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THE EFFECT OF BENZODIAZEPINES ON THE STRESS-INDUCED RESPONSES OF THE NORADRENERGIC AND SEROTONERGIC PROJECTIONS TO THE RAT HIPPOCAMPUS

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ABSTRACT

Microdialysis was used to measure the effect of benzodiazepines (BDZs) on basal levels and on stress-induced increases of 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline (NA), and dihydroxyphenylacetic acid (DOPAC). The stressors used were a 5 min tail pinch or a 10 min period of restraint. A subcutaneous injection of 5 mg/kg diazepam decreased basal levels of 5-HT, 5-HIAA, and DOPAC, but not NA. There was no effect on the stress-induced increase in 5-HT, NA, and 5-HIAA, while the increase in DOPAC was completely abolished. Local infusion of 5 μM flurazepam decreased the basal level of 5-HT but not 5-HIAA and abolished the stress-induced increase of 5-HIAA but not 5-HT. These results suggest that the anxiolytic action of BDZs is unlikely to be attributable to the suppression of stress-induced increases in the release of NA or 5-HT in the hippocampus.

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Keywords: Diazepam, Noradrenaline, Serotonin, Stress, Hippocampus, Microdialysis.

INTRODUCTION

Anxiety in humans is the normal subjective accompaniment of physical and psychological stress; anxiety is pathological when there appears to be no adequate cause. Both forms of stress are relieved by benzodiazepines (BDZs). Although there is widespread clinical use of BDZs as anxiolytics, the mechanism which mediates this effect is incompletely understood.

Studies in both animals and humans provide evidence that noradrenergic and serotonergic neurons are activated by stress[29] and there is a link between anxiety and the activity of noradrenergic[13] and serotonergic[14] neurons. Benzodiazepines enhance the action of γ-aminobutyric acid (GABA) by acting at a specific binding site on the $GABA_A$ receptor complex.[18] The anxiolytic effect of BDZs

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is dose-dependent and is seen with low doses; as the dose is increased the effect of BDZs changes to sedative and muscle relaxing, indicating a progressive increase in GABA-mediated inhibition.

A number of animal studies have shown that BDZs reduce the basal turnover of both 5-hydroxytryptamine (5-HT)[16,30] and noradrenaline (NA).[16,27] Electrophysiological studies have shown that systemic administration of BDZs depresses neuronal firing in the dorsal raphe nucleus in the encephale isole preparation of the rat[15] and the locus ceruleus nucleus of the anesthetized rat.[11] Attempts to localize the receptors responsible for these effects have yielded conflicting results. Acute injections of GABA mimetics into the median and dorsal raphe nuclei reduce 5-hydroxytryptophane (5-HTP) accumulation;[17] microinjection of BDZs potentiate the GABA inhibitory effect on neuronal firing in the raphe nuclei.[8,9] These receptors showed no tonic activity, since the microinjection of GABA antagonists had no effect on 5-HTP accumulation.[17] The absence of effect when drugs were injected into the hippocampus led to the conclusion that the $GABA_A$ receptors and their benzodiazepine (BDZ) binding sites were confined to the cell body region. However, this conclusion was challenged by other work. In experiments with hippocampal synaptosomes both GABA and BDZs produced changes in the release of ^{3}[H] 5-HT[3] and ^{3}[H] NA.[7] Similar effects were obtained with brain slices.[4] Studies using microdialysis have shown that local infusion of 10 µM flurazepam decreased the basal level of 5-HT but not 5-HIAA, an effect that was reversed by the BDZ antagonist flumazenil and GABA antagonist picrotoxin. Picrotoxin alone produced an increase in the level of 5-HT, indicating a tonic GABA activity.[21] These studies all suggest that there are both $GABA_A$ and BDZ receptors in the region of the hippocampal serotonergic terminals.

Although there are a number of recent studies on the anticonvulsant effect of BDZs in hippocampal glutaminergic[5,23] and cholinergic systems,[25] few of them address its anxiolytic[12] effect. There have only been a few previous studies on the effect of BDZs on the stress-induced increases in noradrenergic and/or serotonergic function. In experiments using microdialysis the increase in NA in response to tail pressure was unaffected[1] while the increase produced by foot shock was completely abolished[24] by diazepam. The increase in extracellular 5-HT in the ventral hippocampus of rats exposed to an elevated X-maze was reduced by diazepam;[31] similar experiments with guinea pigs showed no statistically significant reduction of the rise in cortical 5-HT by diazepam.[22]

The aim of the present study was to measure the effect of both systemic and local administration, through the dialysis probe, of benzodiazepines on both the basal activity and the stress-induced changes of the noradrenergic and serotonergic projections to the hippocampus. We used microdialysis in the freely moving rat to measure changes in noradrenaline, serotonin and their metabolites dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) during basal conditions and in response to mild stress.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats, bred in the laboratory, weighing 200-300 g at the time of the experiments were used. Rats were housed six to a cage under conditions of controlled temperature (21±1°C), a 12 h light/dark cycle and with free access to food and water.

Surgery and implantation of microdialysis probe

For general anesthesia rats were injected with chloral hydrate (500 mg/kg, i.p.). During induction of surgical anesthesia rats were left in their home cage for a period of 15 min. Before surgery, the state of anesthesia was checked using leg pinch and corneal reflex. Respiration and heart rate were checked and the airway kept open by careful positioning of the tongue. A small area of the scalp was shaved. Rats were then mounted in a stereotaxic frame. Iodine solution was used as an antiseptic agent for the area of incision. A surgical scalp spreader was used to open the skin (about 1.5 cm^2), and after making a hole (2.5 mm) in the skull, the dura was cut using a precision scalpel.[28]

A U-shaped dialysis probe (4 mm length, 300 µm outer diameter, using a GF80M Hollow fiber dialyser membrane, 5000 m.w. cut-off) was prepared. The cellulose membrane was used in preference to Hospal dialysis membrane, which had been shown to present difficulties with the measurement of 5-HT.[13] Rats were anesthetized and the dialysis probes were stereotaxically implanted into the

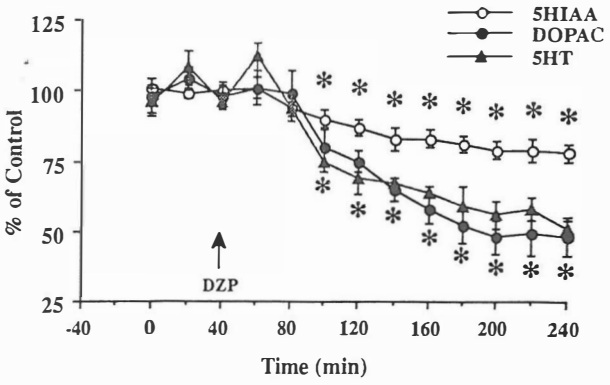

Fig. 1. The effect of 5 mg/kg diazepam (s.c.) on basal levels of 5HT, 5-HIAA and DOPAC. Basal values before diazepam injection in fmol/40 µL. 5HT=40±5.6; 5-HIAA=4627±346; DOPAC=227±32. *p<0.05 compared to basal by paired t-test using absolute values (n=6).

A.

Table I. Increases in fmol/pmol above basal levels measured as the area under the curve.

Stressor	5-HT Increase in fmol	5-HIAA Increase in pmol	NA Increase in fmol	DOPAC Increase in fmol
Vehicle + Res	70±8	0.9±0.3	114±31	183±41
DZP+Res	61±23	0.4±0.3	127±41	Abolished
Flu+Res	80±15	Abolished	No Data	Abolished
Control TP	55±11	1.3±0.4	No Data	106±31
DZP+TP	32±7	0.6±0.2	No Data	Abolished

Res: 10 minute restraint
DZP: diazepam, 5 mg/kg i.p.
Flu: flurazepam, 5 μM solution
TP: 5 min tail pinch.

ventral hippocampus via surgical procedure and the following coordinates relative to bregma and the dura: rostro-caudal, -4.0 mm; medio-lateral, +4.6 mm; and dorso-ventral, -8.5mm. The probe was secured in place with dental cement and skull screws. At the end of the experiments, the brains were dissected out and the localization of the dialysis probe was verified using a microtome cryostat and a viewing lens.

Neurochemical measurements

During the experiments, the dialysis probe was continuously perfused (2 μL/min) with Ringer solution (Na^+ 147 mM, K^+ 4mM, Cl^- 155.6 mM, pH 6.0) through polyethylene tubing connected to a 1 mL syringe mounted on a microinfusion pump (CMA/100, CMA Microdialysis, Stockholm).

Samples were collected every 20 min and were directly injected into the HPLC system with electrochemical detection. The HPLC system consisted of an ACS 300/02 series isocratic pump equipped with a 7125 Rheodyne 50 μL injection valve, an electrochemical detector and a Pantos Unicorder. The HPLC analytical column was a 15 cm length Dynamax Microsorb C18 column. The electrode (BAS LC17) was carbon paste held at 0.50-0.65 mV (Ag/AgCl) by a laboratory-built potentiostat.

Two separate buffer systems were used. One, which measured 5-HT, 5-HIAA and DOPAC, consisted of NaH_2PO_4 (0.15M), EDTA (0.5mM), octane sulphoric acid (0.05mM) and 14% methanol (v/v) adjusted to pH 4.55 using phosphoric acid. The other, which measured NA, DOPAC and 5-HIAA, consisted of NaH_2PO_4 (0.10 M), EDTA (0.2 mM), octane sulphoric acid (2 mM) and 12% methanol at a pH of 4.55-4.70.

Administration of drugs

Diazepam 5 mg/kg or vehicle consisting of 40% propylene glycol in water was given by subcutaneous injection to either test or sham group of animals. Flurazepam 5 μM was added to the perfusion medium for local application.

Experimental protocol

After surgery (see above), the rats were allowed 12 h to recover in their home cages with access to food and water. All experiments were carried out on awake, freely moving rats 12 to 24 h after surgery. Rats were placed in a large plastic bowl and were connected to a microinfusion pump through a liquid swivel which allowed them free movement. Animals were divided into control, sham (vehicle injected), and experimental (diazepam injected) groups in systemic experiments. Since there were two sets of experiments for assaying 5-HT and NA, the number of rats were either 6 or 5 in each group (these were indicated in the result section).

Animals were subjected to the following mild stressors: A) an i.p. injection of 1mL saline, B) a 5 min tail pinch which was delivered by attaching a paper clip (3.2 cm long) to the rat's tail approximately 3 cm from the tail tip, and C) a 10 min period of restraint which was effected by placing the rat in a specially constructed perspex box (for details see ref. 28).The welfare of the animals was assessed in accordance with published guidelines[8] and all procedures were specifically licensed under the Animal Scientific Procedures Act 1989.

Statistical analysis

All results were shown as the concentration of the neurochemicals in a 40 μL sample. Samples were collected until there was a steady baseline. The mean of the last three samples was then taken as the baseline. Changes were expressed either as a percentage of the mean baseline value, statistical significance being calculated from absolute values using the Student's paired t-test, or as the increase, in absolute units, calculated from the area under the curve using those samples which showed a statistically significant difference from the baseline. Comparisons between treatments were made using the increase by ANOVA and Fisher's PLSD.

RESULTS

Serotonergic neurons

Rats were given an intraperitoneal injection of 5 mg/kg diazepam. This produced a brief rise in 5-HT due to the stress of injection which was followed by a decrease in the level of 5-HT to 52±4% ($p<0.0007$, n=6) of control value

by 220 min after the diazepam injection (Fig. 1).

We used two forms of mild stress: a 5 minute tail pinch or a 10 minute period of restraint applied 80 min after the administration of diazepam, when the baseline 5-HT level had fallen to 68±6% ($p<0.0001$, n=6). In order to allow for the stress of intraperitoneal injection we gave a separate control group (sham group) of rats an injection of the diazepam vehicle. Figure 2 shows the response to the 10 min restraint of a group of vehicle injected (sham) and a group of diazepam injected (experiment) rats. The diazepam injection is followed by a decrease in the baseline, but there is no difference in the response to the restraint stress between the diazepam injected and vehicle injected rats, measured either as the percentage rise of 5-HT or the increase in the amount of 5-HT in the dialysate; this is shown in Table I.

A similar result was obtained when, instead of restraint, a 5 min tail pinch was used as stressor. This increased 5-HT to 188±11% ($p<0.03$. n=5) in control rats; in the diazepam treated rats there was a decrease in baseline and the tail pinch produced an increase of 256±79% ($p<0.04$, n=6) from this baseline. The maximum increase in 5-HT both as the increase in the amount of 5-HT in the dialysate and as the percentage increase above baseline was not statistically different in vehicle- and diazepam-injected rats (see Table I).

We also studied the effect of local application of BDZs. For this we used the water soluble BDZ flurazepam which was added to the perfusion medium in a concentration of 5 µM. This caused a reduction in the baseline level of 5-HT without the rise seen after the intraperitoneal injection. The level of 5-HT 80 minutes after the addition of flurazepam was reduced to 73±9% ($p<0.01$, n=5) of control, which is not statistically different from the effect of the systemic dose of diazepam. A 10 min restraint increased 5-HT to 212±22% ($p<0.001$, n=5) and the flurazepam-treated rats to 398±93% ($p<0.006$, n=5) from the decreased baseline. The increase in absolute figures is shown in Table I; it is not different from that seen after systemic diazepam or vehicle injection.

We also measured the changes in the metabolite 5-HIAA. The systemic administration of diazepam caused a decrease in the level of 5-HIAA, which is considerably smaller than that of 5-HT. It was reduced to 80±3% ($p<0.0005$, n=6) of control by 220 minutes and to 87±3% ($p<0.0001$, n=6) by 80 minutes (Fig. 1). A 10 min restraint after vehicle injection increased 5-HIAA to 114±4% ($p<0.02$, n=6) of baseline. In the diazepam-injected group there was a decrease in baseline and restraint increased 5-HIAA to 110±4% ($p<0.03$, n = 6) from this baseline (Fig. 2). Flurazepam had no effect on 5-HIAA baseline but abolished the effect of restraint. Tail pinch in control rats increased 5 HIAA to 114±3% ($p<0.03$, n=5) of control baseline and diazepam-treated rats to 106±2% ($p<0.003$, n=5) from the decreased baseline. Changes expressed in absolute units are shown in Table I; there is no statistically significant difference between the vehicle-injected and diazepam-injected rats.

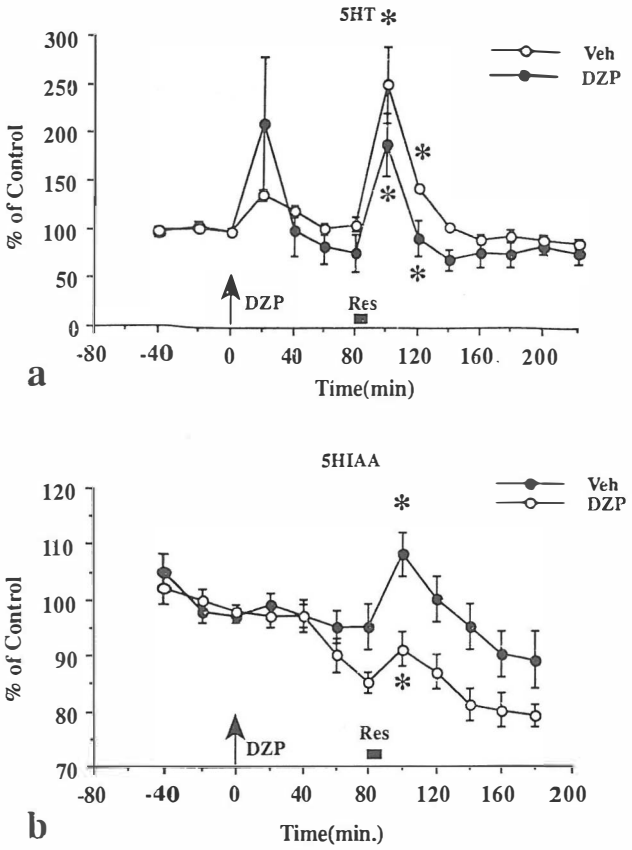

Fig. 2. Effect of 5 mg/kg diazepam (s.c.) on stress-induced changes in 5HT and 5-HIAA. Inj=injection of either vehicle or diazepam; DZP= 5 mg/kg diazepam (s.c.); Res=10 min restraint. * $p<0.05$ compared to last baseline value before stress by paired t-test using absolute values (n=6).

a. Changes in 5HT. Baseline value before injection of vehicle=38±4.8 fmol/40µL; before injection of diazepam = 45±6.3fmol/40 µL.

b. Changes in 5-HIAA. Baseline value before injection of vehicle=4.354±0.435 pmol/40 µL; before injection of diazepam=5.042±0.833 pmol/40µL.

Noradrenergic neurons

We carried out similar experiments using the buffer for assaying NA. Systemic injection of 5 mg/kg diazepam had no effect on the baseline level of NA. A 10 min restraint in vehicle-injected rats increased NA to 224±32% ($p<0.003$, n=5) and in diazepam-injected rats to 229±43% ($p<0.03$, n=5) of baseline. A comparison of the increase in NA in vehicle-injected and diazepam-injected rats (Fig. 3) shows that there was no effect on the stress-evoked increase in NA.

There was a striking difference however in the effect on

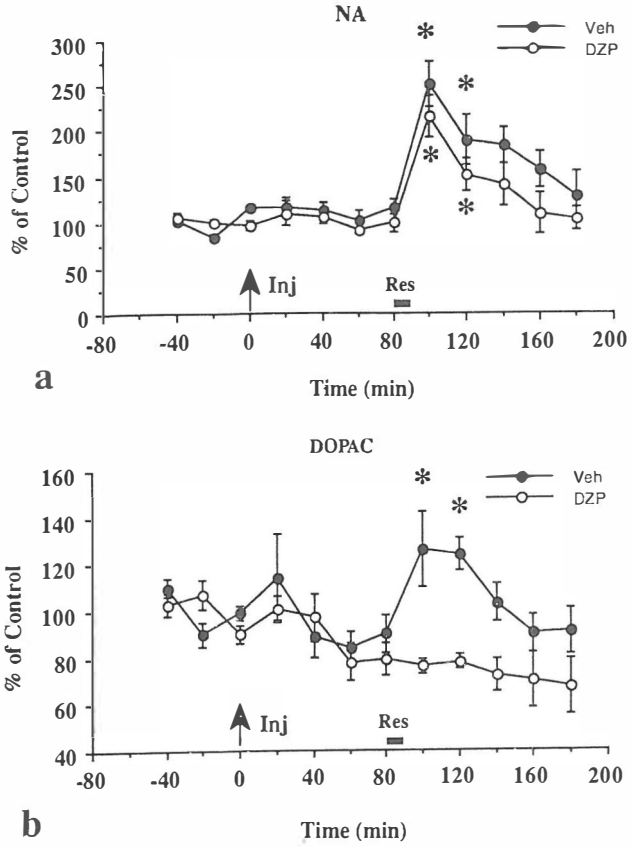

Fig. 3. Effect of 5 mg/kg diazepam (s.c.) on stress-induced changes in noradrenaline and DOPAC. Inj=injection of either vehicle or diazepam; DZP= 5 mg/kg diazepam (s.c); Res= 10 min restraint. * $p<0.05$ compared to last baseline value before stress by paired t-test using absolute values (n=6).

a. Changes in noradrenaline. Baseline value before injection of vehicle = 84±21 fmol/40µL; before injection of diazepam = 91±18 fmol/40µL.

b. Changes in DOPAC. Baseline value before injection of vehicle=183±74 fmol/40 µL; before injection of diazepam=184±69 fmol/40 µL.

DOPAC. An intraperitoneal injection of 5 mg/kg of diazepam reduced basal DOPAC concentration to 54±7% ($p<0.004$, n=5) of control (Fig. 1). A 10 min restraint increased DOPAC to 145±26% ($p<0.03$, n=5) of baseline. In diazepam-injected rats this increase was completely abolished (Fig. 3 and Table I).

DOPAC can also be measured in the dialysate assayed with the 5-HT buffer; the perfusion fluid contained 1µM citalopram which had no effect on the concentration of DOPAC. In these experiments also the increase in DOPAC induced by restraint after both systemic diazepam and local infusion of flurazepam was completely suppressed. Tail pinch increased DOPAC to 263±64% ($p<0.03$, n=5) in control animals; in diazepam-injected rats tail pinch produced no change in DOPAC (Table I).

DISCUSSION

The present results show that BDZs, whether administered systemically or locally through the dialysis probe, have different effects on basal activity and stress-induced changes of noradrenergic and serotonergic projections to the hippocampus.

The effect on basal levels of 5-HT and 5-HIAA of 5 mg/kg systemic diazepam and 5 µM local flurazepam are similar to those produced by 10 mg/kg diazepam and 10 µM flurazepam in a previous study;[21] 5-HT levels were depressed by BDZs given by both routes of administration, whereas 5-HIAA was depressed only by systemic but not by local administration. This suggests that, whereas 5-HT release can be depressed by presynaptic alone, 5-HT synthesis, measured by the concentration of 5-HIAA in the presence of an uptake blocker, is depressed only when neuronal firing is depressed by receptors in the cell body region. Other microdialysis studies reported no change in hippocampal 5-HT in rats after 2.5 mg/kg diazepam[31] but a reduction in cortical 5-HT in guinea pigs after 1.0 mg/kg diazepam.[22] In contrast to the serotonergic neurons, in noradrenergic neurons the level of transmitter was unaffected, while that of the metabolite DOPAC was depressed by diazepam; in another study NA levels in the cortex were depressed by a smaller dose of diazepam.[1] This could be due to a difference in the distribution of presynaptic receptors on terminals of locus ceruleus neurons in different brain areas or to differences in the experimental protocol (the rats in the present study were briefly handled before surgery).

The two forms of stress, a 5 min tail pinch or a 10 min period of restraint, produced increases in both 5-HT and 5-HIAA; these increases were unaffected by systemic administration of diazepam. Local administration of flurazepam had no effect on the stress-induced increase in 5-HT but abolished the increase in 5-HIAA. Similarly, the increase in NA produced by the 10 min restraint was unaffected by 5 mg/kg diazepam. In striking contrast was the complete abolition of the stress-induced increase of DOPAC. This was seen not only in the experiments where the NA buffer was used and desipramine was present in the perfusion medium, but also in the experiments with the 5-HT buffer and citalopram in the perfusion medium; the increase in DOPAC in response to either tail pinch or to restraint was abolished by both systemic diazepam and local flurazepam.

The failure of BDZs to depress the stress-induced increase in 5-HT could be due to a number of factors. There is evidence that stress produces changes in the turnover of GABA as well as the $GABA_A$ receptor and the BDZ binding site. These changes vary with the form of stress, the brain region, and also show marked variation in time course.[26] Little is known about the short term changes that accompany

acute stress, since at present most measurements are carried out after the death of the animal. In one recent study which examined the effect of 5 min of immobilization–a stress similar to that used in the present study – no changes in GABA content, turnover or uptake were found in the hippocampus. However the authors point out that only enormous changs in GABA turnover can be detected and negative results therefore do not necessarily imply that no changes have occurred.[20] It is possible therefore that stress-induced changes in the activity of GABAergic neurons in $GABA_A$ receptors or BDZ binding sites could explain the failure of diazepam to depress the stress-induced increase in 5-HT.

The effects on noradrenergic neurons suggest that although there is no effect on NA release, NA synthesis at the tyrosine hydroxylation step is inhibited. DOPAC is the deaminated product of the dopamine (DA) precursor of NA and there is extensive evidence that levels of DOPAC in noradrenergic neurons are closely coupled to neuronal activity[2,10,19] and are an index of the activity of the enzyme tyrosine hydroxylase. The activity of this enzyme is regulated by a number of protein kinases which are activated by presynaptic receptors.[6] It is possible that the effect of diazepam, although applied locally, is indirect rather than direct. It could alter the release of neurotransmitters which, by acting on presynaptic receptors, can modulate the synthesis of NA. Such a depression of synthesis could have a delayed effect on transmitter release.

These results suggest that suppression of stress-induced increases in NA and 5-HT in the hippocampus do not account for the anxiolytic action of BDZs.

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