The effects of diazepam (0.2 mg/kg/ during 21 days, i.p.) on behaviour, pituitary-adrenocortical and sympa-tho-adrenomedullary system of socially isolated and group-housed adult male rats additionally exposed to immobilization, were studied. Social isolation led to a shorter duration of grooming and longer latency to start grooming. Diazepam in social isolated rats reduced incorrect transitions percentage, but the number of grooming bouts, duration and latency to start grooming remained unchanged. Long-term isolation significantly elevated plasma ACTH and corticosterone, while not affecting noradrenaline and adrenaline. Diazepam decreased only plasma ACTH. Social isolation and immobilization significantly elevated all examined hormones. Immobilization of diazepam-treated isolated rats enhanced plasma ACTH, the increase being significantly lower, comparing to isolated vehicle-treated rats. Immobilization significantly increased plasma adrenaline, noradrenaline and corticosterone of diazepam- or vehicle-treated socially isolated rats. No differences in adrenaline, noradrenaline and corticosterone level between these two groups were observed. This indicates that chronic diazepam treatment of socially isolated rats changes some grooming behaviour parameters, but insignificantly affects stress-related adrenomedullary and adrenocortical alterations.

Key words: diazepam, social isolation, grooming, catecholamines, corticosterone, ACTH

INTRODUCTION

The hypothalamic-pituitary-adrenocortical (HPA) and sympa-tho-adrenomedullary (SAM) systems help to maintain homeostasis during exposure of animals to stressors. Activation of the HPA results in an increase of circulating adrenocorticotropic hormone (ACTH) and corticosterone (COR), while activation of SAM leads to the release of noradrenaline (NA) and adrenaline (A) into the circulation. These two physiological systems are of interest in the research of stress as plasma levels of the above hormones depend on the intensity of the stressful situation and can therefore be suitable stress markers (Marti and
Armando, 1998). Social interactions are an important source of stress. Social isolation and acute environmental changes 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$_A$-barbiturate-chloride ionophore receptor complex (Carrasco and Van der Kar, 2003). Systemic administration of high benzodiazepine doses was shown to prevent stress-induced increase of NA and dopamine turnover in rat brain (Tanaka et al., 2000; Feenstra et al., 2003; Dalley et al., 1996; Petty et al., 1997). Some authors reported that low diazepam doses were not effective in reducing stress-induced COR levels, but similar doses produced anxiolytic effects on behavioural measures of fear and anxiety (Keim and Sig, 1977; Barlow et al., 1979). Moreover, Miyamoto et al. (2000) found that only low diazepam doses of 0.125 and 0.25 mg/kg given i.p. reduced the freezing behaviour, whereas higher doses of 0.5 and 1.0 mg/kg, produced no such effect. Several authors demonstrated different effects of acute and chronic administration of benzodiazepine on HPA and SAM activity.

Acute, but not long-term diazepam treatment, increased COR levels, while acute and chronic diazepam treatments acted by antagonizing stress-induced activation of the dopaminergic and noradrenergic systems (Hegarty and Vogel, 1995; Lazzarini et al., 2003; Da Silva et al., 2003). Also, Matzen et al. (1993) reported that diazepam inhibits ACTH but does not inhibit plasma catecholamine responses to non-hypotensive head-up tilt. Self-grooming is a particularly important part of rodent’s behavioural repertoire (Berridge and Whishaw, 1992; Van Erp et al., 1994). In rodents, grooming is a complex process, with a rich ritual consisting of several stages, including licking the paws, fur and legs, washing movements over the head and cleaning of tail and genitals (Fentress, 1977; Berridge and Aldridge, 2000; Egilbar et al., 2003). 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 behaviours including grooming (Nutt and Malizia, 2001; Bartos et al., 1994).

Given the role of GABA in the regulation of anxiety and grooming, and the influence of stress on grooming behaviour, it was of interest to assess the effects of chronic administration of a low diazepam dose on grooming behaviour and activity of HPA and SAM systems in rats exposed to social isolation for 21 days and 2 hours of immobilization as an additional stress.

MATERIALS AND METHODS

Animals

Adult Wistar rat males weighing 280 - 320 g at the onset of the experiment were used. They were maintained under standard conditions in a temperature controlled room (21±1.0 °C) and 12 h/12 h light/dark cycle. Before exposure to stress, the animals were housed in groups of four individuals per cage and
randomly allotted into four groups. The first group consisting of four rats per cage receiving i.p. the vehicle for 21 days. The second group of four animals per cage was treated i.p. with diazepam for 21 days. The rats individually housed for 21 days and i.p. injected every day with vehicle represent the third group. In the fourth group, rats individually housed for 21 days were i.p. administered diazepam throughout the entire period of social isolation. On the day before blood sampling, a catheter was inserted into the tail artery of each individual under pentobarbital (40 mg/kg, i.p) anesthesia, thus allowing blood samples collection from animals unstressed by blood sampling manipulations. After collection of the base line samples, the rats were treated as described above and exposed to an additional 2 hours immobilization stress.

**Drugs and treatment**

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

**Grooming analysis**

The grooming and visceral behaviour measurements were done by the actimeter test. The actimeter test was a glass cylinder (diameter 20 cm, length 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 10% ethanol solution and dried with paper toweling. The tests were performed on day 21 of social isolation or group housing. The observer recorded the duration and number of grooming behaviours according to Kalueff and Tuohimaa (2004). Three gross measures of grooming activity were evaluated: latency period to start grooming, number of grooming bouts and total time the animal spent grooming.

**Immobilization**

The rats were subjected to 2-h-immobilization by fixing all four limbs to a board with adhesive tape. The head was also fixed by a metal loop over the neck area, thus limiting the motion of the head. In addition to the base line samples, blood was collected 15, 30, 60 and 120 min after the onset of immobilization.

**Biochemical analyses**

Catecholamines were measured by a modification of the radioenzymatic assay after Peuler and Johnson (1977). Catecholamines present in the blood plasma aliquots were converted to their labelled O-methylated derivatives by S-[^3H]adenosylmethionine (Amersham, Buckinghamshire, UK) and lyophilized catechol-O-methyltransferase isolated from rat liver. The O-methylated derivatives of the amines were then extracted along with unlabelled carrier compounds. After prior extraction, plasma COR was measured directly by RIA using commercial kits.
Plasma ACTH concentration was determined by the chemiluminescent method using an IMMULITE automatic analyzer (DPC, Los Angeles, CA, USA).

Statistics
Differences NA, A, COR and ACTH concentrations and grooming analysis were analyzed by one-way ANOVA. The effects of diazepam treatment were analyzed by two-way 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

Grooming parameters
The duration of grooming bouts was shorter and the latency 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 and the latency period to start grooming in both 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 behaviour was markedly affected by stress, manifested in more forepaw grooming patterns and less tail and genitals grooming in individually housed rats as compared to the vehicle-treated group (p<0.05). Diazepam-treated rats of both socially isolated and group-housed rats spent significantly (p<0.05) less time grooming the forepaws and more time grooming the tail and genitals (Table 1).

Biochemical parameters
The effects of diazepam and additional immobilization stress on blood plasma levels of NA and A of individually and group-housed rats are presented in Fig.1. Neither 21 days of social isolation nor diazepam treatment affected the basal plasma level of NA and A. Additional immobilization stress significantly increased circulating NA and A levels in all investigated groups (p<0.001). Immobilization stress also led to increased plasma NA and A levels in group-housed and individually housed rats treated with diazepam, therefore diazepam did not change the concentration of these catecholamines in both groups.

From Fig. 2 it can be seen that the basal plasma ACTH level was significantly elevated in long-term isolated animals treated with vehicle only (p<0.001). However, in individually housed rats treatment with diazepam led to lower basal plasma levels of ACTH, as compared to individually housed animals treated with vehicle only (p<0.05). Two-way ANOVA analysis showed a significant effect of social isolation F(1,20)=14,467, p<0.05 and diazepam treatment F(1,20)=5,659, p<0.05 on basal plasma ACTH concentrations. A significant decrease of ACTH levels (p<0.05, Tukey test) was found in the isolation+diazepam animals vs.
group housed+diazepam rats. Likewise was the group housed+vehicle vs.
group housed+diazepam (p<0.05) comparison. Immobilization resulted in an
increase of plasma ACTH (p<0.001) in both group-housed rats treated with
diazepam or vehicle. Stress also led to an increased plasma ACTH level of socially
isolated rats treated with diazepam, but this increase was significantly lower
(p<0.05) throughout the entire immobilization period than that recorded in
socially isolated rats treated with vehicle only. Basal plasma COR levels were
significantly elevated (p<0.001) in the long-term isolated group. Chronic
administration of low diazepam dose did not affect elevated basal plasma level of
COR in socially isolated rats. When group-housed and individually housed rats
treated with diazepam were exposed to immobilization, plasma COR level was
increased throughout the entire period of immobilization.

Table 1. Behavioural alterations in grooming activity of socially isolated and group-
housed rats long-term treated with diazepam as obtained by the actimeter test

<table>
<thead>
<tr>
<th>Grooming behaviour parameters</th>
<th>Group-housed + diazepam</th>
<th>Group-housed + vehicle</th>
<th>Isolation + diazepam</th>
<th>Isolation + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to start grooming (s)</td>
<td>78.0 ± 10.08</td>
<td>82.4 ± 16.11</td>
<td>148.80 ± 13.53</td>
<td>178.5 ± 23.14</td>
</tr>
<tr>
<td>Total number of bouts</td>
<td>1.20 ± 0.11</td>
<td>1.00 ± 0.10</td>
<td>0.50 ± 0.16</td>
<td>0.70 ± 0.16</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 ± 3.43</td>
<td>20.33 ± 1.48</td>
</tr>
<tr>
<td>Total number of transition</td>
<td>34.1 ± 5.23</td>
<td>37.5 ± 8.19</td>
<td>28.40 ± 3.63</td>
<td>31.40 ± 2.84</td>
</tr>
<tr>
<td>Percent age of incorrect transitions (% of total T)</td>
<td>0.77 ± 0.40</td>
<td>0.79 ± 0.41</td>
<td>2.19 ± 0.61*</td>
<td>4.72 ± 0.97</td>
</tr>
<tr>
<td>Percent age of interrupted bouts (% of total bouts)</td>
<td>14.28 ± 9.70</td>
<td>20.00 ± 13.33</td>
<td>22.22 ± 14.70</td>
<td>44.44 ± 17.58</td>
</tr>
</tbody>
</table>

Regional distribution

| Forpaws (% of total number of patterns) | 33.93 ± 1.94* | 38.68 ± 2.85 | 40.90 ± 1.93* | 47.23 ± 0.94 |
| Face (% of total number of patterns)    | 32.42 ± 2.36* | 40.89 ± 1.72 | 38.48 ± 2.13* | 45.10 ± 1.63 |
| Head (% of total number of patterns)    | 18.97 ± 1.07  | 11.44 ± 1.14 | 8.32 ± 1.12   | 8.96 ± 1.36   |
| Body (% of total number of patterns)    | 13.68 ± 1.94* | 6.76 ± 0.59  | 4.61 ± 0.30*  | 2.51 ± 0.25   |
| Tail and genitals (% of total number of patterns) | 4.42 ± 0.64* | 2.43 ± 0.78  | 1.89 ± 0.22*  | 0.83 ± 0.09   |

The values are means ± S.E.M of 10 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
Figure 1. The effects of chronic diazepam treatment on plasma noradrenaline (NA) and adrenaline (A) levels (pg/mL) in the control and socially isolated rats additionally exposed to immobilization. The animals were receiving diazepam (0.2 mg/kg b.w., i.p.) for 21 days. The values are means ± S.E.M of 6 animals. +++ p<0.001 as compared to zero point.
Figure 2. The effects of long-term diazepam treatment on plasma adrenocorticotropic hormone (ACTH) (pg/mL) and corticosterone (COR) (ng/ml) in the control and socially isolated rats additionally exposed to immobilization. The animals were receiving diazepam (0.2 mg/kg b.w., i.p.) for 21 days. The values are means ± S.E.M of 6 animals. +++ p < 0.001 as compared to zero point; *p < 0.05 diazepam vs. vehicle, # p < 0.01, ### p < 0.01 21 days isolation vs. vehicle-treated group housed rats. Two-way ANOVA revealed a significant effect of diazepam on basal plasma ACTH concentration during social isolation, x p < 0.05 significantly different from the group receiving diazepam.
The effects of chronic administration of diazepam, a representative of anxiolytic drugs, in a dose of 0.2 mg/kg on behavioural and neuroendocrine functions of adult Wistar male rats exposed to individual housing for 21 days were studied. The results showed that social isolation evoked various behavioural and endocrine alterations. 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 analysed grooming behavioural microstructure following chronic treatment with diazepam by using a grooming analysis algorithm which has been recently reported to be a reliable tool for neurobehavioural stress research in mice and rats (Kalueff and Tuohimaa, 2005a). Our results showed that social isolation led to a reduced duration of grooming and a prolonged latency period to the start of grooming. This is in accordance with the data of Liu et al. (2005) who found that rats exposed to chronic unpredictable mild stress are readily induced into depression for 3 weeks, showing a significantly lower weight gain, reduced open-field exploration, rearing, grooming indicative of lethargy, apathy and bodily neglect. On the contrary, Kalueff and Touhimaa (2005b) reported an increased number and duration of grooming in stressed-anxious rats, while the latency to start grooming was shorter. This discrepancy between our results and the data of these authors could be explained by a pronounced depression during social isolation of the animals. Diazepam did not alter grooming activity measures in group-housed and socially isolated rats, but decreased the percentage of incorrect transitions in both groups, and the animals spent more time grooming the tail and genitals. The results of the present behavioural study demonstrate that the low diazepam dose of 0.2 mg/kg produced partially anxiolytic effects on grooming behavioural microstructure in rats exposed to long-term social isolation. The observed behavioural alterations were not accompanied by a greater extent by the changes in pituitary-adrenocortical and sympatho-adrenomedullar activities. We have found previously that long-term social isolation produced a significant increase of basal plasma ACTH and COR but not of plasma NA and A levels. When socially isolated rats were exposed to additional immobilization or cold stress, they produced exaggerated plasma levels of all four hormones in comparison with the control and long-term crowded rats, as well as rats exposed to long-term forced swimming (Dronjak and Gavrilovic, 2005; Gavrilovic and Dronjak, 2005). During stress conditions, brain benzodiazepine receptors appear to participate in the physiological regulation of adrenocortical and neurosympathetic activities (De Boer et al., 1990). Moreover, many authors demonstrated that benzodiazepine receptor ligands with an anxietyt action, such as diazepam, can prevent stress-induced activation of HPA and sympatho-adrenomedullary system (De Boer et al., 1991; Feenstra et al., 1995; Pericic and Pivac, 1996; Wilson et al., 2004). However, our data showed that long-term diazepam treatment of individually housed rats acted by decreasing elevated basal plasma ACTH levels, whereas elevated basal plasma COR remained unchanged in comparison with the corresponding controls. Chronic treatment with diazepam of group-housed rats did not affect
basal plasma concentrations of these hormones. Evidently, the low diazepam dose used throughout the present study did not affect the basal activities of SAM and HPA axes in group-housed rats, but acted partly decreasing the activity of the HPA axis, seen as a reduced ACTH level in socially isolated rats. Similar results were obtained by Kalman et al. (1997) who applied both high and low diazepam doses in conjunction with a stressor and found that the doses of 1.5 and 3 mg/kg were not effective in reducing COR levels, while those in the range of 6 mg/kg were effective in reducing stress-induced levels of COR. Interestingly, in our experiments, plasma levels of COR were not significantly affected in spite of a reduced ACTH secretion. A possible explanation, suggested by Bernet et al. (2000), would be that ACTH levels observed in diazepam-treated rats subjected to stress were nevertheless sufficient to fully stimulate the adrenal cortex.

The present results clearly show that chronically applied diazepam was unable to suppress immobilization-induced increase of circulating NA and A in both socially isolated and group-housed rats. The exposure of group-housed rats, treated with diazepam or vehicle, to immobilization stress led to enhanced plasma ACTH level. Immobilization also resulted in an increase of plasma ACTH levels in socially isolated rats treated with diazepam, but this increase was significantly lower than that in socially isolated rats injected with the vehicle. It has been shown that stress induces a decrease of GABA content in some brain structures. Benzodiazepine receptor represents a part of a macromolecular complex with GABA receptor and Schofield et al. (Schofield et al., 1987) found that GABA represents one of the principal inhibitors of ACTH release. CRH neurons in the paraventricular nucleus (PVN) have inhibitory GABAergic inputs which regulate CRH release (Tasker and Dudek, 1993). GABA_A receptor blockade in the dorsomedial hypothalamic nucleus of male rats was shown to increase heart rate, blood pressure and plasma levels of both ACTH and COR (Keim and Shekhar, 1996). These GABA receptor-mediated central effects of diazepam could explain reduced plasma ACTH during stress, observed in socially isolated rats treated with diazepam for 21 days. On the other hand, when socially isolated rats and group housed rats treated with diazepam were exposed to immobilization, an increase in plasma level of COR was noticed. Obviously, low diazepam dose applied throughout this work could not attenuate the additional stress-induced increase of plasma COR.

In conclusion, the obtained results show that chronic treatment with diazepam of socially isolated rats leads to changes in some parameters of grooming behaviour, decreasing at the same time only basal plasma ACTH level. Exposure to immobilization as an additional stressor produced the lowest increase of plasma ACTH in comparison with that observed in socially isolated rats treated with vehicle.

The current data indicates that chronic treatment of socially isolated rats with a low diazepam dose did not significantly affect stress-related adrenomedullary and adrenocortical alterations.

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PONAŠANJE I ENDOKRINI ODGOVOR SOCIJALNO IZOLOVANIH PACOVA TOKOM TRETMANA DIAZEPAMOM

DRONJAK SLADANA, SPASOJEVIĆ NATAŠA, GAVRILOVIĆ LJUBICA I VARAGIĆ V

SADRŽAJ

Izučavano je dejstvo diazepamova (0,2 mg/kg, 21 dan, i.p) na promene u ponašanju, hipofizno-adrenokortikalni i simpatiko-adrenomedularni sistem kod socijalno izolovanih, odraslih mužjaka pacova, koji su dodatno još izloženi imobilizaciji. Socijalna izolacija dovodi do kraćeg trajanja gruminga i produžava vreme započinjanja gruminga. Diazepam kod socijalno izolovanih pacova redukuje procenat nekorektnih tranzicija, ali ne menja broj, trajanje i vreme započinjanja gruminga. Dugotrajna izolacija značajno povećava ACTH i kortikosteron u plazmi, dok ne menja nivo noradrenalina i adrenalina. Tretman diazepamom kod socijalno izolovanih pacova smanjuje samo nivo ACTH u plazmi. Socijalna izolacija i imobilizacija značajno povećavaju koncentracije svih ispitivanih hormona. Imobilizacija kod izolovanih pacova tretiranih diazepamom povećava koncentraciju ACTH u plazmi. Ovo povećanje je značajno niže u poređenju sa izolovanim pacovima tretiranim placebo. Imobilizacija značajno povećava koncentraciju noradrenalina, adrenalina i kortikosterona u plazmi socijalno izolovanih pacova tretiranih i diazepamom i placebo. Nisu uočene razlike u nivou noradrenalina, adrenalin i kortikosterona između ove dve grupe. Ovo ukazuje da hroničan tretman diazepamom kod socijalno izolovanih pacova menja neke parametre u gruming ponašanju ali neznačajno utiče na stresom izazvane promene u adrenomedularnom i adrenokortikalnom sistemu.