Pergamon

Pharmacology Biochemistry and Behavior, Vol. 49, No. 2, pp. 359–362, 1994

0091-3057/94 $6.00 + .00

0091-3057(94)E0138-8

Diazepam Withdrawal Increases [^{3}H]-5-HT Release From Rat Amygdaloid Slices

CATHY FERNANDES, NICK ANDREWS AND SANDRA E. FILE[1]

Psychopharmacology Research Unit, UMDS Division of Pharmacology, Guy's Hospital, London SE1 9RT

Received 28 September 1993

FERNANDES, C., N. ANDREWS AND S. E. FILE. *Diazepam withdrawal increases [^{3}H]-5-HT release from rat amygdaloid slices.* PHARMACOL BIOCHEM BEHAV **49**(2) 359–362, 1994.—The release of [^{3}H]-5-HT and [^{14}C]-GABA from hippocampal and amygdaloid slices was studied in a group of rats in which an anxiogenic response had been found on withdrawal of chronic diazepam treatment (2 mg/kg/day for 21 days). Basal release and uptake of [^{3}H]-5-HT and [^{14}C]-GABA and K^+-evoked release of [^{14}C]-GABA were not significantly changed in either brain region following diazepam withdrawal. However, there was a significant increase in K^+-evoked [^{3}H]-5-HT release from the amygdala, but not from the hippocampal, slices. These results demonstrate that increased 5-HT release from the hippocampus is not necessary to mediate the anxiogenic withdrawal response, and that raised 5-HT release in the amygdala may be sufficient to mediate this response. The results are discussed with respect to conditions, such as noise during diazepam treatment, that might produce regionally specific changes in 5-HT tone and hence modify the pattern of changes found during diazepam withdrawal.

Amygdala Hippocampus 5-HT GABA Anxiety Benzodiazepines

PREVIOUS studies have found increases in K^+-evoked [^{3}H]-5-HT release from hippocampal, but not from cortical, slices taken from rats withdrawn from chronic diazepam treatment (8,2). Although no behavioural measures of the withdrawal response were carried out, it was suggested that these increases in 5-HT release from terminal regions in the hippocampus mediated the anxiogenic behavioural response during benzodiazepine withdrawal (2). Consistent with this idea were the reversals of the anxiogenic withdrawal response by a number of ligands reducing 5-HT function (1). The causal link between raised 5-HT release in the hippocampus and the increase in anxiety is difficult to establish, but was strengthened by the finding that a group of chronically treated animals that showed no increase in anxiety on withdrawal, also showed no increase in 5-HT release in the hippocampus (1). Although the increase in 5-HT was regionally specific in that no significant changes were detected in the cortex, the role of other limbic areas, such as the amygdala, has not yet been explored.

The amygdaloid complex, which receives a major serotonergic input from the dorsal raphé nucleus, has been proposed to play a pivotal role in anxiety and probably represents an important site of diazepam's action (4,9). It has also been demonstrated that the anxiogenic withdrawal response, following chronic benzodiazepine administration, is reversed after injection of the 5-HT_3 receptor antagonist ondansetron into either the amygdala or the dorsal raphé nucleus (3).

In the present study, we therefore examined the release and uptake of [^{3}H]-5-HT and [^{14}C]-GABA from slices of the amygdala and hippocampus, taken from rats that displayed an anxiogenic withdrawal response in the elevated plus-maze test of anxiety, following withdrawal from chronic diazepam treatment.

METHOD

Animals

Male, hooded Lister rats (Harlan UK Ltd, Bicester, Oxon, UK), weighing approximately 200 g were housed in pairs, in a room maintained at 22°C, with lights (30 scotopic lx) on from 0700 to 1900 h. Food and water were freely available.

Drugs and Chemicals

Diazepam (Roche Products Ltd., Welwyn Garden City, Herfordshire, UK) was ultrasonically dispersed in distilled water, containing a drop of Tween-20, for 20 min prior to injection, to give a final concentration of 1 mg ml^{-1}. Rats received daily morning injections of 2 mg kg^{-1} administered intraperi-

[1] To whom request for reprints should be addressed.

TABLE 1

MEAN (± SEM) % NUMBER OF CLOSED ARM ENTRIES, % NUMBER OF ENTRIES ON TO, AND TIME SPENT ON THE OPEN ARMS OF THE PLUS-MAZE BY RATS TREATED FOR 21 DAYS WITH VEHICLE OR WITHDRAWN FROM DIAZEPAM (2 mg/kg/day)

	No. of Closed Arm Entries	% No. of Open Arm Entries	% Time Spent on the Open Arms
Vehicle (n = 8)	12.9 ± 0.8	35.8 ± 3.3	39.5 ± 5.2
Diazepam withdrawal (n = 12)	9.3 ± 1.5	18.8 ± 5.2*	19.4 ± 5.5*

*$p < 0.05$ compared with vehicle group.

toneally, in a volume of 2 ml kg^{-1} for 21 days. Control animals received equal volume IP injections of vehicle (distilled water/Tween-20) daily for 21 days.

For the neurotransmitter release studies, 4-amino-*n*-[U-^{14}C]-butyric acid ([^{14}C]-GABA, 213 mCi/mmol) and 5-[1,2-^{3}H *N*)]-hydroxytryptamine creatinine sulphate ([^{3}H]-5-HT, 12.7 Ci/mmol) were obtained from Amersham International (Amersham, Buckinghamshire, UK). The Krebs bicarbonate buffer contained (mM): NaCl 118; KCl 4.8; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; and glucose (Sigma Chemical Co., Poole, Dorset, UK) 11.1. The Krebs buffer was continuously gassed with 95% O_2–5% CO_2 and also contained (μM): amino-oxyacetic acid (Sigma) 50; pargyline (Sigma) 50; ascorbic acid (Sigma) 100; and EDTA 35. All other chemicals were obtained through BDH Ltd (Dagenham, Essex, UK) unless stated otherwise.

Apparatus

The plus-maze was made of wood and had two open arms (50 × 10 cm) with transparent perspex ledges (12 mm high) and two enclosed arms of the same size with walls 40 cm high. It was elevated 50 cm above the ground. The arms were connected by a central square (10 × 10 cm) and thus the maze formed a plus sign. The light level in the test room was <50 scotopic lx.

Procedure

Animals were randomly allocated to either chronic (21 days) diazepam (n = 12) or vehicle (n = 8) treatment groups. Rats were tested 18 h after the last chronic injection and 30 min following an IP vehicle injection. Each rat was placed in the central square of the plus-maze, facing an enclosed arm, and allowed 5 min to freely explore the maze. The rat was observed on a video monitor from an adjacent room and the numbers of entries onto, and times spent on, open and enclosed arms were scored by an observer blind to the chronic treatment.

All testing took place under quiet conditions, in an order randomised for previous drug treatment, between 0930 and 1200 h. Animals then received at least one more day of treatment and were sacrificed 18 h after their last chronic injection and 30 min following an IP vehicle injection.

Measurement of Release

Rats were stunned and killed by cervical dislocation. The brain was rapidly removed and hippocampus and amygdala dissected. Slices (0.2 mm) of both areas were cut using a MacIlwain tissue chopper (Mickle Lab. Engineering Co., Gom-

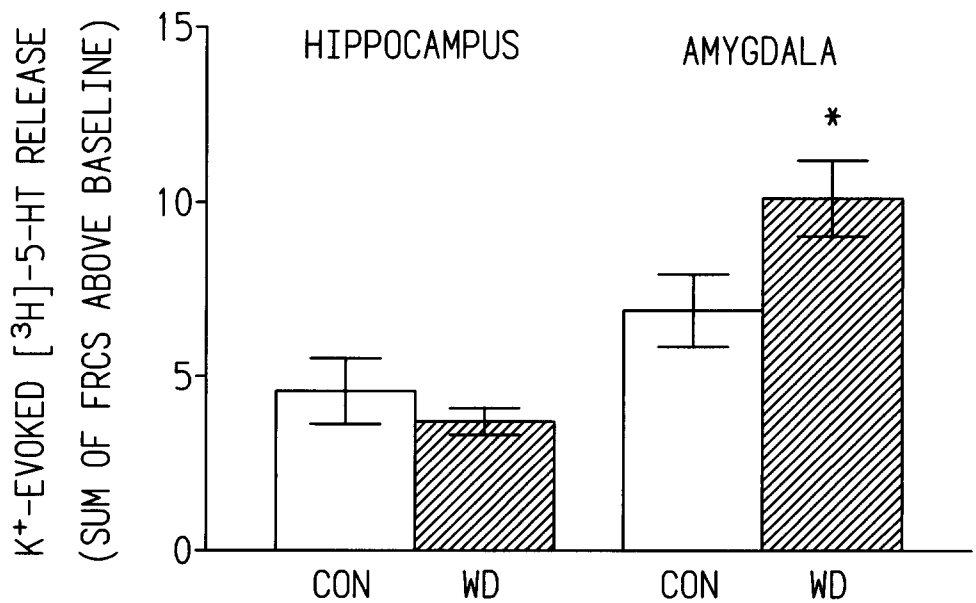

FIG. 1. Mean (±SEM) K^+-evoked [^{3}H]-5-HT release (Σ FRCs above baseline) from hippocampal and amygdaloid slices taken from rats treated for 21 days with vehicle (CON) or withdrawn from diazepam (2 mg/kg/day for 21 days; WD) 18 h following the last chronic injection. Rats were killed 30 min after i.p. injection with vehicle. *$p < 0.05$ compared with vehicle group.

shall, Surrey, UK) and the tissues (five slices equating to approximately 10–15 mg wet weight) incubated separately in 5 ml Krebs bicarbonate buffer at 37°C for 10 min.

The release and uptake of [^{3}H]-5-HT and [^{14}C]-GABA from slices of the amygdala and hippocampus were measured by a superfusion technique, described by Andrews et al. (2).

Statistics

The data were analysed with one-way analyses of variance (ANOVA).

RESULTS

Behaviour in the Plus-Maze

The rats withdrawn from 21 days of diazepam (2 mg/kg/day) showed a significant decrease in the percentage number of entries onto, and the percentage time spent on, the open arms of the elevated plus-maze, compared with controls, [$F(1, 18) = 6.0$ and 6.3 respectively; $p < 0.05$ in each case], see Table 1. This is indicative of an anxiogenic withdrawal response. The decrease in the number of closed arm entries by diazepam-withdrawn rats compared with controls failed to reach significance ($p = 0.07$), see Table 1.

[^{3}H]-5-HT Release and Uptake

The total K^+-evoked release of [^{3}H]-5-HT from amygdaloid slices was significantly increased in diazepam-withdrawn rats compared with control-treated rats [$F(1, 18) = 4.1, p = 0.05$], see Fig. 1. There were no significant changes in basal release or in uptake of [^{3}H]-5-HT from amygdaloid slices when control-treated rats were compared with the diazepam-withdrawn group. There were no significant differences in [^{3}H]-5-HT release or uptake from hippocampal slices between these two groups, see Table 2.

TABLE 2

MEAN (± SEM) BASAL (FRCS) AN K^+-EVOKED (Σ FRCS ABOVE BASELINE) AND UPTAKE (DPM × 10^{-3}) OF [^{14}C]-GABA AND [^{3}H]-5-HT FROM HIPPOCAMPAL AND AMYGDALOID SLICES TAKEN FROM RATS TREATED FOR 21 DAYS WITH VEHICLE OR WITHDRAWN FROM DIAZEPAM (2 mg/kg/day)

	Control (n = 8)	Diazepam Withdrawal (n = 12)
[^{14}C]-GABA Hippocampus		
Basal release	0.7 ± 0.1	0.7 ± 0.1
K^+-evoked	26.7 ± 3.8	30.8 ± 3.9
Uptake	21.2 ± 1.8	18.0 ± 0.9
[^{14}C]-GABA Amygdala		
Basal release	0.8 ± 0.1	0.9 ± 0.1
K^+-evoked	28.7 ± 2.2	41.4 ± 6.2
Uptake	20.1 ± 3.0	20.1 ± 2.7
[^{3}H]-5-HT Hippocampus		
Basal release	0.8 ± 0.1	0.8 ± 0.1
Uptake	166.5 ± 25.2	170.7 ± 11.5
[^{3}H]-5-HT Amygdala		
Basal release	0.7 ± 0.1	0.7 ± 0.1
Uptake	173.6 ± 16.6	185.4 ± 15.0

[^{14}C]-GABA Release and Uptake

The increase in K^+-evoked [^{14}C]-GABA release from the amygdaloid slices did not reach significance ($p = 0.12$) and there were no other significant changes in basal or K^+-evoked release or in uptake of [^{14}C]-GABA between control-treated and diazepam-withdrawn rats, in either area investigated, see Table 2.

DISCUSSION

Rats treated for 21 days with diazepam (2 mg/kg/day) displayed changes in the elevated plus-maze test, indicating an anxiogenic response, when they were tested 18 h after their last chronic dose. These results are in agreement with previous findings that increased anxiety can be detected in animal tests when rats are in spontaneous withdrawal from chronic treatment with a wide range of benzodiazepine doses (6). An increase in K^+-evoked [^{3}H]-5-HT release from amygdaloid slices was detected in these diazepam-withdrawn rats, compared with controls. These results once again support the hypothesis that during benzodiazepine withdrawal there is an increase in anxiety and an increase in 5-HT release (1). However, in contrast with previous findings (2), there was no change in [^{3}H]-5-HT release from hippocampal slices after diazepam withdrawal. This highlights the important finding that raised 5-HT release in the hippocampus is not necessary to mediate the anxiogenic responses seen in the plus-maze on withdrawal from chronic diazepam treatment. Since the studies reporting increased [^{3}H]-5-HT release from the hippocampus (8,2) did not include behavioural measures or examine release in the amygdala, we do not know whether an increased 5-HT release in either limbic area is sufficient to mediate behavioural changes, or whether it is the changes in the amygdala that are crucial. The absence of changes in hippocampal 5-HT release in the present experiment may be related to changes in the basal tone of the serotonergic and GABAergic systems within these areas. For example, Fernandes and File (5) found striking increases in GABA release from amygdaloid and hippocampal slices taken from rats housed in conditions of chronic noise, whereas there was increased 5-HT release only in the hippocampus. The present study also took place during exposure to intermittent noise of moderate intensity (ranging between 70–100 dB), produced by construction work. The levels of [^{3}H]-5-HT and particularly [^{14}C]-GABA release from both regions in our control rats were comparable with those previously found following noise exposure. It is possible that the elevation in hippocampal 5-HT release caused by the construction noise prevented a further significant increase on withdrawal from diazepam. Indeed, we have also found that when the chronic diazepam treatment was given under conditions of more extreme noise this completely prevented the development of tolerance to the anxiolytic effects of diazepam and an anxiogenic response on diazepam withdrawal (7). It remains to be established whether there is any change in amygdaloid [^{3}H]-5-HT release under these conditions. This will be a crucial test of the importance of increased amygdaloid 5-HT release to the anxiogenic response following withdrawal from chronic benzodiazepine treatment.

ACKNOWLEDGEMENT

These experiments were supported by a grant from the Wellcome Trust.

REFERENCES

1. Andrews, N. A.; File, S. E. Neurochemical and behavioural evidence that increased 5-HT release mediates the anxiogenic response during benzodiazepine withdrawal. Psychopharmacology 112:21–25; 1993.
2. Andrews, N. A.; Zharkovsky, A.; File, S. E. Raised [^{3}H]-5-HT release and $^{45}Ca^{2+}$ uptake in diazepam withdrawal: Inhibition by baclofen. Pharmacol. Biochem. Behav. 41:695–699; 1992.
3. Costall, B.; Jones, B. J.; Kelly, M. E.; Naylor, R. J.; Onaivi, E. S.; Tyers, M. B. Sites of action of ondansetron to inhibit withdrawal from drugs of abuse. Pharmacol. Biochem. Behav. 36:97–104; 1990.
4. Davis, M.; Hitchcock, J. M.; Rosen, J. B. Anxiety and the amygdala: Pharmacological and anatomical analysis of the fear-potentiated startle paradigm. In: Bower, G. H., ed. The Psychology of Learning and Motivation, vol. 21. New York: Academic Press; 1988:263–305.
5. Fernandes, C.; File, S. E. Beware the builders: Construction noise changes [^{14}C]-GABA release from hippocampal and amygdaloid slices in the rat. Neuropharmacology 32:(12)1333–1336; 1993.
6. File, S. E. The history of benzodiazepine dependence: A review of animal studies. Neurosci. Biobehav. Rev. 14:135–146; 1990.
7. File, S. E.; Fernandes, C. Noise stress and the development of benzodiazepine dependence in the rat. Anxiety 1:8–12; 1994.
8. Hitchcott, P. K.; File, S. E.; Ekwuru, M.; Neal, M. J. Chronic diazepam treatment in rats causes long-lasting changes in central [^{3}H]-5-hydroxytryptamine and [^{14}C]-γ-aminobutyric acid release. Br. J. Pharmacol. 99:11–12; 1990.
9. Shibata, K.; Kataoka, Y.; Gomita, Y.; Ueki, S. Localization of the site of the anticonflict action of benzodiazepines in the amygdaloid nucleus of rats. Brain Res. 234:442–446; 1982.