obtained results showed that social isolation evoked various alterations, including changes in body weight, defecation, urination, vertical rears, performance in the elevated platform test, and grooming. Diazepam affected non-grooming behavior, causing decrease in the number of defeactions and urinations, shortening of the period of reluctance to step down from the platform, and increase in the number of vertical rears in socially isolated rats. Self-grooming in rodents is stereotypically sequenced and naturally occurs after stress, which provokes a disorganization of grooming sequences (Komorowska and Pellis, 2004; Audet et al., 2006). We analyzed grooming behavioral microstructure after chronic treatment with diazepam using a grooming analysis algorithm recently reported to be a reliable tool for neurobehavioral stress research in mice and rats (Kalkeff and Tuohimaa, 2004). Our results showed that social isolation led to reduced duration of grooming and prolongation of the period of reluctance to start grooming. This is in accordance with the data of Liu et al. (2005), who found that rats exposed to chronic unpredictable mild stress for a period of 3 weeks experience depression in the quise of significantly lower weight gain, reduced open-field exploration, fewer rears, and grooming indicative of lethargy, apathy, and bodily neglect. Contrary to this, Kalkeff and Tuohimaa (2005b) reported an increased number and duration of grooming actions in stressed anxious rats, while the period of reluctance to start grooming was shorter. This discrepancy between our results and the data of these authors may be attributable to stronger depression during social isolation of the animals. Diazepam did not alter grooming activity in group-housed and socially isolated rats, but decreased the percentage of incorrect transitions in both groups. Moreover, we also found that animals spent more time grooming the tail and genitals. Although these interesting phenomena need to be further investigated, we can venture an ‘anxiolytic’ interpretation of increased caudal grooming which is, in line with the predominantly rostral nature of rodent grooming evoked by stress (Komorowska and Pisula, 2003; Kalkeff and Tuohimaa, 2004). The obtained data support the notion that changed grooming activity does not always reflect higher levels of stress and clearly support the idea that grooming behavioral microstructure in rats is very sensitive to the level of stress. The results of the present behavioral study demonstrated that a low dose of diazepam dose of 0.2 mg/kg produced anxiolytic effects on non-grooming behavior and some aspects of grooming behavioral microstructure in rats exposed to long-term social isolation.

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Dejstvo hrONICHNOg TretmanA DiAzepamom na promene u ponashaњu kod SOCIjalNO izolovanih pacova

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У овој студији испитивано је дејство хроничног третмана ниске дозе диазепама на понашање: телесну тежину, дефекацију и уринирање, вертикално издизање, тест подигнуте платформе и груминг код муњака пацова изложених 21 дан социjalnoj izolaciji. Пацови су били третирани 21 дан диазепамом (0,2 мг/кг. и.п.) или плацебо раствором. Социjalna izolacija smaњuje tелесну tежину и вертикална излизанja, повећава дефекацију и уринирање, продужава време спласка са платформе, скраћује трајање груминга и продужава време започињања груминга. Хронични третман диазепамом код социjalno изолованих пацова повећава телесну тежину, вертикали излизанja, смањује броj дефекациjа и уринирања и скраћује време спласка са платформе. Диазепам не мења броj, трајање и време започињања груминга, али смањује процент некоректних транзициjа. Добијени подаци показуju да хроничан третман диазепамом код социjalно изолованих пацова не-груминг понашањe и неке параметре груминга понашањa.