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Social separation and diazepam withdrawal increase anxiety in the elevated plus-maze and serotonin turnover in the median raphe and hippocampus

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Abstract

The present work aimed to evaluate the effects of social separation for 14 days (chronic stress) and of withdrawal from a 14-day treatment with diazepam (acute stress) on the exploratory behaviour of male rats in the elevated plus-maze and on serotonin (5-hydroxytryptamine) turnover in different brain structures. Social separation had an anxiogenic effect, evidenced by fewer entries into, and less time spent on the open arms of the elevated plus-maze. Separation also selectively increased 5-hydroxytryptamine turnover in the hippocampus and median raphe nucleus. Diazepam withdrawal had a similar anxiogenic effect in grouped animals and increased 5-hydroxytryptamine turnover in the same brain structures. Chronic treatment with imipramine during the 14 days of separation prevented the behavioural and neurochemical changes caused by social separation. It is suggested that the increase in anxiety determined by both acute and chronic stress is mediated by the activation of the median raphe nucleus-hippocampal 5-hydroxytryptamine pathway.

Keywords

social separation, anxiety, elevated plus-maze, serotonin, diazepam withdrawal, imipramine

Introduction

The serotonergic pathway that originates in the median raphe nucleus (MRN) and innervates the dorsal hippocampus has been implicated in adaptation to both acute and chronic stress. In acute stress, serotonin (5-hydroxytryptamine, 5-HT) is supposed to enhance the functioning of Gray's 'behavioural inhibition system' that generates anxiety (Gray, 1982, 1987; Gray and McNaughton, 2000). Under chronic stress, 5-HT would foster hippocampal processes that build up resilience, the failure of which results in depression (Deakin and Graeff, 1991).

The general purpose of the present study has been to test these hypotheses by measuring anxiety in the elevated plus-maze (EPM) (Pellow et al., 1985) and in the activity of the MRN-hippocampal 5-HT pathway through assessment of the 5-HT turnover in rats under either acute or chronic stress. Twelve hours withdrawal from chronic administration of diazepam has been used for acute stress and social separation for 14 days, for chronic stress.

Abrupt withdrawal of a benzodiazepine (12, 24 or 48 h) following prolonged administration (7–21 days) has been shown to induce anxiety in rats or mice, measured in different animal models: plus-maze (Assié et al., 1991; Bhattacharya et al., 1995; File and Hitchcott, 1991; File et al., 1987; Fontanesi et al., 2007; Martijena et al., 1996; Pokk and Zharkovsky, 1998; Wright et al., 1991), social interaction (Andrews and File, 1993; Andrews et al., 1997) or in both (Begg et al., 2005), and light-dark test (Souza Pinto et al., 2007). Withdrawal for 24 h from chronic diazepam treatment (21 days) has been related to increased 5-HT release in the hippocampus (Andrews and File, 1993; Andrews et al., 1997). Social separation in adult rats has also been shown to increase anxiety, in which 5-HT and gamma-aminobutyric acid have been implicated (Maisonnette et al., 1993; Rex et al., 2004; Sherif and Oreland, 1996).

Reported evidence indicates that anxiety is linked to an increase of 5-HT activity in the MRN-hippocampal pathway. Thus, electrolytic as well as neurotoxic (5,7-dihydroxytryptamine) lesions of the MRN have been shown to determine an anxiolytic effect in the EPM and light–dark box tests (Andrade and Graeff, 2001); the same lesions have impaired inhibitory avoidance in the elevated T-maze, characterizing an anxiolytic effect (Andrade et al., 2004). In the same direction, micro-injection of 5-HT_{1A} agonists into the MRN, mainly 8-OH-DPAT, reducing the activity of 5-HT neurons by stimulating 5-HT_{1A} autoreceptors (Dourish et al., 1986; Hannon and Hoyer, 2008; Kalsner, 2000; Mongeau et al., 1997; Ögren et al., 2008), has been shown to decrease anxiety in several animal models, such as, plus-maze (De Almeida et al., 1998), elevated T-maze (Dos Santos et al., 2005),

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social interaction (Andrews et al., 1994), dark-light transition (Carli and Samanin, 1988; Vicente et al., 2008), contextual conditioning (Avanzi and Brandão, 2001; Avanzi et al., 2003; Borelli et al., 2005; Silva et al., 2004) and ultrasonic vocalization (Schreiber and De Vry, 1993a, 1993b). Accordingly, further results have shown that the anxiolytic effect of intra-MRN estradiol in ovariectomized female rats tested in the EPM is antagonized by local pre-treatment with a selective 5-HT_{1A}-receptor blocker, Way 100635 (Andrade et al., 2005). In all these cases, impairment of the MRN-hippocampal 5-HT pathway resulted in anxiety reduction.

Therefore, in the present study we predicted that social separation and diazepam withdrawal would increase anxiety indices in the EPM as well as 5-HT activity in the MRN-hippocampal pathway. To assess this activity, we measured 5-HT turnover in the MRN and dorsal hippocampus. For comparison, turnover was also measured in the medial prefrontal cortex and the amygdala as the latter brain structure is mainly innervated by the dorsal raphe nucleus (Azmitia and Segal, 1978), and the medial prefrontal cortex by both the dorsal and median raphe nuclei (Meloni et al., 2008).

Imipramine is an antidepressant agent that inhibits 5-HT and noradrenaline reuptake, and has been shown to improve both generalized anxiety disorder and panic disorder after prolonged administration (McLeod et al., 2000). However, inconsistencies have been found with animal models of anxiety. In the EPM, chronic treatment with imipramine does not have reliable anxiolytic effects (Cole and Rodgers, 1995; File and Johnston, 1987). However, in the elevated T-maze, the same treatment has been shown to impair both inhibitory avoidance of the open arms and one-way open-arm escape, two tasks supposedly related to generalized anxiety disorder and panic disorder, respectively (Borsini et al., 2002; Dombrowski and Andreatini, 2006; Teixeira et al., 2000).

Imipramine seems to interfere with the functioning of the MRN-hippocampal pathway, since electrophysiological studies have shown that at the early phase of daily drug administration, functioning is reduced by the stimulation of somatodendritic 5-HT_{1A} autoreceptors. Twenty-one days after, these receptors are fully desensitized while post-synaptic, hippocampal 5-HT_{1A} receptors become over responsive to 5-HT, resulting in increased functioning of the MRN-hippocampal pathway (Blier and Montigny, 1999; Mongeau et al., 1997). On this basis, we have further verified whether daily administration of imipramine during the 14-day period of social separation would affect the behavioural and neurochemical consequences of separation.

Material and methods

Animals

Male Wistar rats weighing 200 g (about 2.5 months old) at the beginning of the experiments were used. The animals were housed in either groups of five or individually in polypropylene cages (32 cm × 38 cm × 18 cm, for grouped; 28 cm × 17 cm × 13 cm, for singly housed). It is important to notice that the socially separated animals still had distant auditory, visual and olfactory contact with other rats (Maisonnette et al., 1993, Rex et al., 2004). The animals had free access to food and water during the whole period of the experiment until the behavioural evaluation in the EPM. Room temperature was kept at 21 ± 2°C. Lights were on at 07:00 h and off at 19:00 h. The animals were manipulated three times a week to clean the cages. The experiments reported in this paper were performed in compliance with the recommendations of the Brazilian Society of Neuroscience and Behaviour which, in turn, are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apparatus

The EPM was made of wood and consisted of two open arms (50 cm × 10 cm) crossed at right angles with two opposed arms of the same size and enclosed by 40 cm high walls; these arms were 50 cm above the floor (Pellow et al., 1985). To prevent the rats from falling, a rim of Plexiglas (0.5 cm high) surrounded the perimeter of the open arms. Brightness at the level of the floor maze was 50 lux. The experimental sessions were recorded by video camera interfaced with a video monitor and a video recorder placed in an adjacent room. The experimenter stayed outside the room, and the behaviour of the rat was recorded on videotape, which was later analysed using standardized software (Noldus Observer, Wageningen, The Netherlands).

Drugs

Diazepam (Roche, Brazil) and imipramine (Sigma, USA) were dissolved in sterile 0.9% saline. The suspension of the diazepam in the vehicle was aided by the addition of Tween 80 (1%).

Procedures

Animals were randomly allocated to separate or grouped housing, and to three treatment groups for each condition (separated or grouped housing): (1) saline control; (2) diazepam (2 mg/kg); (3) imipramine (15 mg/kg). Rats received two daily intraperitoneal injections of either drug or saline, at 12-h intervals for 14 days, in a volume of 1 ml/kg. Doses of the drugs were chosen according to previous reports (Andrews and File, 1993; Andrews et al., 1997; Cole and Rodgers, 1995; File and Johnston, 1987; Fernandes et al., 1999).

Twelve hours after the last injection, animals were tested in the EPM. Each rat was taken directly from the home cage and placed at the centre of the plus-maze heading an enclosed arm, and allowed to explore the environment for 5 min. Before testing the next rat, the maze was cleaned with a 20% ethanol solution and dried with a cloth. The number of open or closed arm entries (with the four paws) and time spent on the arms were recorded. Data were expressed as percentage of open/total arm entries and of time spent on the open arms.

The concentration of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) was assayed by reverse-phase high performance liquid chromatography with electrochemical detection, as earlier described (Mayer and Shoup, 1983). Briefly, a C18 reverse phase column (Hypurity Elite, CLC-ODS 250 mm × 4.6 mm), an amperometric detector L-ECD-6A

(Shimadzu) and a liquid chromatography workstation LC10 AD (Shimadzu) were used. The animals were decapitated immediately after the behavioural evaluation in the EPM; their brains were removed, placed in ice-cold distilled water and allowed to cool for 1 min. The hippocampus, frontal cortex, amygdala and MRN were dissected out and the tissues were kept at −70°C for up to one week until biochemical assessment. The frozen tissue was weighted and homogenized in 0.2 M perchloric acid + 2,3-dihydroxybenzoic acid (internal standard). After centrifugation (15,000 rpm, 15 min, 6°C), 20 µl of the supernatant was injected into the chromatograph. The mobile phase flow rate was 1.2 ml/min and its composition was: 14.14 g chloroacetic acid, 4.66 g sodium hydroxide, sufficient sodium hydroxide to bring the pH value to 3.0, 208 mg octyl sodium sulphate, 250 mg ethylenediaminetetraacetic acid, 35 ml acetonitrile and 24.8 ml tetrahydroflurane. Detector sensitivity was 2 nA and the oxidation potential was fixed at 0.85 V using a glassy carbon-working electrode versus an Ag/AgCl reference electrode. The concentration of 5-HT and 5-HIAA in the tissue was obtained by comparison with standard solutions of those substances. The values obtained were expressed as ng/mg of wet tissue. The 5-HIAA/5-HT ratio was used as an index of 5-HT turnover.

Statistical analysis

Two-way analysis of variance (ANOVA) was used for both behavioural and neurochemical data. The factors were housing condition (grouped × separated), and drug treatment (saline, diazepam or imipramine). When needed, variances were homogenized by logarithmic transformation. Post-hoc relevant comparisons were made with the Newman–Keuls test. Data are reported as mean ± SEM. The level of significance was set at $p < 0.05$; a value of $p > 0.05$ and <0.1 was considered as a trend.

Results

Behaviour

Figure 1 shows the percentage of entries (upper panel) and of time (lower panel) on the open arms of the EPM in relation to the total.

In regard to open arm entries, ANOVA showed a significant interaction between housing and drug treatment ($F(2,44) = 6.48$; $p = 0.003$). Post-hoc comparisons with the Newman-Keuls test showed that social separation decreased open arm entries ($p < 0.05$); diazepam withdrawal decreased open-arm entries in grouped ($p < 0.01$), but not in separated animals that already had a very low baseline; and imipramine increased open-arm entries in separated ($p < 0.001$), but not in grouped animals.

As to the percentage of time spent on the open arms, ANOVA showed a significant main effect of drug treatment ($F(2,44) = 15.26$; $p < 0.001$), but no effect of housing ($F(1,44) = 0.96$; $p = 0.333$). There was a nearly significant interaction between the factors ($F(2,44) = 2.90$; $p = 0.066$). Post-hoc comparisons showed that social separation decreased the time spent on open arms ($p < 0.05$); diazepam

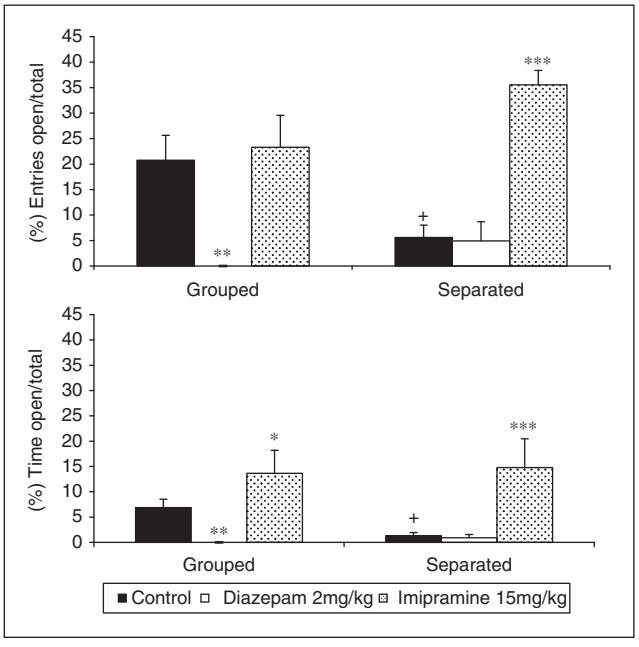

Figure 1. Mean ± SEM of the percentage of entries on to and time spent on the open arms of the elevated plus-maze in relation to the total, in rats grouped (five per cage) or single-housed, given two daily intraperitoneal injections for 14 days of either saline (grouped $n = 12$, separated $n = 12$), 2 mg/kg diazepam (grouped $n = 6$, separated $n = 9$) or 15 mg/kg imipramine (grouped $n = 5$, separated $n = 6$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate differences in relation to the control group in the same condition and $^{+}p < 0.05$ for comparisons between the control groups. Analysis of variance followed by the Newman-Keuls test.

withdrawal decreased time spent on open arms in grouped ($p < 0.01$), but not in separated animals; and imipramine increased time spent on open arms in separated ($p < 0.01$) and in grouped animals ($p < 0.05$).

Therefore, with the two indexes of anxiety measured in the EPM both social separation and diazepam withdrawal increased anxiety, the anxiogenic effect of separation being prevented by imipramine.

Neurochemistry

Table 1 shows the changes in 5-HT turnover in the brain structures analysed.

In the hippocampus, there was a significant housing × drug treatment interaction ($F(2,30) = 5.71$; $p = 0.008$). Post-hoc comparisons showed that social separation increased 5-HT turnover in control rats ($p < 0.05$), an effect that was antagonized by imipramine ($p < 0.001$). In addition, diazepam withdrawal increased 5-HT turnover in grouped animals ($p < 0.05$).

In the MRN, ANOVA showed a significant interaction between the factors ($F(2,27) = 13.60$; $p < 0.001$). Post-hoc comparisons showed that separation increased 5-HT turnover ($p < 0.01$), that was antagonized by imipramine ($p < 0.001$). Moreover, diazepam withdrawal increased 5-HT turnover in grouped rats ($p < 0.001$).

In the frontal cortex, ANOVA showed a significant main effect of housing on the 5-HT turnover ($F(1,29) = 16.02$; $p < 0.001$), but no main effect of drug treatment ($F(2,29) = 2.38$; $p = 0.111$), nor a significant interaction between the factors ($F(2,29) = 0.72$; $p = 0.496$). The figures of Table 1 show that social separation generally decreased 5-HT turnover.

In the amygdala, ANOVA showed a main effect of drug treatment ($F(2.29) = 9.74$; $p = 0.001$), and a trend towards a significant main effect of housing ($F(1,29) = 3.95$; $p = 0.056$). There was no significant interaction between the factors ($F(2,29) = 0.53$; $p = 0.594$). It can be seen in Table 1 that diazepam withdrawal generally increased, whereas imipramine generally decreased 5-HT turnover, independent of housing. The latter condition had little effect on 5-HT turnover in control rats, the above trend towards a housing main effect probably being due to the lower values verified in drug-treated animals.

Therefore, a similar pattern of changes occurred in the hippocampus and MRN, 5-HT turnover rate being increased by separation, and this effect antagonized by chronic imipramine treatment. Also in these structures, diazepam withdrawal increased 5-HT turnover in grouped rats, only. In the frontal cortex, separation had an opposite effect, generally decreasing 5-HT turnover, drug treatments being ineffective. In the amygdala, separation also decreased 5-HT turnover in drug-treated, but not in control animals. In addition, diazepam withdrawal increased, whereas imipramine decreased 5-HT turnover in both housing groups.

Discussion

The behavioural results of the present study showed that social separation for a period of 14 days increases spatiotemporal indices of anxiety in the EPM. These results are in agreement with reported results showing that social separation in the adult rat similarly reduces the frequency of open-arm entries and the time spent on the open arms of the EPM (Haller and Halász, 1999; Maisonnette et al., 1993; Rex et al., 2004; Sherif and Oreland, 1996).

In the same way as social separation, diazepam withdrawal was presently shown to have an anxiogenic effect in the EPM, also in agreement with previously reported results (Assié et al., 1991; Begg et al., 2005; Bhattacharya et al., 1995; File and Hitchcott, 1991; File et al., 1987; Fontanesi et al., 2007; Martijena et al., 1996; Pokk and Zharkovsky, 1998; Souza Pinto et al., 2007; Wright et al., 1991). However, in the former studies the anxiogenic effect was observed 24–48 h

Table 1. 5-hydroxytryptamine turnover estimated by the 5-hydroxyindoleacetic acid/5-hydroxytryptamine ratio in different brain structures of rats that have explored the elevated plus-maze

Brain structure	Drug	Grouped	Separated
Hippocampus	Control	1.05 ± 0.08	$1.73 \pm 0.29^{+}$
	Diazepam	$1.87 \pm 0.20^{*}$	2.13 ± 0.35
	Imipramine	1.00 ± 0.08	$0.31 \pm 0.04^{+***}$
Median raphe nucleus	Control	2.51 ± 0.16	$5.83 \pm 0.53^{++}$
	Diazepam	$9.55 \pm 2.09^{***}$	$5.27 \pm 0.69^{+++}$
	Imipramine	2.64 ± 0.33	$1.30 \pm 0.08^{***}$
Amygdala	Control	2.56 ± 0.46	2.36 ± 0.34
	Diazepam	3.28 ± 0.42	2.56 ± 0.36
	Imipramine	1.72 ± 0.40	$0.72 \pm 0.34^{**}$
Frontal cortex	Control	1.11 ± 0.15	0.81 ± 0.12
	Diazepam	1.43 ± 0.10	0.85 ± 0.16
	Imipramine	1.04 ± 0.10	0.69 ± 0.10

Figures represent mean ± SEM; $N = 5$–6. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ are significant differences between drug treatment and the respective control; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ are significant differences between grouped and separated animals. Drugs were administered twice a day for 14 days; doses were 2 mg/kg for diazepam and 15 mg/kg for imipramine. Separated animals were single housed for 14 days; grouped animals were five per cage.

after the last injection of diazepam, while in the present work the abstinence period was only 12 h. The behavioural changes induced by discontinuation of chronic diazepam seem to be time-dependent, because after 72 h they have been shown to disappear (Martijena et al., 1996).

The main neurochemical finding of the present study is that the chronic stress of social separation selectively increased 5-HT turnover rate in the hippocampus and MRN. The same change occurred following acute stress by diazepam withdrawal in grouped rats only, probably because values were already high in separated animals. These results indicate that the MRN-hippocampal pathway is activated by both acute and chronic stress.

Although the above behavioural and neurochemical changes may be independent phenomena, there is reported evidence pointing to a causal connection between these events. As mentioned in the Introduction, several manipulations that decrease the activity of the MRN-hippocampal pathway decrease anxiety in several animal models (Andrade and Graeff, 2001; Andrade et al., 2004, 2005; Andrews et al., 1994; Avanzi and Brandão, 2001; Avanzi et al., 2003; Borelli et al., 2005; Carli and Samanin, 1988; De Almeida et al., 1998; Dos Santos et al., 2005; Schreiber and De Vry, 1993a, 1993b; Silva et al., 2004; Vicente et al., 2008). In the opposite direction, reported evidence showed that electrical or chemical stimulation of the MRN produces behavioural changes suggestive of anxiety that are mediated by 5-HT (Dos Santos et al., 2005, 2008; Graeff and Silveira Filho, 1978; Vicente et al., 2008). In addition, microinjection of the 5-HT_{1A} receptor agonist 8-OH-DPAT into MRN, stimulating inhibitory autoreceptors, has been shown to reverse the anxiogenic effect of diazepam withdrawal, which indicates that increased 5-HT neurotransmission in the MRN-hippocampal pathway mediates increased anxiety (Andrews and File, 1993; Andrews et al., 1997).

The same mechanism may be responsible for the increase in anxiety determined by social separation, since chronic treatment with the 5-HT_{1A} agonist gepirone has been shown to inhibit the anxiogenic effect of a two-week period of social separation (Maisonnette et al., 1993). However, no specification of the 5-HT pathway involved is possible because the drug was given systemically.

Within this context, the present results showing that daily administration of imipramine for 14 days simultaneously reduced both the anxiogenic effect in the EPM and the increase in 5-HT turnover in the MRN and in the hippocampus determined by social separation are highly suggestive that such anxiogenesis is mediated by activation of the MRN-hippocampal pathway.

Electrophysiological and neurochemical studies have shown that 5-HT raphe neurons recover their firing rate (Blier and Montigny, 1994; Blier et al., 1990), and enhanced 5-HT concentrations are then found in the terminal areas only after 3 weeks of treatment with antidepressants (Artigas, 1993). In agreement with these findings, our results showed that 5-HT turnover has not been altered in any of the analysed structures of grouped animals following 14-day administration of imipramine. In spite of this, the drug regimen was able to prevent the behavioural and neurochemical effects of separation. Therefore, although there is reported evidence that imipramine progressively enhances the responsiveness of postsynaptic 5-HT_{1A} receptors in the hippocampus (Blier and Montigny, 1999), under chronic stress, the protective action of imipramine seems to be mainly due to a pre-synaptic action.

Together with the lack of change in 5-HT turnover, the present results showed that 14-day treatment with imipramine did not change EPM anxiety indices in grouped animals. These data are in line with previous reports showing no effect of chronic treatment with imipramine in grouped rats or mice also tested in the EPM (Cole and Rodgers, 1995; File and Johnston, 1987). A recent study in rats (Pinheiro et al., 2008) has similarly shown that 14-day administration of imipramine does not affect inhibitory avoidance in the elevated T-maze, a task that has predictive value for clinical anti-anxiety drug action (Graeff et al., 1998). Nevertheless, in the same study (Pinheiro et al., 2008) as well as in previously reported results (Dombrowski and Andreatini, 2006; Teixeira et al., 2000; Zanovelli et al., 2005) 21-day administration of imipramine has been shown to impair the same inhibitory avoidance, considered as an anxiolytic effect.

In conclusion, the present results show that the acute stress of diazepam withdrawal had anxiogenic-like effects, accompanied by increases in 5-HT activity in the MRN and hippocampus. Similar behavioural and neurochemical changes were also observed in rats following the chronic stress of 14-day social separation, which were prevented by concurrent daily treatment with imipramine. It is suggested that activation of the MRN-hippocampus 5-HT pathway mediates the anxiogenic effects of both acute and chronic stress.

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