A comparison of rat brain amino acid and monoamine content in diazepam withdrawal and after exposure to a phobic stimulus

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1 The content of amino acids (taurine, glycine, glutamic acid, γ -aminobutyric acid (GABA) and aspartic acid) and monoamines (5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline) in homogenates of rat cortical and hippocampal tissue were measured by high performance liquid chromatography (h.p.l.c.) with fluorescent and electrochemical detection respectively, after two anxiogenic treatments: exposure to a phobic stimulus (cat odour) and withdrawal from chronic diazepam treatment.

2 In neither of the two anxiogenic situations was there a significant change in any amino acid content, in either brain area.

3 In the group withdrawn from chronic diazepam, cortical 5-HT and 5-HIAA levels and hippocampal 5-HT levels were significantly increased. Noradrenaline content was significantly decreased in the hippocampus.

4 The changes in 5-HT and 5-HIAA levels following cat odour exposure were area-specific in that they decreased in the hippocampus, but increased in the cortex.

5 Following cat odour exposure, noradrenaline levels appeared not to change in either area studied. However during exposure to cat odour, it was found that half the animals avoided the odour source and half were indifferent. The animals showing marked avoidance had significantly higher cortical noradrenaline content and this was significantly different from control, whereas hippocampal noradrenaline levels were not dependent upon the differences in avoidance of the odour source.

6 The results show clearly different neurochemical changes in the rat following exposure to a phobic stimulus and withdrawal from diazepam. It is hoped comparative studies such as this will enable better understanding of anxiety states in the rat which could parallel the different classes of anxiety recognised in the clinic

Keywords: Cat odour; diazepam withdrawal; noradrenaline (NA); 5-hydroxytryptamine (5-HT); amino acids

Introduction

Several different types of anxiety disorder are recognised clinically and these show differential sensitivity to pharmacological treatments. For example, generalised anxiety disorder is still mainly treated with benzodiazepines, whereas panic disorder responds best to tricyclic antidepressants, and simple phobias are often not effectively treated by drugs, although they sometimes respond to monoamine oxidase inhibitors. This differential diagnosis and pharmacological treatment suggests that the various anxiety disorders have different neurobiological substrates.

Blanchard *et al.* (1989) described the relative lack of effects of diazepam to the response of rats to the odour of a predator, and we have recently proposed (Zangrossi & File 1992a) that the responses of rats to cat odour could provide a model of phobic anxiety. In contrast, we believe that the anxiety produced by benzodiazepine receptor agonist withdrawal is closer to that of generalised anxiety disorder. The purpose of the present experiments was to compare the effects of these two manipulations on brain neurotransmitter content.

Methods

Animals

Male, hooded Lister rats (Olac, Bicester), approximately 250 g in weight were housed in groups of five, until five days before the experimental session at which time they were housed singly. Food and water were freely available at all times. The room was maintained at 22°C with lights on from 07 h 00 min to 19 h 00 min.

Drugs and chemicals

Diazepam (Roche U.K. Ltd.) was suspended (by use of a sonicating water bath) at a concentration of 1 mg ml⁻¹ in distilled water containing 1 drop of Tween-20 per 20 ml of water. It was administered intraperitoneally (2 mg kg⁻¹, i.p.) once per day for 21 days. Controls received i.p. vehicle daily for 21 days. All injections were in a volume of 2 ml kg⁻¹.

Procedure

Diazepam withdrawal Eighteen animals were randomly allocated to either chronic vehicle ($n = 9$) or diazepam ($n = 9$) treatment for 21 days. On the test day, 24 h after the last chronic injection, all the animals received an i.p. injection of vehicle 30 min before they were killed.

Exposure to cat odour Seventeen rats were randomly allocated between neutral ($n = 8$) and cat ($n = 9$) odour conditions. The cat odour was obtained by rubbing a damp cloth

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vigorously against the fur of a laboratory-housed domestic cat for 5 min. This procedure was carried out 1 h before the experimental session. The cat odour cloth was kept in a sealed plastic bag. Each cloth was used for 4 exposures only. Damp pieces from the same original cloth were used for the neutral odour.

All odour exposures took place in a separate small, dimly lit room and the neutral odour exposures always preceded the cat odour exposures in order to prevent any traces of cat odour influencing the neutral odour group. Before the first cat odour exposure, an impregnated cloth was left in the test room for 10 min. Each rat was carried to the exposure room in its home-cage which was placed next to an empty cage, with the odour cloth wedged between the cage tops, at the opposite end from the food and water containers. The duration of odour exposure was 5 min and the rats were videotaped for later scoring. A cloth contact was defined as a direct contact or sniffing ≤ 5 cm from the cloth; sheltering was defined as and when the rat was underneath the food and water compartments. For further details see Zangrossi & File (1992b). Immediately after the exposure the animals were returned to the animal house and 30 min later they were killed. This time interval between odour exposure and death was selected because it was the time at which marked changes in behaviour in the social interaction and plus-maze tests were detected after exposure to cat odour (Zangrossi & File, 1992b).

Tissue preparation Animals were stunned and killed by cervical dislocation and the brains removed. Frontal cortex (approximately 25 mg) and hippocampus (approximately 100 mg) were dissected out on an ice-cooled petri-dish and immediately placed in 200 μ l and 400 μ l respectively of homogenizing solution (0.2 M perchloric acid containing 100 μ g μ l⁻¹ dihydroxybenzylamine hydrobromide). The tissue was then sonicated using a soniprep soniprobe for 8 s. The resulting homogenate was then divided in half, with one aliquot for amino acid analysis and one aliquot for the monoamine analyses.

Amino acid analysis After storage at -20°C for at least 2 h, and centrifugation for 1 min at 8,800 g in an Eppendorf bench top centrifuge (model 5413), the resulting supernatant was decanted and stored at -20°C until assay. The samples were analysed for γ -aminobutyric acid (GABA), aspartic acid, glycine, glutamic acid and taurine on a Varian 5000 HPLC (high performance liquid chromatograph) with fluorescent detection following pre-column derivatisation with *o*-phthalaldehyde (Turnell & Cooper, 1981).

Indole- and catecholamine analysis The h.p.l.c.-electrochemical detection (e.c.d.) system comprised an isocratic pump (BAS PM-80, Solvent Delivery System) which was connected to an analytical column (Spherisorb 5 ODS; 150 $\times$ 4.6 mm; HPLC Technology) via a Rheodyne injector. The analytical column was protected by a C₁₈ guard column. The effluent from the analytical column was passed into an electrochemical detector (BAS LC-4C Amperometric Detector; glassy carbon electrode potential + 700 mV versus silver/silver chloride reference electrode). The output from the electrode was monitored with a recording/plotting integrator (JCL6000 Jones Chromatography). The h.p.l.c.-e.c.d. system, with the exception of the integrator, was maintained at a constant temperature of 4°C inside a glass-fronted cool cabinet. The optimised mobile phase for the simultaneous detection of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in homogenates (methanol 11% v/v; disodium hydrogen orthophosphate 106 mM; citric acid 36 mM; tetraethylammonium bromide 2 mM; pH 6.2–6.3; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) or noradrenaline (NA) following alumina extraction (see Anton & Sayre, 1962, for detailed methodology) from the brain homogenate (methanol 11% v/v; citric acid 100 mM; sodium

Figure 1 Mean ($\pm$ s.e.mean) amounts (ng mg⁻¹ protein) of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in hippocampus and cortex of rats following 24 h withdrawal from vehicle (21 days; Con) or diazepam (2 mg kg⁻¹ daily for 21 days; With) (a) or 30 min following exposure to a neutral (Neu) or cat (Cat) odour (b). Group sizes were $n = 7-9$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with vehicle control group or with neutral odour group.

octylsulphate 0.225 mM; sodium EDTA 0.05 mM; pH 2.4–2.6; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) were delivered to the analytical column at a rate of 1.4 ml min⁻¹. Injections of external standards were made in order to identify and calibrate the peaks resulting from the injection of the dialysates.

The results of all analyses were finally expressed in terms of the amount per mg protein in the tissue sample, as determined by the method of Lowry *et al.* (1951).

Figure 2 Mean ($\pm$ s.e.mean) amounts (ng mg⁻¹ protein) of noradrenaline in hippocampus (a) and cortex (b) of rats exposed to neutral or cat odour (with either low (Cat low) or high (Cat high) behavioural responses to the odour). Group sizes were $n = 7-9$. * $P < 0.05$ compared with neutral odour.

Table 1 Amino-acid content ($\mu\text{g mg}^{-1}$ protein) in hippocampal and cortical tissue of rats either 24 h following withdrawal from chronic (21 days) vehicle (Control) or 2 mg kg^{-1} diazepam (Withdrawal) or 30 min following exposure to neutral (Neutral) or cat (Cat) odour

	Aspartate	Taurine	Glycine	Glutamate	GABA
<i>Hippocampus</i>					
Control	1.23 $\pm$ 0.12	4.01 $\pm$ 0.37	0.66 $\pm$ 0.07	7.09 $\pm$ 0.67	0.90 $\pm$ 0.06
Withdrawal	1.21 $\pm$ 0.11	3.92 $\pm$ 0.32	0.64 $\pm$ 0.05	7.16 $\pm$ 0.33	0.93 $\pm$ 0.05
Neutral	1.67 $\pm$ 0.11	4.23 $\pm$ 0.48	0.68 $\pm$ 0.10	8.57 $\pm$ 0.78	1.00 $\pm$ 0.12
Cat	1.57 $\pm$ 0.11	3.84 $\pm$ 0.26	0.56 $\pm$ 0.04	7.85 $\pm$ 0.37	0.93 $\pm$ 0.06
<i>Cortex</i>					
Control	2.19 $\pm$ 0.24	4.84 $\pm$ 0.53	0.89 $\pm$ 0.08	10.41 $\pm$ 1.04	0.86 $\pm$ 0.07
Withdrawal	2.18 $\pm$ 0.19	4.60 $\pm$ 0.56	0.77 $\pm$ 0.06	10.91 $\pm$ 1.01	0.83 $\pm$ 0.08
Neutral	1.98 $\pm$ 0.26	4.32 $\pm$ 0.85	0.78 $\pm$ 0.09	9.80 $\pm$ 1.13	0.63 $\pm$ 0.13
Cat	2.11 $\pm$ 0.14	4.17 $\pm$ 0.38	0.77 $\pm$ 0.06	10.81 $\pm$ 0.69	0.64 $\pm$ 0.08

Values are mean $\pm$ s.e.mean. Group sizes were $n = 6$.

Statistics

The data from the diazepam withdrawn and cat odour exposed animals were compared with their own respective controls by independent t tests, and it is the significances of these that are shown in Figure 1 and Table 2. When the cat odour data were divided into low and high responder groups the results were analysed with analysis of variance, followed by Duncan's tests for significance of individual groups compared with the controls. It is the significance that is shown in Figure 2.

Results

Neurochemical consequences following diazepam withdrawal

There were no changes in amino acid content in either the hippocampal or cortical samples of rats withdrawn from chronic diazepam (see Table 1). However, Figure 1a shows the levels of 5-HT and 5-HIAA to be greater in the samples of cortex and hippocampus from withdrawn animals compared with control. The levels of noradrenaline were significantly lower in the hippocampus, but not the cortex of withdrawn animals, see Table 2.

Neurochemical consequences following cat odour exposure

There were no changes in amino acid content in either cortical or hippocampal homogenates in the animals exposed to cat compared with neutral odour (Table 1). However, a marked decrease in the levels of 5-HT and 5-HIAA was observed in the hippocampus of these animals compared with the animals exposed to neutral odour (see Figure 1b). Figure 1 also shows that in the cortex of rats exposed to cat odour, the levels of 5-HT and 5-HIAA were significantly increased.

The levels of noradrenaline were not significantly different between the two groups in either area investigated. However, some differences emerged when the behavioural scores of the individual rats were considered. Exposure to cat odour is characterized by the animals spending the majority of the exposure time sheltering under the food and water hoppers (Zangrossi & File, 1992b). In this particular experiment the animals formed two distinct groups based on their time spent sheltering. One group ($n = 5$) was highly responsive to the cat odour (mean of time sheltering = 219.8 ± 30.7 (s)) and the other ($n = 4$) was insensitive to the cat odour (mean of time sheltering = 52.3 ± 14.5 (s)), compared with the neutral odour exposed group (mean of time sheltering = 40.8 ± 14.4 (s)). When the noradrenaline levels of these two groups were compared with the levels of the animals exposed to neutral odour it was found that in the cortex, but not the hippocampus,

Table 2 Noradrenaline content (ng mg^{-1} protein) of cortical and hippocampal tissue of rats 24 h following withdrawal from either chronic (21 day) vehicle (Control) or diazepam (Withdrawal) treatment

	Cortex	Hippocampus
Control	0.26 $\pm$ 0.05	6.00 $\pm$ 0.70
Withdrawal	0.19 $\pm$ 0.02	3.30 $\pm$ 0.40**

Values are mean $\pm$ s.e.mean. Each group contained 7–9 animals. Student's t test was used to analyse for statistically significant differences within groups.

** $P < 0.01$.

pus, the group highly responsive to cat odour showed a significant increase in the level of noradrenaline (Figure 2). The results of 5-HT, 5-HIAA and amino acid analyses were not changed by dividing the cat odour results in this way.

Discussion

The diazepam-withdrawn rats did not differ from their control in their cortical or hippocampal amino acid content. This suggests that amino acid transmitters do not play a major part in the withdrawal responses from this low dose of diazepam, and we found no change in GABA release and uptake in rats withdrawing from the same dose (Andrews *et al.*, 1992). However, the conclusion that amino acids are not important must be viewed with some caution since whole tissue levels do not always reflect alterations in extracellular levels. No changes in the levels of amino acids were found in either cortical or hippocampal tissue after exposure to cat odour. Yet we found increased [^{14}C]-GABA release and decreased uptake from both hippocampal and cortical slices from animals exposed to cat odour (File *et al.*, 1992). We do not at present know the reason for this discrepancy.

5-HT and 5-HIAA content were increased following diazepam withdrawal in both cortex and hippocampus. We have already suggested that the increased hippocampal 5-HT release underlies the increase in anxiety during diazepam withdrawal (Andrews & File, 1992), but we have no evidence to date of the behavioural changes that may be mediated by increased cortical 5-HT function.

Noradrenaline levels were significantly decreased only in the hippocampus following withdrawal from diazepam treatment. This finding agrees with that of Rastogi *et al.* (1976). It is not possible to say whether these changes reflect an increased or decreased noradrenergic function, but the present results suggest further investigations of noradrenaline release and uptake would be warranted.

Prior exposure to cat odour had differential effects on 5-HT and 5-HIAA according to the brain area analysed. Thus in the hippocampus, 5-HT and 5-HIAA levels were

lowered whereas in the cortex they were raised relative to levels of neutral odour exposed rats. Although there were significant changes in 5-HT and 5-HIAA, it is interesting that these were not dependent on the animal's actual behavioural response during the odour exposure.

In contrast the changes in noradrenaline content in the cortex were crucially dependent on the rat's response during the odour exposure. Thus animals showing high avoidance of the odour also had high noradrenaline content in cortical tissue. Gorman *et al.* (1989) have recently hypothesized that phobic avoidance frequently observed in panic patients may involve the activation of the pre-frontal cortex, while anticipatory anxiety and panic attacks involve activation of limbic and brain-stem structures respectively. The changes in noradrenaline content following exposure of rats to cat odour further suggests the involvement of the cortical noradrenergic system in the development of phobic avoidance.

In conclusion, this study has shown that the two treatments give rise to different changes in 5-HT and noradrenaline content. We also have evidence from release studies for differences between these two treatments. Hippocampal

5-HT release is increased during diazepam withdrawal (Andrews *et al.*, 1992), but is not altered following exposure to cat odour (File *et al.*, 1992). We have also found that drugs capable of inhibiting 5-HT function reverse the anxiogenic effects of diazepam withdrawal, but only a few studies have investigated the effects of 5-HT drugs on the behaviour of rats exposed to cat odour. Blanchard's group has shown that the 5-HT₂ receptor antagonist, ritanserin, and the 5-HT_{1A} receptor agonist, gepirone, failed to show reliable anxiolytic effects during the presentation of cat odour (Shepherd *et al.*, 1992; Blanchard *et al.*, 1990). More detailed behavioural pharmacology studies of the responses to cat odour are needed, and the results from this study would suggest that drugs acting on the noradrenergic system might be effective in modulating responses to phobic stimuli.

The results of the present study lend support to there being neurochemical differences between anxiety generated by a phobic stimulus and that induced by diazepam withdrawal.

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