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BRIEF COMMUNICATION

Diazepam Reverses the Effects of Pentylenetetrazole in Rat Pups by Acting at Type 2 Benzodiazepine Receptors

AMANDA L. JOHNSTON,* SANDRA E. FILE,†[1] JASPER DINGEMANSE‡ AND KARI ARANKO§

**MRC Neuropharmacology Research Group, The School of Pharmacy, University of London*
†Psychopharmacology Research Unit, UMDS Division of Pharmacology
University of London, Guy's Hospital, London SE1 9RT
‡Center for Bio-Pharmaceutical Sciences, Division of Pharmacology, Leiden, The Netherlands
§Department of Pharmacology and Toxicology, University of Helsinki, Finland

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JOHNSTON, A. L., S. E. FILE, J. DINGEMANSE AND K. ARANKO. *Diazepam reverses the effects of pentylenetetrazole in rat pups by acting at type 2 benzodiazepine receptors.* PHARMACOL BIOCHEM BEHAV **32**(3) 823–825, 1989.—Pentylenetetrazole (75 mg/kg) induced a characteristic coarse body tremor (accompanied by limb extension) and hyperactivity in 4-day-old rat pups. These effects were reversed by diazepam (0.5 and 2 mg/kg) but not by CL 218,872 (10 and 20 mg/kg) which is selective for type 1 benzodiazepine receptors. Diazepam did not affect the brain concentrations of pentylenetrazole, indicating that the reversal was not based on a pharmacokinetic interaction. Neither diazepam nor CL 218,872 had significant effects on the behavior of the rat pups, although diazepam (2 mg/kg) tended to increase locomotor activity. The results suggest that diazepam displays an anticonvulsant effect in the neonatal rat which is mediated by type 2 receptors.

Hyperactivity Tremor Anticonvulsant Benzodiazepine Type 2 receptors

PENTYLENETETRAZOLE (PTZ) has been reported to induce hyperlocomotion and seizure-like activity in rat pups (4, 12, 13). In this investigation we tried to antagonise the effects of PTZ in 4-day-old rat pups. Antagonism studies were carried out using the 1,4-benzodiazepine, diazepam, and CL 218,872 (3-methyl-6[3 (trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-6]pyridazine), which is selective for type 1 benzodiazepine receptors (9). Diazepam was used to determine whether the effects of PTZ in neonatal rats can be reversed by a benzodiazepine. In the adult rat, PTZ acts at the picrotoxinin site on the GABA-benzodiazepine receptor complex and diazepam acts at the benzodiazepine receptor on this same complex (14,15). However, it is not known whether this complex functions in the same way in neonatal rats and it is not known whether diazepam is able to reverse the effects of PTZ in the neonate. This is an interesting question because of the evidence that low doses of benzodiazepines can themselves induce seizure-like activity in the neonate (1, 7, 12). In the newborn rat, type 2 benzodiazepine receptors are present at birth, whilst type 1 receptors develop after the first week (3). CL 218,872 was therefore included to confirm that any actions of diazepam were due to activity at type 2 receptors. In addition, brain concentrations of the three drugs (and, for diazepam, its metabolite, temazepam) were assessed to exclude any pharmacokinetic interactions.

A pilot dose-response study was carried out to choose a suitable dose of PTZ which would produce reliable behavioral changes. A dose range of 50–100 mg/kg was investigated. PTZ was found to induce hyperactivity and a very characteristic coarse body tremor accompanied by the extension of all four limbs. A dose of 75 mg/kg was chosen for all further studies. Doses of diazepam and

[1]Requests for reprints should be addressed to Dr. S. E. File.

CL 218,872 were chosen on the basis of a previous study on their effects in neonates (7) and on the basis of their anticonvulsant doses in adult animals (6).

METHOD

Drugs

PTZ (Sigma) was dissolved in distilled water. Diazepam (Hoffmann-La Roche) and CL 218,872 (Lederle) were suspended in distilled water with a drop of Tween-20. Control animals received either distilled water or distilled water to which a drop of Tween 20 had been added. All drugs were injected intraperitoneally in concentrations to give an injection volume of 4 ml/kg.

Animals

Male and female offspring of hooded Lister rats (Olac Ltd, Bicester) were used. The pups were left undisturbed with their mothers until day 4 (the day of birth being day 0) when testing was carried out. The pups weighed 5.6–9.1 g on the test day.

Procedure

On day 4, rat pups were randomly allocated (within each litter and within the sexes) to the following drug conditions, n = 8/group: control, PTZ (75 mg/kg) alone, and in combination with diazepam (0.5 and 2 mg/kg); and control, PTZ (75 mg/kg) alone, and in combination with CL 218,872 (10 and 20 mg/kg). All of the pups received two injections of vehicle or drug, as appropriate.

The pups were injected with their allocated drug treatment and immediately placed on a paper lined tray and observed for 15 min by an observer who was blind to the drug treatment. The test room was maintained at a constant temperature of 28°C. The incidence of jerks was scored and the duration of the following behaviors recorded: coarse body tremor (accompanied by limb extension) and locomotor activity (including forward walking, paddling and climbing up the sides of the tray). Duration was defined as follows: 1, for a brief occurrence; 2, for a duration of 2–10 sec; and 3, for a duration of more than 10 sec. This scoring method was based on that used by File and Wilks (4).

Immediately after observation the pups were decapitated and their brains rapidly removed and stored at −15°C until analysis of the drug concentrations.

Analysis of Drug Concentrations

The brains were homogenised in 1:1 dichloromethane/petroleum ether. Pentylenetetrazole was measured using the gas chromatographic method of Dingemanse *et al.* (5). Diazepam, temazepam and CL 218,872 were determined by HPLC, using lormetazepam and desmethyldiazepam as the internal standard, respectively, and water/acetonitrile/acetic acid as the mobile phase (5).

RESULTS

Table 1 shows the number of jerks made and the scores for coarse body tremor and locomotor activity.

In both groups PTZ (75 mg/kg) significantly increased the scores for coarse body tremor (accompanied by limb extension) and locomotor activity (forward walking, paddling and climbing up the sides of the tray) (see Table 1). PTZ had no effect on the number of jerks observed. The effects of PTZ on coarse body tremor and locomotor activity were completely reversed by the addition of diazepam (0.5 and 2 mg/kg). Diazepam alone had no significant effects on any of the behaviors scored, although there was a tendency for the higher dose of diazepam (2 mg/kg) to increase locomotor activity scores. CL 218,872 (10 or 20 mg/kg) failed to prevent either the coarse body tremor or the hyperactivity induced by PTZ. CL 218,872 alone had no significant effect on any of the behaviors scored.

TABLE 1

THE MEDIAN SCORES OF JERKS (SCORES REFLECT INCIDENCE), COARSE BODY TREMOR AND LOCOMOTOR ACTIVITY (SCORES REFLECT INCIDENCE AND DURATION) FOR RAT PUPS TREATED WITH EITHER CONTROL OR PTZ (75 mg/kg) ALONE OR IN COMBINATION WITH DIAZEPAM (0.5 AND 2 mg/kg) OR CL 218,872 (10 AND 20 mg/kg)

Treatment mg/kg	Jerks	Coarse Body Tremor	Locomotor Activity
Control	2.5	2.5	1
PTZ 75	0	31†	22‡
Diazepam 0.5	7	2	1
Diazepam 2	2	1	7.5
PTZ 75 and Diazepam 0.5	1	0§	0#
PTZ 75 and Diazepam 2	4.5	4.5§	0#
Control	1	0	0
PTZ 75	0	29.5‡	13‡
CL 218,872 10	2	0	1
CL 218,872 20	4	0	4
PTZ 75 and CL 218,872 10	0	34.5‡	10‡
PTZ 75 and CL 218,872 20	0	43.5‡	11.5†

*$p \leq 0.05$, †$p \leq 0.01$, ‡$p \leq 0.001$, significantly different from controls; §$p \leq 0.05$, #$p \leq 0.001$, significantly different from PTZ alone, Mann-Whitney U-tests.

Table 2 shows the mean brain concentrations of PTZ, diazepam, temazepam and CL 218,872. The concentrations of diazepam and of its metabolite, temazepam, were dose-related, but those of CL 218,872 were not. This was probably due to limited absorption from the injection site. It is unlikely to reflect an extraction problem since calibration graphs with in vitro spiked brain samples were linear with $r > .99$. Diazepam had no effect on the brain levels of PTZ. The mean (±sem) PTZ levels (μg/g) were: 83.4 ± 8.0 in the PTZ alone group; 81.6 ± 6.5 in the PTZ and diazepam 0.5 mg/kg group; and 80.6 ± 5.1 in the PTZ and diazepam 2.0 mg/kg group.

DISCUSSION

Consistent with previous studies on the effects of PTZ in neonatal rats, we found that PTZ produced hyperactivity and induced a coarse body tremor (4, 11, 12). PTZ did not cause an increased incidence of jerks and this finding is again consistent which those of other investigators (4,11). When given in combination with PTZ, diazepam completely reversed the effects of the former compound. The brain concentration of the drugs indicate

TABLE 2

MEAN (±sem) BRAIN CONCENTRATIONS OF PTZ, DIAZEPAM, TEMAZEPAM (A MAJOR METABOLITE OF DIAZEPAM) AND CL 218,872

PTZ:	PTZ levels = 83.4 (± 8.0) μg/kg
Diazepam 0.5 mg/kg:	Diazepam levels = 107 (± 21) ng/g
	Temazepam levels = 21 (± 7) ng/g
Diazepam 2 mg/kg:	Diazepam levels = 282 (± 58) ng/g
	Temazepam levels = 59 (± 14) ng/g
CL 218,872 10 mg/kg:	CL 218,872 levels = 15 (± 4) μg/g
CL 218,872 20 mg/kg	CL 218,872 levels = 17 (± 3) μg/g

that diazepam did not prevent the action of PTZ by a pharmacokinetic mechanism, since the brain levels of PTZ given alone were not different from those in pups which had received both PTZ and diazepam. Diazepam alone had no significant effect on any of the behaviors which were scored, although there was a tendency for the higher dose of diazepam (2 mg/kg) to increase locomotor activity. Diazepam has been reported to induce hyperlocomotion and to increase the incidence of jerks in rat pups of a similar age (7,13).

Could the failure of CL 218,872 to reverse the effects of PTZ be due to the choice of inappropriate doses? It is most unlikely that the doses were too high to be effective against PTZ. In the adult mouse, high (20 mg/kg) doses are needed to antagonise seizures induced by PTZ (6,11); and the effective anticonvulsant dose is about ten times higher than the effective dose of diazepam. However, it is possible that in the neonate rat an even higher dose would be needed. In this study, we used the same doses of CL 218,872 and of PTZ in the rat pups, as we had used in our previous studies in adult animals; thus the highest dose of CL 218,872 was 40 times higher than the lowest dose of diazepam (0.5 mg/kg) that was effective against PTZ in the pups. There are no data on the pharmacokinetics of CL 218,872 in the neonate, but the PTZ response has been found to be the same in neonate and adult animals (10) and the effects of diazepam the same or greater in the neonate (8,10). Higher doses of CL 218,872 could not be used because they would have produced brain concentrations in the range at which CL 218,872 loses selectivity for the type 1 receptors. In the five-day-old rat pup, the IC_{50} for Ro 15-1788 binding in the presence of GABA was 3.6 nM for diazepam and 233 nM for CL 218,872 (2), reflecting the relative selectivity of the latter for the type 1 receptors. Our brain concentrations of CL 218,872 were about 57 times higher than those of diazepam and higher concentrations would have had effects on the type 2 receptors.

In conclusion, within the doses investigated, CL 218,872 had no effect on any of the behaviors in the rat pups, indicating that the effects of diazepam were likely to be mediated by type 2 receptors. Whilst we cannot exclude the possibility that higher doses would have counteracted the effects of PTZ, the effects of such doses would most likely be due to an action on the type 2 receptors. If the coarse body tremor elicited by PTZ in the rat pup is indicative of seizure activity, this suggests that diazepam displayed anticonvulsant activity in the neonatal rat and that this effect is mediated by type 2 receptors. This confirms an earlier brief report that diazepam displayed anticonvulsant activity against PTZ in newborn rats (age and exact behavior measured was unspecified) (6).

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